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Preface

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Preface

It is our pleasure to welcome you to the 61st International Meat Industry Conference (MEATCON2021) to be held at Mt. Zlatibor, Serbia, on September 26-29th 2021, traditionally hosted by the Institute of Meat Hygiene and Technology, Belgrade, Serbia.

For many years, this Conference has been the converging point for food/feed scientists, experts from the industry and legislation sphere, members of the professional associations, and other entities striving towards a common goal: **healthy food for the present and future**. The rapid development of scientific and technological research in the areas of food/feed technology, safety, quality, regulatory, environmental, and numerous other fields imposed the necessity for a synthetic approach to food-related challenges. Such an approach is primarily governed by understanding the complexity of food science that is taking place within the scientific community for the past two decades. Hence our commitment to the multidisciplinary nature of the Conference.

This three-day gathering will try to maintain its holistic character, presenting the latest research in food and feed production and technology, quality and safety issues, risk-assessment, consumer-related concerns, governmental actions, and strategies – with 111 contributions submitted for publication. Each paper has been peer-reviewed through a rigorous process that included conference committee members and international reviewers, and an English language editorial service. We are excited to publish all the contributions to the Conference in this volume, hoping that its contents shall be helpful to Conference participants and other interested readers.

The 61st International Meat Industry Conference also provides the opportunity for fellow scientists to meet, exchange experiences, and perhaps agree on future joint scientific endeavours. Carefully selected speakers from Serbia and abroad will present their latest research through plenary lectures, invited lectures on specific topics, and poster presentations. Formats such as round tables and workshops are also available to disseminate knowledge and experience.

The Conference has been supported by: Ministry of Education, Science and Technological Development of the Republic of Serbia, Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia – Veterinary Directorate and Chamber of Commerce and Industry of Serbia. Co-organizers of the Conference are the Faculty of Veterinary Medicine, University of Belgrade, Faculty of Agriculture, University of Belgrade, and Institute of Food Technology, University of Novi Sad.

Target audience:

- Food scientists and researchers

- Food technologists

- Scientists and researchers in the area of food quality and safety

- Food professionals from manufacturing, retail, foodservice industry…

- Governmental officials and policymakers in the area of food quality and security

On behalf of the Programme Committee, we would like to thank all contributing authors, reviewers, speakers, participants, Organizing Committee, and sponsors who contributed to the success of the MEATCON2021.

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Peer review declaration

All papers published in this volume of IOP Conference Series: Earth and Environmental Science have been peer reviewed through processes administered by the Editors. Reviews were conducted by expert referees to the professional and scientific standards expected of a proceedings journal published by IOP Publishing.

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- **Number of submissions received: 111**
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Goat meat products

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Abstract. In general, goat meat is not inferior to other meat types regarding nutritional and biological value – it has a high protein content (up to 29%), and it is a good source of minerals, vitamin B-complex, and essential amino acids. However, the meat of older and culled goats is less juicy, less tender, has a characteristically different odour and taste compared to kids' goat meat (and meat of other animals), and thus tends to be less desirable. Different meat products could be produced using goat meat (including culled goat meat): dry-fermented sausages (e.g. sucuk), dry-cured meats (Violino di capra – goat prosciutto), frankfurters, mortadella, etc. without adverse effects on products` technological properties. The negative impact of goat meat on the properties of meat products is mainly associated with the use of goat fatty tissue. However, this could be overcome by using fatty tissue of other animals (e.g. pork back fat or beef fatty tissue).

1. Introduction

Goats are small ruminants that live in small or large herds in different areas and environments across the world, with the exception of extremely cold areas. According to Devendra [1], there are 1156 different breeds of goats. The total number of goats in the world is over 1,000 billion, whereby around 95% are bred in Africa and Asia [2] and are considered suitable for meat production [3]. Contrary to this, in Europe, especially in the Mediterranean countries (where the highest goat population is located), goats are kept primarily for milk production.

From the 2000s, the global number of goats has increased by 30% while goat meat production has risen by almost 50% [2]. In Serbia, about 200,000 goats are kept primarily for milk production [2].

Goat meat consumption is not limited by religious restrictions and cultural habits. Despite that, goat meat consumption is lower compared to beef [4]. However, in developing countries goat meat is the main source of red meat [5].

Kids' goat meat is gladly consumed and is used for the preparation of different traditional dishes in the Mediterranean countries, while meat of older and culled goats is less acceptable and has low commercial value [6, 7]. However, the meat of such animals has been used for centuries for the production of salted/cured and dried meat products, e.g. *cecina de cabra* [6]. Moreover, some recent research indicates that goat meat, including culled goat meat, can be used in the production of some well-known industrial meat products such as mortadella [8], frankfurters [9, 10] etc.

In general, numerous studies indicate that goat meat (including culled goat meat), in addition to being used for the production of traditional products, is also used in the production of industrially processed

non-heat treated (including non-heat treated for heat processing) and heat-treated meat products, as well as for the production of meat products with improved functional/nutritional properties within all groups [8-11].

The aim of this paper is to give an overview of goat meat usage in the production of different meat products.

2. Goat meat characteristics

Goat meat is consumed as kids' meat and goat meat. As mentioned above, kids' goat meat is very acceptable and appreciated, so that kids bred in the Mediterranean EU countries are recognized as brands with protected designation of origin and protected geographical indications [7]. However, the meat of older and culled goats is less juicy, less tender, has a characteristically different odour and taste compared to kids goat meat (and meat of other animals) and, thus, tends to be less desirable [4, 12, 13].

On the other hand, goat meat generally has a high protein content (up to 29%) and is a good source of minerals (iron and potassium), vitamin B-complex, and essential amino acids, e.g. lysine, threonine and tryptophan [12, 14]. Moreover, goat meat generally has a low fat content and could have a more desirable unsaturated/saturated fatty acid ratio compared to beef and pork [14, 15]. All in all, goat meat is not inferior to other meat types regarding nutritional and biological value.

Regarding technological properties, goat meat can be successfully used in different types of meat products. Dry-fermented sausages and dry-cured meat can be produced using goat meat without an adverse effect on the technological properties of products [15, 16]. Because of the similar content of salt-soluble proteins compared to beef, goat meat can be used in the formulation of emulsified meat products [4].

The negative impact of goat meat on meat product properties is mainly associated with the use of goat fatty tissue. Goat fatty tissue decreases emulsion stability and reduces the palatability of meat products [17], while in dry-fermented sausages, the use of goat fatty tissue decreases taste acceptability [16]. This unwanted influence of goat fatty tissue could be overcome during the production process by using the fatty tissue of other animals with better technological properties (e.g. pork backfat, beef tail fat etc.) and/or by using nonmeat ingredients (e.g. spices).

3. Dry and cured goat meat products

Dry and cured meat products are prepared from whole meat pieces (bone-in or deboned) or ground meat and fatty tissue (sausages), which are salted/cured, sometimes smoked and air-dried.

3.1. Dry-cured meats

Dry-cured meats have been traditionally prepared for centuries by salting (or curing) different parts of animal carcasses (or even the whole carcass). Many of them are considered national products and are protected as quality brands.

Cecina de cabra and *Cecina de castron* are dry-cured meat products made from goat legs, and are traditionally produced in Spain; they are also called goat ham [6]. The whole process (3–8 months) is done in six stages [18]: shaping, salting with coarse salt (0.3–0.6 day/g at 2–5 °C and RH 80–90%), washing with warm water, post-salting (30–45 days at 3–5 °C and RH 85–90%), smoking and ripening (at 12–20 °C and RH 65–80%).

Violino di capra, called "goat prosciutto", also a dry-cured meat, is produced in Alpine valleys of Northern Italy from the legs of female goats from dairy herds, and is flavoured with garlic and different herbs [4, 6]. The whole production process lasts several months and the final product has a violin-like shape, hence its name. This "goat prosciutto" is characterized by specific sensory properties and is high in mono- and polyunsaturated fatty acids with a very favourable n-6/n-3 ratio of 1.7 [19].

In Serbia, Ivanovic *et al.* [15] examined the characteristics of dry-cured and smoked goat ham from the Balkan breed, reared in three regions: mountain, hilly and plain. The results indicated that diet had a significant impact on the quality of dry-cured goat ham. The products from goats reared in the mountainous region had the lowest fat and saturated fatty acid contents, while the content of

polyunsaturated fatty acids was the highest and indicated a more favourable fatty acid profile in those hams. Moreover, the results of sensory evaluation pointed to the ham from goats reared in the mountainous region as the most acceptable.

3.2. Dry-fermented sausages

Fermented sausages are meat products that have been manufactured for centuries and are highly valued for their sensory characteristics. Almost every region in the world is known for some typical type of sausage classified into this group.

Among them, sucuk is one of the most popular fermented sausages prepared without using pork meat. Sucuk formulations differ regionally, but in general, beef and beef fat and/or sheep tail fat is mostly used [16]. In research by Stajić *et al.* [16], the use of goat meat (and goat fatty tissue) was examined and compared with all-beef and all-mutton sucuks. Moreover, the influence of the addition of commercial starter cultures in production conditions similar to traditional production was examined. Technological properties (weight loss, basic chemical composition, pH changes and instrumental colour) were very similar between treatments made from the different meats. The use of starter cultures could shorten the production process and could enhance safety (faster pH drop). However, all-goat sucuk received poorer grades in terms of the sensory evaluation of taste and texture, which could be explained by the specific properties of goat fatty tissue. The authors suggested recipe modifications and use of beef fatty tissue to overcome this negative influence of goat fat.

Regarding the implementation of goat meat in the formulation of salami-type products, a combination of 25% goat meat with 75% pork can provide products with desirable properties. Also, very good consumer acceptance of fermented products was achieved by using 80% adult goat meat and 20% pork meat [4].

4. Non-heat treated goat meat products for heat processing

Minced meat products, e.g. burgers, patties, fresh sausages, are non-heat treated products which are intended for heat processing before consumption.

An off-flavour was noticed in burgers prepared with goat meat compared to beef burgers, which could be overcome by using 25% of beef/pork or using liquid smoke after moulding to improve flavour and colour [4].

Goat meat was successfully used as the only meat source in patty formulation [17]. However, the dispersion of goat fat globules was not uniform in patties, unlike chicken fat and vegetable oil which were used in other treatments. Moreover, patties with goat fat had significantly lower sensory scores for flavour and overall acceptability, which the authors corelate with a smeary and greasy mouth-coating sensation that panelists felt when evaluating patties containing goat fatty tissue. Patties with chicken fat and vegetable oil received the highest scores regarding all examined sensory properties.

Culled goat meat was used in fresh sausage production, and compared to the sausages made of sheep meat, they were defined as harder and more fibrous, though they were generally very well accepted by consumers [20]. Also, Leite *et al.* [7] reported that fresh sausages prepared with culled goat meat and 30% of pork fat had good acceptability.

5. Heat treated goat meat products

Among heat-treated meat products, emulsion-type sausages (e.g. frankfurters, mortadella) are popular worldwide and gladly consumed. As mentioned above, goat meat has a similar content of salt-soluble proteins as beef and could be used in the formulation of emulsion-type sausages. However, due to bad emulsion properties and a negative impact on taste and texture properties, goat fatty tissue should be avoided.

In the research by Stajić *et al.* [10], culled goat meat was used for partial and complete replacement of beef in all-beef frankfurters (beef and beef fat). Cooking loss, purge loss during storage, basic chemical composition and instrumental texture parameters were not affected by the amount of goat meat used in frankfurter formulation, while pH values were progressively higher with the increase in the

amount of goat meat, but were within the values for emulsion-type sausages. A progressive decrease of saturated fatty acid content and a progressive increase of polyunsaturated fatty acids (PUFA), especially long chain PUFA, was observed with the increase of culled goat meat content in frankfurter formulation. This led to a more favourable n-6/n-3 ratio in frankfurters with the higher amount of culled goat meat, reaching 6.63 in frankfurters with 100% goat meat. On the other hand, the main effect was observed regarding instrumental colour parameters – with the increase of culled goat meat content (and decreased beef content), frankfurters were lighter and less red, which led to higher values of total colour difference values. Since the colour of meat products is of great importance regarding consumer preferences [21], it would be expected that these observed differences are noticed by consumers. The check-all-that-apply (CATA) analysis confirmed that hypothesis, since the frequency of consumers that marked light surface and light pink surface as present progressively increased in goat frankfurters. However, this was not negatively perceived, because more than 80% of consumers marked pleasant colour as present in both beef frankfurter and in goat frankfurters. Panellists also perceived goat frankfurters as lighter than beef frankfurters and pointed to the frankfurter with 50% of beef and 50% of culled goat meat as the most preferred.

Guerra *et al.* [8] used goat meat from culled animals to produce mortadella with different pork fat levels (10–30%). All treatments showed good emulsion stability and water holding capacity, whereby mortadella with 30% of pork fat showed the highest values of these parameters. On the other hand, consumers gave the highest scores to mortadella with 10% of pork fat in all examined sensory properties except for texture. Moreover, about 70% of consumers said they would purchase goat mortadella.

6. Conclusions

Numerous research studies indicated that goat meat, including meat from culled animals, could be used in the formulation of different types of meat products. Some of them, such as *Violino di capra*, could be of importance as traditional meat products, while some, such as goat frankfurters, goat mortadella, etc., could be included in industrial manufacturing. Moreover, some value could be added to culled goat meat by using it in the formulation of these products. On the other hand, goat fatty tissue is marked as the main influence on the sensory properties of goat meat products, especially the ones made of culled goat meat. This could be overcome by using the fatty tissue of other animals (e.g. pork backfat or beef fatty tissue).

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Uncertainty of measurement and conformity assessment

Uncertainty of measurement and conformity assessment

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Abstract. Knowledge of the measurement uncertainty of test results is fundamentally important for laboratories, their customers and all parties using and interpreting these results. In conformity assessment, a measurement result is used to decide if an item of interest conforms to a specified requirement. Because of measurement uncertainty, there is always the risk of incorrectly deciding whether or not an item conforms to a specified requirement based on the measured value of a property of the item. Conformity assessment can be quite challenging when the entity measured is so close to the tolerance limits of the specification that its uncertainty, however estimated, critically affects decision-making. In such cases, different decision rules can be used to make statements of conformity. The aim of this paper is to provide a survey of methods for the evaluation of measurement uncertainty in testing, as well as to stress the need for appropriate estimation of measurement uncertainty. This paper also aims to assist testing laboratories in understanding the different decision rules used in conformity assessment and level of risk (such as false accept and false reject) associated with the decision rule employed.

1. Introduction

Credibility and reliability of analytical data has never caught the public eye more than today. They are used extensively by regulators for the public benefit in the provision of services that promote safe food, clean water, an unpolluted environment, energy, health and social care services [1]. Unreliable results bring a high risk of incorrect decisions and could lead to higher costs, health risks, and illegal practices. Hence, the goal of any analytical measurement is to obtain consistent, reliable, and accurate data. For this purpose, the results of laboratories accredited according to ISO/IEC 17025 are used [2].

The result of each real measurement is not perfect. It is only an estimate of the value of the test item's characteristic being measured. In order to make adequate decisions, through the conformity assessment, which demonstrates that specified requirements relating to a product, process, system, person or body are fulfilled [3], it is necessary that these data contain evaluated measurement uncertainty, a parameter associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand [4]. Proper consideration of uncertainty is imperative when testing a sample against legal/compositional limits, especially when the entity measured is close to the tolerance limits of the specification. The new edition of the standard ISO/IEC 17025 has introduced the concept of decision rules [2]. It is defined as "a rule that describes how measurement uncertainty will be accounted for when stating conformity with a specified requirement" [2]. Conformity with a requirement is inherently connected to the decision rule employed [1]. The introduction of this concept clarifies that no single decision rule can be applied to all conformity assessment to specifications,

because it significantly depends on the test area itself. Also, whether a measurement could result in a decision on conformity (acceptance) using one decision rule and rejection using a different decision rule needs to be considered. It is, therefore, expected that the decision rule is agreed before the measurements are taken [2]. When performing conformity assessment, there are probabilities related to two types of incorrect decisions, one for the supplier and one for the consumer [5].

Therefore, the aim of this study is to provide a survey of methods for the evaluation of measurement uncertainty in testing, and to stress the need for appropriate estimation of measurement uncertainty. This paper also aims to assist testing laboratories in understanding the different decision rules used in conformity assessment and level of risk (such as false accept and false reject) associated with the decision rule employed.

2. Measurement uncertainty

All measurements are affected by a certain error. The classical approach starts from the assumption that by measuring, we determine the true value of the measured quantity and its errors, which can be random or systematic in nature. The main difficulty is that neither the true value nor the measurement error can be perfectly known [6]. Hence, the new approach, the uncertainty approach, omits the term "true value" and, in accordance with the definition, considers the interval in which that value is. This interval, which includes the best estimate of the measured quantity, is in fact the measurement uncertainty. Reporting is required when information on uncertainty is relevant to the validity or application of the test results, when the client requires it, or when the uncertainty affects conformity with a specification limit [2]. ILAC-G17:01/2021 [7] recognized that there are situations where the requirement for reporting measurement uncertainty may not be obvious.

There are many possible sources of uncertainty in testing, such as sampling, sample effects, storage conditions, instrument and operator effects, reagent purity, measurement conditions, assumed stoichiometry, computational effects, blank correction, random effects etc. [8, 9]. The required depth of the uncertainty estimations can be different in different fields. The contributions of all the above factors to the total value of the measurement uncertainty constitute the budget of the measurement uncertainty related to a particular test [8]. The basis for the evaluation is a measurement and statistical approach, where the different uncertainty sources are estimated and combined into a single value. Most of the information needed to evaluate the uncertainty is likely to be already available, like quality control charts, validation, proficiency testing, certified reference material, handbooks etc. [8, 9, 10].

2.1. Evaluating measurement uncertainty

Measurement uncertainty is assessed through two methods that are essentially just concepts for processing different types of measurement results: the type A method and the type B method. Both types of estimates are based on probability distributions. Type A standard uncertainty is calculated from a series of repeated observations and is equal to the square root of the statistically estimated variance. The type A method is called the standard deviation [8, 9]. However, the uncertainty component can also be determined without actual observations, through experience based on available information. Such an estimate is called a type B method, and the derived uncertainty is referred to as standard type B uncertainty [8, 9]. The pool of information could include previous measurement data, data provided in calibration certificates, manufacturer's specifications, uncertainty assigned to reference data taken from handbooks, experience with or general knowledge of the behaviour and properties of relevant materials and instruments etc. [8, 9].

Several approaches to obtaining an uncertainty estimate are described [8, 9, 10]. According to the EURACHEM concept, it is necessary to specify measured quantities, identify and group sources of measurement uncertainty, quantify uncertainty components (convert components to standard deviations), and finally calculate the combined, uc, and expanded, U, measurement uncertainty [8]. It should also be clear whether a sampling step is included within the procedure or not. If it is, estimation of uncertainties associated with the sampling procedure need to be considered [9]. In the Nordtest model, within laboratory reproducibility, standard deviation is combined with estimates of the method and

laboratory bias [10]. Alternatively, according to ISO 21748 [11], the combined standard uncertainty, uc, can be directly estimated from the reproducibility between laboratories, sR [10]. The Nordtest model covers all steps in the analytical chain from the arrival of the test sample in the laboratory to the reporting of the analytical result, but sampling, sample transportation, equipment and possible gross errors during data storage/retrieval are not included [10].

2.2. Reporting uncertainty

For most purposes in food analysis, an expanded uncertainty should be used, which is obtained by multiplying the combined standard uncertainty, uc, by a coverage factor, k. The choice of the factor k is based on the level of confidence desired. For an approximate level of confidence of 95%, k is 2 [8, 9, 12]. The test result, y, and the expanded uncertainty, U, should be reported as $y \pm U$ and accompanied by a statement of confidence [8, 10, 12].

Not all the uncertainty sources identified during an uncertainty evaluation will make a significant contribution to the combined uncertainty. Whether the contribution of uncertainty will be neglected depends on the relative size of the largest and smallest contributions, its impact on overall uncertainty, user requirements or regulations. Failure to properly consider all sources of uncertainty leads to a lower assessment of uncertainty.

3. Conformity assessment

Conformity assessment is any activity undertaken to determine, directly or indirectly, whether a product, system, process, body or person meets relevant standards and fulfils specified requirements [13]. It is a common activity performed in testing, inspection and calibration, being defined to establish confidence for consumers and for the safety and quality of life [14]. Over time, in practice, many statements of conformity to a specification or standard have been confused with opinions and interpretations, although these are two completely different terms. Statements of conformity can serve as a basis in the processes of giving opinions and interpretations. In conformity assessment, a measurement result is used to decide if an item of interest conforms to a specified requirement [13]. To establish procedures in order to perform conformity assessment in practical situations, objective criteria are needed in what is called "decision rule" [2, 14]. The development of a probabilistic approach in measurement, introducing measurement uncertainty as a parameter to express the variability of measurement, had a significant impact in decision process [14]. Due to measurement uncertainty, there is always the risk of an incorrect decision, especially when the result is close to the specification limit [13]. Such incorrect decisions are of two types. First, with false acceptance, an item accepted as conforming could actually be non-conforming. The probability of such an incorrect decision is called consumer's risk, because the cost associated with such a mistake is often borne by a consumer [13]. Second, with false rejection, an item rejected as non-conforming could actually be conforming. The probability of such an incorrect decision is called producer's risk, because the cost associated with such a mistake is often borne by a producer who cannot sell an item that has failed a test of conformity [13, 14]. These errors are also known as Type I (α) and type II (β) errors, respectively, meaning that conforming products are incorrectly rejected or non-conforming products are incorrectly accepted [14]. General rules for calculation of conformance probability and risk of false decision are given in ISO/IEC Guide 98-4:2012(E) [13]. The evaluated probabilities depend on the measuring system and the production process. Where the decision rule is prescribed by the customer, regulations or normative documents, a further consideration of the level of risk is not necessary [2].

3.1. Guard bands

Tolerance limits, specified requirements for a measurand of interest, consist of limiting values, and separate intervals of permissible values of the measurand, tolerance intervals, from intervals of non-permissible values. Tolerance intervals can be either one-sided, with either a lower or an upper tolerance limit, or two-sided, with both lower and upper tolerance limits [13]. Most of this paper for simplicity deals with an upper tolerance limit. When the analytical result is close to the tolerance limit, acceptance

or rejection of an item can be an incorrect decision and lead to undesirable consequences. The use of guard bands can reduce the risks of incorrect accept/reject conformance decisions by defining an acceptance interval of permissible measured values of a measurand [13, 15]. They represent a safety factor built into the measurement decision process. The length of the Guard Band (w) is the Tolerance/specification Limit (*TL*) minus the Acceptance Limit (*AL*) or $w=TL - AL$ (Figure 1) [15].

Acceptance limits and corresponding decision rules are chosen in such a way as to manage the undesired consequences of incorrect decisions.

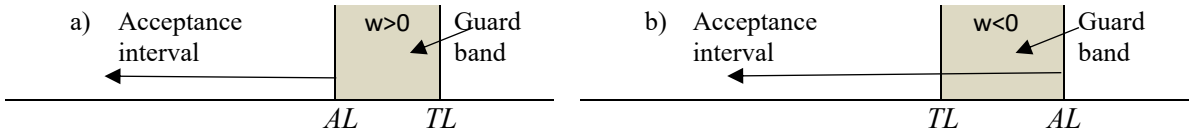

Figure 1. a) acceptance limit *AL* inside the tolerance interval *TL* and b) acceptance limit *AL* outside the tolerance interval *TL* according to ISO/IEC Guide 98-4:2012(E) [13].

The length parameter, w, is taken to be a multiple of the expanded uncertainty for a coverage factor k = 2, U = 2u. A common choice is r = 1, hence w = U. The probability of accepting a nonconforming item in that case is at most 2.3% (assuming a normal PDF for the measured quantity) [13]. There are cases where a multiplier other than 1 is more appropriate. Also, customers can define r [15].

It should be taken into account that the measurement could result in a decision on conformity (acceptance) using one guard band, or non-conformity (rejection) if a larger guard band is used. Therefore, compliance with the requirements is inherently related to the applied decision rule. Hence, the decision rule is expected to be agreed before measurements are made [2]. No single decision rule can address all statements of conformity across the diverse scope of testing and calibration.

3.2. Decision rules

An important and widely used decision rule is known as simple acceptance or shared risk. Under such a rule, where a guard band has a length equal to zero, w = 0, the producer and consumer (user) of the measurement result agree to accept as conforming (and reject otherwise) an item whose property has a measured value in the tolerance interval [13, 15]. When a measurement result is exactly on the tolerance limit (assuming a symmetric normal distribution of the measurements), the probability of being outside the tolerance limit could be as high as 50%. Hence, there would be a 50% chance of an incorrect decision [13, 15]. Either of these probabilities can be reduced by choosing acceptance limits that offset the tolerance limits. The risk of accepting a non-conforming item can be reduced by setting an acceptance limit *AL* inside the tolerance interval (guarded acceptance decision rule), and the risk of rejecting a conforming item can be reduced by setting an acceptance limit *AL* outside the tolerance interval (guarded rejection decision rule), as shown in Figure 1a and 1b, respectively. For a guarded acceptance decision rule, the guard band is w > 0, and for a guarded rejection decision rule, the guard band is w < 0. It is not possible to set the acceptance limits to minimize both the consumer's and producer's risks simultaneously. Decreasing one will increase the other [13]. Very importantly for the proper definition of a decision rule, the following question must be answered: What should be proved by the conformity assessment: compliance or non-compliance with a specification? Based on the answer, either the supplier's risk (α) or the consumer's risk (β) has to be specified [14]. Binary decision rules, acting to reduce the producer's risk (supplier's risk), will always increase the consumer's risk [15].

In conformity assessment, a binary decision rule exists when the result is limited to two choices (pass or fail), and a non-binary decision rule exists when multiple terms can express the result (pass, conditional pass, conditional fail, fail) [15].

3.2.1. Binary Statement for Simple Acceptance Rule (w=0). Reported as: Fail – the measured value is above the acceptance limit, $AL=TL$ and Pass – the measured value is below the acceptance limit, AL = TL [15].

3.2.2. Binary Statement with Guard Band. Reported as: Fail – the measurement result is above the acceptance limit, $AL = TL - w$ (rejection based on guard band); and Pass – the measurement result is below the acceptance limit, $AL = TL - w$ (acceptance based on guard band) [15].

3.2.3. Non-binary Statement with Guard Band. Reported as: Fail - the measured result is above the tolerance limit added to the guard band, $TL + w$; Pass - the measured result is below the acceptance limit, $AL = TL - w$; Conditional Fail - the measured result is above the tolerance limit but below the tolerance limit added to the guard band, in the interval $[TL, TL + w]$; Conditional Pass - the measured result is inside the guard band and below the tolerance limit, in the interval $[TL - w, TL]$ [15].

4. Conclusion

Awareness of the need to evaluate the measurement uncertainties is increasing in particular within the framework of accreditation. Evaluation of measurement uncertainty has still not matured equally well in all areas of testing, and many laboratories feel that uncertainty estimation is a laborious and intellectually challenging task to perform. Incorrect estimation the uncertainty associated with the result of conformity assessment measurement will lead to an incorrect accept/reject decision, so measurement uncertainty has to be considered and judged to be acceptable for the intended purpose. The need for statements of conformity with specifications has developed greatly, together with documents on the concept of decision rules used to make such statements. Decision rules need to be compatible with the customer, regulation or standard requirements. Where choices of decision rules are available, customers and laboratories will need to discuss levels of risk regarding the probability of false acceptance and false rejects associated with available decision rules. Customers are not sufficiently informed and involved in the choice of decision rules.

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Culinary preparation and processing of meat with wooden breast myopathy

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Culinary preparation and processing of meat with wooden breast myopathy

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Abstract: Recently, poultry meat production and consumption has become increased worldwide. Decades of intensive selection in poultry breeding resulted in fast-growing broilers, improved food conversion, low production costs, and high yield of breast meat, as the most valuable part of the carcass. Some side-effects of intensive production and rapid growth of broilers include the appearance of myopathies in breast muscle. Increasing attention has been paid to the defect known as "wooden breast" (WB) due its incidence and severity of anomaly. WB is characterized by the hardness and pale colour of the fillet. These changes lower the consumer acceptance of the meat, and a pronounced WB is unsuitable for culinary and industrial processing. Different procedures can be used to tenderize the meat and include physical and chemical procedures, often combined in industry. Physical procedures comprise the application of heating, mechanical force, ultrasound, electric stimulation, hydrodynamic shock wave-pressure technology, high pressure processing, and pulsed electric field. Chemical procedures include marinating, exposure to the endogenous enzymes, and the use of exoenzymes. In the future, it is necessary to develop optimal tenderizing techniques or combinations of different tenderizing techniques to achieve better sensory quality and improved nutritional value of WB.

1. Poultry meat production

Over the last decades, the production of food has managed to meet the needs of increasing world population. The five-year average meat production (2016-2020) in the world was 323.25 million tons mt) and poultry meat had the biggest share (37.99%). From 2000 to 2020, world poultry meat production has increased from 40 mt to 132 mt, with an annual increase from 3.5% to 4.7%. The average annual consumption of poultry meat is 15 kg per capita, being the most widely consumed type of meat in the world [1]. The reasons for the increasing poultry meat production should be sought in its exceptional nutritional value (low fat content and high protein content), the perception that it is healthier than other

meats, pleasant flavour, acceptance by most cultures and religions, quick and easy preparation, affordable price, and utilization of all carcass parts in industrial processing [2, 3]. One of the important features of poultry meat is its possibility to be used as a functional food by formulating diet for poultry nutrition. Thus, by adding selenium, especially organic, with or without vitamin E in poultry diet, higher content of Se and vitamin E can be achieved in meat. Moreover, use of feeds rich in polyunsaturated fatty acids (PUFA), n-3 fatty acids or conjugated linoleic acid (CLA), results in higher PUFA content, balanced ratio of n-6 to n-3 fatty acids and higher CLA content in poultry meat with beneficial health effects for humans [4, 5, 6].

2. Myopathies in poultry

Myopathies are defined as progressive and degenerating neuromuscular diseases that are characterized by hypotonia, muscle fibre damage and muscle atrophy [7]. So far, several causes of myopathies in poultry have been described, like genetics, nutrition, metabolic disorders, toxins, infectious agents, environmental factors and unknown aetiology [8]. In congenital myopathies, different genes have been identified as associated with the various phenotypic and histological changes in muscles. Causes for some poultry myopathies are still not known, and intensive work is being done on their identification [9].

Genetic selection of poultry has been carried out in order to shorten the fattening period, increase the growth rate, obtain higher final body weight and breast yield, and improve feed conversion. Better growth performance results have been accompanied by undesirable occurrence of myopathies in breast muscle [10, 11, 12]. To date, five myopathies associated with rapid growth of poultry (broilers and turkeys) are known and described: deep pectoral myopathy (DPM), pale, soft, exudative meat (PSE), white striping (WS), wooden breast (WB), and “spaghetti” meat (SM) [2]. WB myopathy, unlike the others mentioned, has not been found in turkey breast meat. Myopathies do not pose a safety risk to human health, since they are not biological, chemical or physical hazards. In effect, myopathies deteriorate meat quality (sensory, nutritional and functional properties) and reduce the economic value of meat. Economic losses can be remarkable and depend on the type of defect, the incidence of occurrence, as well as the intensity of its severity. Economic losses occur as a consequence of consumers’ refusal to buy meat with pronounced myopathy, and it must be used for the production of meat products [3, 13].

WB is the most common myopathy in broilers. It is manifested by the hardness of the pectoral muscle, especially in the cranial part. The muscle is pale, covered with viscous contents and petechial lesions [2, 13]. Other muscles of the carcass are unaffected. Histological analysis determines fibre atrophy, variations in fibre shape and size, multifocal degeneration and necrosis, loss of striation, mononuclear cell infiltration, interstitial inflammation, oedema, deposition of extracellular collagen, and thickening of the perimysial connective tissue [3]. The changes are very similar to the WS. These two defects often occur simultaneously [11, 14]. The defect is usually graded as mild (focally diffused and light firmness), moderate (focally diffused with extensive firmness), severe (more than 75% of breast is firm), and extremely severe (firm breast) [3]. From normal breast WB differs in colour (bright red, blurred), higher pH value and lower water holding content (WHC), higher drip loss, higher protein content in exudate, and higher MDA value and carbonyl content [15]. The aetiology of WB is still unknown, but it is mainly associated with the genetics, age, higher final weight, higher dressing percentage and breast weight, and nutrition [3, 12]. WB myopathy has been found in many countries around the world, and data on the incidence of this defect show it is becoming more common [15]. In Italy, WB was found in 53.2% of broiler carcasses [16], and in the USA in 50% of cases [17]. Tijare *et al*. [18] identified this defect in 90.1% of the samples. The WB defect is, according to the findings of Xing *et al*. [15], found in 30.8% of broilers in China. This defect remains pronounced even after heat treatment.

WB is the subject of intensive research in the world. Research refers to examination of morphometric parameters of breasts (weight, yield, length, width, height of different parts of breast), chemical composition (the content of moisture, protein, fat, ash, collagen, fatty acids, malondialdehyde, protein

carbonyls), functional properties (L, a, b values, pH, shear force, hardness, cooking loss, thawing loss, salt-induced water uptake, drip loss), sensory properties (appearance, colour, texture, odour, and taste), nutritional value and consumer acceptability. Important conditions for the appearance of this defect were examined, as well as the application of different meat processing procedures with WB [3].

3. Meat tenderization

Skeletal muscles consist of two fractions of proteins, myofibrils, involved in muscle contraction and sensitive to endogenous enzymes, and collagen, the main constituent of connective tissue responsible for fixed toughness [19]. The purpose of meat tenderization, both in culinary preparation and processing, is to disrupt the structure of myofibrils, sarcoplasmic proteins and collagen, and consequently to facilitate and improve the digestibility of nutrients. Meat tenderization procedures can be divided into premortal and *postmortem*. Premortal procedures were applied in cattle and largely abandoned. Premortal procedures included oral administration or injection of vitamin D that elevated calcium serum level and in turn activated *postmortem* tenderization process in meat [20]. *Postmortem* tenderizing procedures include physical and chemical methods, in practice often combined.

3.1. Physical procedures

Physical procedures include the application of thermal treatment, mechanical force, ultrasound, high-voltage electric stimulation, hydrodynamic shock wave-pressure technology, high pressure processing, and pulsed electric field [20, 21, 22].

One of the physical procedures most often used is heating at different temperatures, with or without the addition of water (cooking, in pressure cooking, microwave cooking, baking, stewing, frying, grilling). Heat treatment of meat contributes to its safety in terms of the absence of biological hazards, reduces the nutritional value, and contributes to the formation of odour and taste. During heating, the texture of the meat changes due to the denaturation of myofibrillar proteins and sarcoplasmic proteins. Hydrolysis of collagen induced by heating leads to meat tenderization, since collagen is degraded to smaller molecules of gelatin. Gelatin has good solubility and the ability to bind water. Meat tenderization sharply increases with exposing meat to temperatures higher than 70 °C [23]. Heat treatment of meat can be applied at lower temperature and for a longer time (low-temperature long-time method and sous vide cooking – processing in vacuum) [21, 24].

Mechanical methods of tenderization (hitting, pressure, cutting, stabbing, tumbling, massaging) disrupt the structure of meat, resulting in the extraction of soluble proteins, but on the other hand reduce cooking time, hardness and chewiness of meat [19]. Mechanical procedures for tenderizing meat also include hitting with a serrated blunt object (culinary processing) and passing through two rotating slitting blades, where the distance between the blades is adjusted to the thickness of the meat [23]. Mechanical processes most often precede marinating processes [25]. For tenderizing poultry meat with WB defect, Tasoniero *et al*. [26] recommended a “blade tenderization” procedure that involved cutting meat with a set of blades that can be used in the household and catering, but also in the meat industry. Blades disrupt the structure of muscle and connective tissue, and in combination with marinating and the application of enzymes, the penetration of the marinade and enzymes is enabled into deeper layers of meat, increasing the tenderness.

Ultrasound has been investigated as a method for meat tenderization [27] and, applied on carcasses, provokes lysosomal rupture and fragmentation of myofibrils and connective tissue [20]. Ultrasound applied to WB myopathy decreased the hardness of breast, confirmed by increased myofibrillar protein degradation [28]. High-voltage electric stimulation (ES) of pre-rigor carcasses has been applied for decades for meat tenderization [20]. ES increases the rate of *postmortem* glycolysis, leading to rapid pH decline and depletion of intracellular calcium, and thus preventing the cold-shortening during carcass chilling. Hydrodynamic shock wave-pressure technology is applied to packaged meat placed in water. Shock waves generated by explosion break down sarcomere proteins, resulting in higher tenderness [20]. The use of high hydrostatic pressure (high pressure processing) in the range of 350-600 MPa for a few minutes destructs the quaternary structure, and even the tertiary structure of proteins. High pressure

disrupts ionic and hydrogen bonds in proteins, induces denaturation of proteins and forms gel consistency. After high pressure treatment, the elasticity of meat increases and it becomes more tender [21, 29].

3.2. Chemical procedures

Chemical procedures include marinating, the activity of endogenous enzymes, and the use of exoenzymes. Marinades usually contain sodium chloride, organic acids (citric, acetic, tartaric acid), phosphates, citrus juices, then oil, vinegar, wine, spices, and sugar. Meat tenderization occurs as a consequence of various physicochemical mechanisms that increase protein hydration, leading to "swelling and weakening" of muscles and water binding. During marination, an increase in proteolysis caused by higher activity of cathepsins and an increase of collagen transformation to gelatin that both contribute to tenderization of meat are observed [21]. Better effects are achieved when marinade is applied by injection technology than by immersion of poultry meat in marinade [30]. Chicken breasts exposed to combined effects of marinade with ultrasound achieved larger myofibril fragmentation and higher tenderness than breasts immersed only in marinade [25].

Enzymatic meat tenderization involves exposing the meat to endoenzymes and exoenzymes. In *postmortem* muscles, endoenzymes have significant roles in meat tenderization during aging. Several endogenous proteolytic enzymes, including calpains, lysosomal proteases and cathepsins, are involved in degrading myofibrillar and cytoskeletal proteins. A large number of studies indicate the calpain system as responsible for *postmortem* proteolysis and tenderization of meat. The role of calpain enzyme system (m-calpain, μ-calpain, and calpain 3) and their calcium-activated inhibitor (calpastatin) in meat tenderization was revealed [22]. Calpains are cysteine proteases that degrade myofibrillar proteins (tropomyosin, roponin T, troponin I, C-protein, connectin, titin, vinculin, and desmin) [31]. Aging of meat is often applied to beef, and it can last up to 55 days in controlled conditions (temperature and humidity) [21].

Exoenzymes used for tenderizing meat are of plant, bacterial or fungal origin. To date, five exogenous enzymes have been "generally regarded as safe" (GRAS) for use in the meat industry [32]. Exogenous proteases extracted from plants are papain (papaya), bromelain (pineapple), and ficin (fig), from bacteria (*Bacillus subtilis*) are subtilisin and neutral protease, and from fungi (*Aspergillus oryzae*) is aspartic protease [19]. Exogenous enzymes require optimal temperature and pH value. Their activities towards the hydrolysis of myofibrillar proteins and collagen are different, so in practice, the simultaneous use of two or more exoenzymes is recommended. The effect of different exoenzymes on degradation of myofibrillar proteins and collagen along with optimal pH and temperature for their activity are presented in Table 1 [33].

Table 1. Optimal pH and temperature for exoenzymes and their effectiveness on muscle proteins

Protease	**Active pH range**	**Optimal pH**	**Active temperature range (^{0}C)**	**Optimal temperature (^{0}C)**	**Hydrolysis of myofibrillar proteins**	**Hydrolysis of collagen**
Papain	4.0–9.0	4.0–6.0	50–80	65–75	Excellent	Moderate
Bromelain	4.0–7.0	5.0–6.0	50–80	65–75	Moderate	Excellent
Ficin	5.0–9.0	7.0	45–75	60–70	Moderate	Excellent
Protease of *A. oryzae*	5.0–9.0	7.0	50–65	55–60	Moderate	Poor
Proteases of *B. subtilis*	2.5–7.0	<6.5	40–60	55–60	Poor	Excellent

Papain is proteolytic enzyme derived from aqueous extract of papaya. The higher activity of enzyme is obtained from immature papaya fruit. It is capable of breaking down large protein molecules to small

peptides and amino acids [31]. Papain cleaves lysine, phenylalanine and arginine peptide bonds, thus tenderizing meat [20]. Side-effects of papain use are "mushy" texture and off-flavours of meat [34]. Bromelain is obtained from fruits of pineapple family. Bromelain is effective in breaking down myofibrillar proteins and collagen, resulting in higher tenderness of meat. Bromelain and papain were used for tenderization of poultry meat (ducks and chickens) [35, 36]. Ficin is extracted from plant of genus *Ficus*. It is a proteolytic enzyme, a class of cysteine or sulfhydryl protease that enhances the solubility of muscle proteins. Although ficin is capable of degrading to some extent collagen and elastin, it is primarily effective in breaking down myofibrillar proteins [19, 31]. *Bacillus subtilis* contains subtilisin and neutral protease of high specific activity against collagen and elastin, but had no significant effect on myofibrillar proteins [31]. *Aspergillus oryzae* produces aspartic protease that preferentially affects myofibrillar proteins, with almost no effect on collagen [31].

Exoenzymes can also be used with other additives (acids, phosphates, salts) and mechanical processing procedures (tumbling, massaging, injection, blade tenderization) [19, 31]. Paddy oat fruit peel [37], kiwifruit [38] and ginger root [31] can also be used to tenderize meat. Actinidin derived from kiwifruit breaks down myofibrillar proteins and it is also involved in activation of m-calpain. Actinidin applied in high concentration moderately tenderize meat preventing the occurrence of mushy meat [31].

The efficiency of tenderizing procedures can be determined by various methods (sensory analysis, electrophoresis, electron microscopy, histological analysis, proteolysis index, pH value, WHC, peptide content, etc.) [39].

4. Conclusion

The WB defect is a novel condition to the poultry industry, and it is associated with huge economic losses. To alleviate negative consequences of WB occurrence, different strategies have been proposed. One of them is to include affected chicken breasts in various processed products. The other way to minimize adverse effects of genetic selection and improve sensory characteristics of WB is to apply different tenderizing techniques. Meat tenderization could allow the poultry industry to put on the market whole breast fillets. Therefore, further research is needed to develop an optimal tenderizing technique or combination of different tenderizing techniques to achieve better sensory quality and improved nutritional value of breasts with WB myopathy.

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Extraction procedure optimization of the method for detecting ethylene oxide and 2-chloroethanol in sesame seed

Extraction procedure optimization of the method for detecting ethylene oxide and 2-chloroethanol in sesame seed

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Abstract: This study describes modifications of the extraction procedure within the European Union Reference method for determination of ethylene oxide and 2-chloroethanol in sesame seed. The method suggests utilisation of uniform stainless steel balls in order to facilitate extraction in small seed samples. Experiment was conducted with combination of balls of different sizes and the extraction efficacy was assessed by measurement of 2-chloroethanol in sesame seed samples from the local market. Increased efficacy of 18.3% for ethylene oxide and 16.2% for 2-chloroethanolwas observed when the combination of two diameters of balls was used compared to samples extracted by the guidelines in original method and alternative approach with uniform balls of the lower diameter..

1. Introduction

Chemical contamination of food is an important aspect of food safety [1]. It is also a very complex field, consisting (among the other) of prevention and control of thousands of compounds from entering the food chain, whether those compounds are intended to be used in food manufacturing/processing, or abused, due to their proven beneficial properties in food, however at the high cost of their harmful effects on humans. Such is the case with ethylene oxide (EO), the compound with one of the highest production rates worldwide. It is a precursor or intermediate in production of many chemicals e.g. polyethylene glycols, PET, cosmetics additives, construction materials, etc.

However, due to the volatility and high reactivity of the EO, one of its uses is also in fumigation of objects, warehouses, sterilization of medical equipment and (before 1980s) food commodities, especially spices and herbs. Being a small molecule, EO can easily penetrate cellular membranes of bacteria, their spores and viruses thus reacting with DNA/RNA and complex molecules resulting in their inactivation.

Although fumigation can be classified as “niche” use of EO accounting for only 0.05% of the global production [2], having in mind its overall production, this is still a significant proportion of the chemical that is being used for this purposes. However, the same properties for which EO represents an excellent fumigant (interactions with genetic material of the microorganisms), are also responsible for its genotoxic, mutagenic and carcinogenic potential. European Chemicals Agency (ECHA) has classified EO in category 1B in respect to carcinogenic, mutagenic and genotoxic properties and category 3A in respect to acute toxicity [3].

After fumigation of food commodities, EO quickly produces several reaction products, of which 2-chloroethanol (2-CE) is the most significant, and legally relevant as well, since it is included in the residue definition scope in the European Regulation 868/2015 [4] (sum of ehylene oxide and 2-chloroethanol expressed as ethylene oxide).

Although EO is not an authorized substance for fumigation in the EU from 1991, it is still used in countries that are large producers of certain commodities such is sesame seed (e.g. India, China, Nigeria), in order to reduce contamination with Salmonella and other faecal bacteria. This was apparent in mid-2020 after numerous Rapid Alert System for Food and Feed (RASFF) notifications concerning EO residues in sesame seed from India. Number of notifications amounted to about 140 by November 2020 [2]. Certain quantities of contaminated sesame seed were imported to Serbia as well resulting in prompt withdrawal from the market.

European Union Reference Laboratory for Single Residue Methods (EURL-SRM) published a comprehensive analytical observations report [2] in December 2020 concerning EO and 2-CE, covering historical, toxicological and analytical aspects of this subject. Detailed analytical method based on QuEChERS method described in ISO EN 15662 [5] and quantitative determination by gas chromatography-tandem mass spectrometry (GC-MS/MS) is provided. One of the important steps in analysis of EO/2-CE, especially in the case of samples containing of small seeds (poppy, sesame) is extraction of as much residue as possible from the matrix consisting of very small (1-3 mm in diameter) units. In the case of more persistent chemicals, this could be accomplished by grinding and homogenization in laboratory mills with or without freezing. However, in the case of EO which is highly volatile this could result in significant analyte loss.

Therefore, EURL-SRM analytical method stipulates utilization of extraction aids (4-5 stainless steel balls, 9.5 mm in diameter) in order to facilitate maceration of small sesame seed and increase availability of EO and 2-CE and speed up the extraction process. Polypropylene tube (50 mL) is to be loaded with 2 g of sesame seed, 10 mL of acetonitrile with 5% of water and stainless steel balls. The tube is to be shaken for 15-30 minutes, centrifuged and an amount of the crude extract is to be subjected to further clean-up. However, during the extraction procedure, we noted that steel balls can be stuck in the tube not performing the seeds maceration efficiently, especially if the shaker speed is low.

The aim of this study is to assess modifications of the EURL-SRM method for determination of EO and 2-CE related to sample extraction and determine whether these modifications can contribute to extraction efficacy.

2. Materials and methods

Analytical standards of EO and 2-CE were purchased from Sigma-Aldrich (St. Louis, MO, USA), as well as HPLC purity acetonitrile and water. QuEChERS mixture for oily samples clean-up (150 mg C_{18}; 150 mg PSA; 900 mg $MgSO_4$) was obtained from Phenomenex (Torrance, Canada). Stainless steel balls (5 mm and 10 mm in diameter) were obtained from the local hardware store, thoroughly washed with detergent, rinsed and submerged in hexane and acetonitrile in ultrasonic bath for 20 minutes. Balls were dried and stored in sealed container until the analysis.

GC-MS/MS system consisted of Shimadzu (Kyoto, Japan) Nexis GC 2030 with split/splitless injector, AOC-20i plus auto sampler and AOC 20s plus auto-injector, coupled to Shimadzu GCMS-TQ 8050NX triple quadrupole mass spectrometer operating in EI mode.

Sesame seed was obtained from the local retail and was subjected to the analysis according to the original analytical method in order to determine absence of residues of EO and 2-CE. Then, 100 g of seeds was weighted in the polypropylene jar and spiked with solutions of EO and 2-CE in order to achieve concentration of 0,05 mg/kg of each compound. Although current MRL for sesame seed is lower (0,02 mg/kg for sum of EO and 2-CE), our intention is to get clear signals in order to interpret the results correctly. The jar was sealed and shaken on the overhead shaker for 1 hour. Tightly sealed jar was left on room temperature for 2 days before the analysis. Six samples were taken and analysed again in order to determine homogeneity of the residues. Established values were satisfactory (cv less than 5%) and this sample was used for further experiments.

Detailed description of the analytical method for GC-MS/MS determination of EO and 2-CE in sesame seed has been presented in the EURL-SRM document [2]. Briefly, 2 g of sesame seed is weighted with accuracy of ± 0.02 g in polypropylene tube, 10 mL of 5% water in acetonitrile was added

as well as stainless steel balls. The tubes were closed and shaken on the Neuation Technologies (Gujarat, India) shaker at 40 revolutions per minute for 25 minutes.

The modifications of the extraction procedure were conducted as follows:

Instead of extracting the sesame seed with 5 stainless steel balls 10 mm in diameter, three batches each consisting of 6 samples of the same sesame seeds were extracted with various size balls. Batch 1 was control batch and was extracted according to the method (four 10 mm balls). Batch 2 used ten 5 mm balls and Bach 3 used mixture of three 10 mm balls and three 5 mm balls. One millimetre balls were available as well, however, initial experiments confirmed that their weight if applied alone in any number is not sufficient to macerate sesame seeds entirely leaving intact seeds in the tube. Therefore, the smallest balls were excluded from the experiment.

After the extraction, samples were centrifuged at 3000g for 5 minutes and 6 mL of the extract was transferred to the 15 mL polypropylene tube with QuEChERS mixture for oily samples clean-up (C_{18}/PSA/$MgSO_4$). The prescribed mass fraction of constituents of the mixture is 25/25/150 mg per mL of extract, therefore, for 6 mL the 150/150/900 mg of each clean-up agent was used. The tubes were manually vigorously shaken for one minute, centrifuged for 5 minutes at 3000g and 1 mL aliquot was transferred into the GC vial.

GC column was Shimadzu SH-Rxi-5MS (30m x 0.25 mm id x 0.25 mm df). Injector temperature was 260°C, splitless injection was performed. Oven program was as follows: start at 50°C, hold 2 min, ramp to 150°C at 40°C/min, ramp to 280°C at 12°C/min, hold till 20 min.

Mass spectrometer was operating in EI mode, source temperature was 270°C, transfer-line temperature was 250°C.

Transitions for the EO were 44>14 (CE 20), 44>28 (CE 5) and 44>29 (CE 5) while for 2-CE transitions were 80>31 (CE 5), 80>43 (CE 5) and 82>31 (CE 5).

Calibration was not performed in these experiments since the aim of the study was to assess extraction efficacy only and for that purpose, quantification was not relevant. Therefore, obtained peaks were integrated and resulting peak areas were compared. One sesame sample was analysed before the analysis of three batches according to the original method (five 10 mm balls) in order to establish initial peak areas for integrated EO and 2-CE peaks. These areas were assigned as number 1 and used for comparison to the obtained results.

3. Results and discussion

Table 1 shows peak areas of EO and 2-CE standardized to areas obtained from the initial sample prepared according to the original method (five 10 mm stainless steel balls as an extracting aid). Since batch 1 consists of six samples of sesame seed extracted in the same manner as initial sample, the results for this batch are expected (92-106% for EO and 94-110% for 2-CE) and can account for the variability of the analytical method. However, batch 2 (ten 5 mm balls) shows significant decrease (76-87% for EO and 76-96% for 2-CE) of the original intensity and batch 3 (combination of three 10 mm balls and 3 5 mm balls) shows significant increase of extraction efficacy (109-125% for EO, in average 18.3% increase and 109-122% for 2-CE, in average 16.2% increase).

Table 1. Standardized peak areas in three batches of sesame seed

	Batch 1 (EO)	Batch 2 (EO)	Batch 3 (EO)	Batch 1 (2-CE)	Batch 2 (2-CE)	Batch 3 (2-CE)
Sample 1	1.06	0.87	1.13	0.99	0.88	1.22
Sample 2	0.96	0.78	1.21	0.94	0.93	1.17
Sample 3	0.92	0.82	1.19	1.1	0.96	1.15
Sample 4	0.96	0.81	1.09	0.97	0.89	1.19
Sample 5	1.05	0.76	1.23	1.06	0.76	1.07
Sample 6	1.03	0.86	1.25	1.08	0.82	1.17

These findings can be explained by the variations in mechanics of stainless steel balls depending of their size and weight. Diameter of 50 mL polypropylene tube is 29 mm and although 4-5 balls (10 mm in diameter) fit into the tube, they can stuck at slower shaker speeds, reducing crushing and maceration of sesame seeds. From the positive side, large diameter balls provide sufficient weight to perform the maceration very well. Combination of three heavy 10 mm balls and three lighter 5 mm ones reduce the possibility of getting stuck, provide adequate weight and therefore crushing of seeds and at the same time the mixing of larger and smaller balls during shaking process is continuous and more efficient. This results in increased efficacy of the extraction process.

4. Conclusion

Presented modification of analytical method for determination of EO and 2-CE in sesame seed in respect to extraction mechanism, increases transfer of these compounds from matrix to the solvent and therefore increase efficacy of the whole process. Further investigation can be performed in order to investigate this modification on other matrices that require utilization of mechanical extraction aids.

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Polish meat products - tradition and modernity

Polish meat products - tradition and modernity

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Abstract. The production and promotion of high-quality traditional food are playing an important role in the European Union. One of the main indications of the production quality is the awarding of signs confirming the quality of the traditional products. Meat and meat products play an important role in preserving the tradition. They are produced according to traditional recipes, passed down from generation to generation, and their preparation uses products typical of the region, from local crops and breeding. Thanks to this, they gain specific taste values, unheard of in other parts of the country.

1. Introduction

Consumers in Poland are increasingly looking for natural products that are minimally processed. However, modern processing methods are most often used to ensure maximum shelf life and food safety. In recent years, the interest in traditional food has increased significantly. This may be due to the fact that the high quality of traditional products is the result of the use of natural raw materials and additives, relatively short, uncomplicated production methods and immediate distribution. The growing interest in traditional food means that increasing numbers of consumers choose traditional products, assuming that 'traditional' is synonymous with 'high-quality'. An important place in Polish regional culture is held by meat and meat products, which for most people have always been something special. Polish cold cuts, sausages, hams, and smoked meats provide an inexhaustible range of highly valued flavors and aromas. Depending on the culinary regions, these products are prepared in different ways. Using various culinary techniques, the method of serving makes each product really special. It all adds up to the uniqueness of the culinary traditions of a given region.

2. Traditional and regional products in Poland

Food referred to as traditional covers different categories of food products that are characterized by specific characteristics of the raw materials, processing methods and place of origin. However, according to the definition adopted by the European Commission, this term refers to food that is on the market for a period indicating its transmission from generation to generation. This period should correspond to the time usually assigned to one generation, at least 25 years [1,2]. In order for a given product to be described as such, it should have specific features that distinguish it from other similar products within the same category in terms of 'traditional ingredients' from which it was made, 'traditional taste' and 'traditional production or processing methods' [1,3].

Figure 1. Graphic symbols of traditional broadcast products [4].

The changes that have taken place in the public awareness over the years have also caused significant changes in the lifestyle and attitudes of modern consumers. Currently, easy access to food is no longer enough, but the quality, variety and attractiveness of the food products on offer are starting to play an increasingly important role [5,6]. The choice of certain foods is also influenced by cultural heritage. Despite the widespread access to food from various exotic parts of the world, or resulting from the use of advanced technology in its production, traditional food and patterns of its consumption are still used to meet various needs, including mental and social needs [7,8]. The growing interest in traditional food is also related to the desire to preserve and display the values of cultural heritage. In Poland this food is perceived by consumers as extremely tasty and high quality [1,7,9].

An important place in Polish regional cuisine is held by meat, which has always been something special for most people. Meat products, especially highly processed and consumed in excess, can have an adverse effect on human health and well-being. However, it is a very important part of the diet, so meat should not be eliminated from it. Therefore, traditional food, the production of which is based on simple ingredients and natural technology, is an ideal solution. Polish cuisine is divided into several culinary regions. In many of them, similar raw materials are used for the production of food, but the specificity of the regional cuisine lies in the unique method of combining raw materials, the use of specific culinary techniques, and the method of serving or naming. It all adds up to the uniqueness of the culinary traditions of a given region.

2.1. Kindziuk or skilandis - Traditional specialty from eastern Poland

Kindziuk is a very tasty and durable product from Podlasie in the eastern part of Poland (Figure 2), and thanks to the addition of salt, it can hang in a cool place, even in hot weather. This meat is an example of how to manage keeping the product fresh without the preservatives and chemicals that are used nowadays. The history of its creation is related to the need to provide food for the harvest season in a time when refrigerators were not known. Meat and cold cuts were prepared in winter, so that they were ready for the time of intensive work in the field (spring and summer). The meat of the ham, sirloin and shoulder blades was cut into 1-3 cm pieces, salted and seasoned abundantly with garlic and pepper, then firmly stuffed into a cleaned pork stomach and sewn up. They were hung for a short time in a warm place by the stove to be dried, and then taken to the attic, where, in an airy place, the kindziuk dried. Sometimes it was smoked. Kindziuk cut into smaller pieces, served with home-made bread and

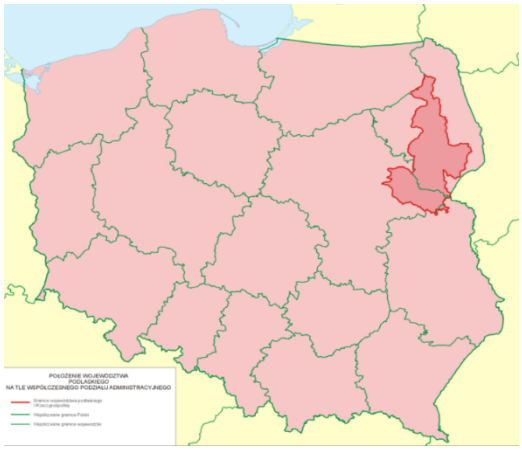

Figure 2. Part of Poland where kindziuk is known as a traditional dish.

butter is considered a delicacy. In summer, kindziuk can be used to cook very tasty borscht and sour soups with a specific aroma [10].

The modern method of making kindziuk resembles the traditional one, except that today it is more often made in a pork bladder than in the stomach. Thanks to this, the products have smaller size. Also nowadays, more spices are added to cure the meat (e.g. allspice, coriander, mustard) [11].

2.2. Biłgorajska sausage

Figure 3. Part of Poland where Biłgorajska sausage is known as a traditional dish.

Biłgorajska sausage is produced in family farms using local, traditional recipes passed down from generation to generation. From the 60-70s the recipe, composition of raw materials, production and smoking methods have never been changed [12].

Biłgorajska sausage consists mainly of crushed meat and fat as raw materials. The natural casing sticks tightly to the stuffing, is brown to dark brown and evenly wrinkled. Jelly and looser binding of meat raw materials is allowed. It is a crispy and brittle sausage. It has the taste and smell characteristic of pork, and smoked and roasted sausages. The aroma of smoking is strongly felt in a traditional smokehouse with the use of alder wood and spices, especially garlic. Biłgorajska sausage is based on raw materials obtained exclusively in the region (Figure 3), from the ecologically clean areas of Roztocze. These are: pork livestock, garlic and alder wood for smoking [13]. Throughout the production process, the most important factor influencing the quality of the final product is the quality of the meat and spices. The raw materials prepared after cutting are mixed, and cooked pork skins are also added. In winter and spring, about 10% more garlic is given due to the loss of aroma during storage. Alder wood is also an important factor, which gives a specific flavor and aroma bouquet. Thermal treatment consists of smoking with hot smoke in a special smokehouse made of burnt bricks fired with alder wood. The smoking process takes about 4-6 hours, then the sausage is air cooled [14].

2.3. Salceson polski from the Karkonosze Region

Figure 4. Part of Poland where salceson polski is known as a traditional dish.

This product entered the list of traditional meat products in 2019 and it comes from the Dolny Śląsk part of Poland (Figure 4). To prepare salceson, pork stomachs or beef bladders are filled with a stuffing made of pork head meat, tongues, hearts, and meat made of pork knuckle with jelly. The spices used to make it are mainly salt, cumin and black pepper. The meats are cured in brine with bay leaf, allspice, garlic and pepper. Then, the stomachs are tied at the ends with yarn, boiled, placed on nets, and pressed with a heavy object in order to obtain the characteristic kidney-shape. Salceson contains clear, amber-colored jelly. The cross-section shows pieces of meat, masks, heads and offal. In consistency, the product is compact, resilient and slightly greasy. In taste, there is noticeable garlic, marjoram, pepper and cumin and its aroma is typical of cured meat and offal [15].

3. Conclusions

The production, protection and promotion of high-quality food plays an increasingly important role in the European Union countries. One of the basic methods of implementing the quality policy in the Community is the implementation of the Strategy for the Identification and Promotion of Traditional Products. The benefit of promoting regional products is to show consumers and producers how rich various regions of Poland are in terms of traditional food production and cultural heritage.

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Development and validation of modified QuEChERS methods for the analysis of fipronil and its metabolites in chicken meat

Development and validation of modified QuEChERS methods for the analysis of fipronil and its metabolites in chicken meat

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Abstract. A sensitive method for the precisely and accurate determining the presents of fipronil and its metabolites in chicken meat was developed and validated using a modified quick, easy, cheap, effective, rugged and safe approach coupled with gas chromatography-mass spectrometry analysis. The solvent acetonitrile was used for the extraction of the samples with the salt phases composed of sodium chloride and magnesium sulphate, and then in the second phase used C18 and anhydrous magnesium sulphate. The linearity of the analytical response across the studied range of concentrations (0.005-0.050 mg kg^{-1}) was excellent, obtaining correlation coefficients higher than 0.99. The average recoveries of the pesticide ranged from 75 to 106% for fortification levels of 0.005, 0.01 and 0.05 mg kg^{-1}. The precision values associated with the analytical method, expressed as RSD values, were less than 11.15%. Matrix-matched solutions were also prepared by serially diluting the intermediate solution with blank chicken meat sample extracts containing none of the tested analytes to perform matrix-matched calibration with the same concentrations as in the solvent. The validated method was used to analyse the target compounds in 30 real samples imported from European countries. The present of fipronil-desulfinyl metabolite was confirmed in four samples.

1.Introduction

Pesticides are constantly used in agricultural and livestock production [1]. But pesticide residues can remain in the environment and pollute surface water, fruits and vegetables. When pesticides are used irresponsibly, disrespecting the waiting period between applications and maximum dosage levels, dangerously high residues of these products can be found in the foods produced from animals [2]. These compounds have massive potential dangers to human and environment health. The contamination routes can lead to bioaccumulation of persistent pesticides in food products of animal source such as meat, fat, fish, eggs and milk. The analysis of pesticide residues in food is very important due to the risks that these compounds offer to human health, besides their persistence in the environment and their ability to bioaccumulate [3]. Different pesticides can be imported into the food chain by usage of veterinary drugs and retained in the meat and meat products.

The situation that broke in Europe in July 2017, when millions of eggs were withdrawn from the shelves of stores across Western Europe due to high levels of the pesticide fipronil, has proved the need for improving and validating methods for accurate and precise analysis of fipronil in all matrices. Fipronil is a broad-spectrum insecticide that belongs to the phenyl pyrazole chemical family [4]. According to the IUPAC nomenclature, its name is

(±)-5-amino-1-(2,6-dichloro-α,α,α,-trifluoro-p-tolyl)-4-trifluoromethylsulfinylpyrazole-3-carbonitrile. Fipronil was first registered for use by the United States Environmental Protection Agency (U.S. EPA) in May 1996. It is used as an active substance in veterinary products against fleas and ticks in dogs and cats. This formula is used in granular, gel and spray formulation for kills unwanted arthropods in horticultural, and also for kills ants and roaches. The two most represented metabolites of fipronil are fipronil sulfone and fipronil desulfinyl, of which fipronil sulfone is the most

widely used. A range of methods and techniques used to determine the levels of fipronil and these two metabolites has been addressed in a number of studies [5]. Also, a large number of studies examined the amount of fipronil in sediment and soil [3, 6], because they are the result of the integration of and chemical that take place in an aquatic ecosystem, affecting all processes in the whole system.

A number of analytical methods for extraction and analysis of multiple pesticide residues have been developed in the middle of the last century and have greatly contributed to agriculture, control and health care. During the 2000s, new advanced technologies incorporated the QuEChERS method that reduces the use of expensive and carcinogenic solvents in large quantities [7]. Different sorbents have different affinities for pesticide residues; for example, magnesium sulphate ($MgSO_4$) is used to reduce the water phase and promote partitioning of pesticides into an organic layer, while sodium chloride (NaCl) is used to dissolve fat globules. Primary secondary amine (PSA) is compound which can be used to remove substances such as fatty acids, organic acids and cholesterol. PSAs are especially useful for removing matrix co-extractants, which can interfere with pesticide determination. QuEChERS methods are increasingly being used because of the simple and quick preparation for a variety of matrices, such as soil, sediment, water [8] and various food matrices, among others, honey [9] and eggs [10]. Recently, the QuEChERS multi-residue procedure has replaced many complex and time-consuming analytical steps in traditional approaches and is being commonly used for the analysis of pesticides in foods. In many cases the main problem during analysis of pesticide residue is the purification process, which is required to isolate the residues from matrix components and reduce matrix effects and it is also essential to enable a long column life [11].

This study describes the use of a quick, easy, cheap, effective, rugged, and safe (QuEChERS) method for extraction and cleanup of fipronil and its metabolites of interest in chicken meat. Current developments involve the use of extraction methods based on modifications to the QuEChERS procedure. The aim of this study was to apply and validate methods for extraction and analysis of pesticide residues in chicken meat. The QuEChERS method was modified in the clean-up step by applying dispersive SPE using only the C18 and magnesium sulphate phase, which was recommended for the determination of drug residues in meat, versus standard dispersive SPEs that also contain PSA. The validation was performed by matrix-matched calibration, to compensate for the matrix effect. The quantification and identification of the pesticides were performed by gas chromatography combined with mass spectrometry (GC-MS). The prepared samples were analysed with GC-MS in the selected ion monitoring mode (SIM) using one target and three qualitative ions for each analyte. The method was validated using quality control material for fipronil in products of animal origin produced by Fapas (UK) in 2019.

2.Materials and methods

Quantification and quantification of analytes was carried out using a GC Clarus 680 PerkinElmer system comprising an autosampler and a gas chromatograph interfaced with an MS Clarus SQ8T instrument under the following conditions: capillary column Elite-5MS (30 x 0.25 mm ID x 0.25 μm df, composed of 95% dimethylpolysiloxane and 5% phenyl), operating in the electron impact mode at 70 eV. Helium (of the highest purity) was used as the carrier gas at the constant pressure of 21.5 psi and an injection volume of 2 μL was employed (a split ratio of 50:1) at the injector temperature of 250 ºC; the temperature of the ion-source was 280 ºC. Mass spectra were taken at 70 eV; the scan interval was 0.2 seconds and fragments were from 50 to 400 Daltons. The initial oven temperature of 70 °C was held for 3 minutes and then increased to 150 °C at 25 °C/min, then increased to 200 °C at 3 °C/min, and further increased to 280 °C at 8 °C/min and held for 10 min. Total GC running time was 41.87 min. The software was adapted to handle mass spectra and the chromatograms were read using Turbo Mass Ver 6.1.0.

Stock standard solution (10 mg L^{-1}) was supplied by DR Ehrenstorfer (LGC, Germany). A stock standard solution was prepared in acetonitrile and stored at -18 °C. The individual stock standard solutions were prepared in ethyl acetate. This solution was used as spike solution for recovery

experiments in the three-concentration level (0.005, 0.01 and 0.05 mg kg^{-1}) and also to prepare the analytical curves solution for linearity studies in solvent and in the matrix. All high purity solvents were obtained from PanReac AppliChem (ITW Reagents, USA). Extraction kits, apropos QuEChERS extraction agents, were manufactured by Phenomenex (USA). In order to investigate the matrix effect and to reduce matrix induced effects during GC, validation was performed by calibration in the matrix, so that the sample and calibration solution had the same concentration of co-extracted matrix components. Matrix-matched solutions were also prepared by serially diluting the intermediate solution with blank chicken meat sample extracts containing none of the tested analytes to perform matrix-matched calibration with the same concentrations as in the solvent. The use of matrix matched calibration solution is necessary to minimize errors associated with matrix induced enhancement or suppression effects during GC-determination. To each aliquot of the blank extract were added corresponding known amounts of pesticide standards mixture (five levels) and known and the same concentration of internal standard (ISTD). Triphenyl phosphate (TPP) was used as an internal standard.

Each sample was homogenized and ground using the laboratory blender and a representative amount of each homogenate (10.0 g) was then placed into a 50 mL polyethylene tube. Homogenates were extracted and cleaned up immediately after sampling using the QuEChERS method. Ten millilitres of acetonitrile were added into each tube. The samples were well shaken using a vortex mixer at maximum speed. Afterward, 4.0 g of anhydrous magnesium sulphate and 1.0 g of sodium chloride were added, then extracted by shaking vigorously on a vortex for 2 min and centrifuged for 5 min at 4000 rpm. An aliquot of 6 mL was transferred from the supernatant to a new clean 15 mL centrifuge tube containing with C18 sorbent, and anhydrous $MgSO_4$ in a simple approach termed dispersive solid-phase extraction (dispersive-SPE) clean-up. The samples were again vortexed for 3 min and then centrifuged for 5 min at 4000 rpm. Final extracts of acetonitrile were concentrated using a gentle stream of nitrogen and reconstituted into ethyl acetate. To achieve the best possible extraction results, extracts were re-purified using the C18 column (100 mg/3 ml) before the analysis. The eluents were collected and were determined by precise GC/MS analysis using the selected ion monitoring mode with ion ratios for confirmation. After all determination parameters have been successfully set, the method was validated according to linearity, recovery, and precision.

3.Results and discussion

Trace analysis of organic contaminants, such as pesticides in food samples, typically consist of following consecutive steps: homogenization of samples, isolation of analytes from the sample matrix, removal of bulk co-extracts from crude extract, identification and quantification of target analytes and examination to ensure there have been no false positive results. Animal-derived food matrices are so chemically complex that sample preparation is extremely important for trace analysis, during which avoiding the interference caused by co-extraction of non-target substances is the biggest challenge and difficulty. Meat products contain a high content of lipids, i.e., more precisely myristic, palmitic and oleic acid, which can negatively affect the recovery of pesticides and damage the column and the baseline response. Modifications to the original QuEChERS method have been made depending on the characteristics of the samples, and in this case, because of the presence of lipids, so we opted for C18 purification without adding water, because we determined satisfactory recovery by optimization. For the method described here, the first phase without addition of water was found to be the most suitable.

Extraction is a highly significant part of sample preparation because it affects the efficiency of the purification process as well as the productivity of the clean-up step [10]. Therefore, due to the presence of proteins and fats, that is saturated and unsaturated fatty acids, additional purification was used using the C18 cartridge. This purification process has led to a higher sensitivity, less impact on the matrix effect, and a lesser presence and effect of fatty acids, esters and cholesterol during chromatogram recording in full scan mode [11]. Although a C18 column cartridge improved recovery of water-soluble pesticides, it was actually used in the last preparation step of the QuEChERS method

in order to protect the GC column and MS detector. The QuEChERS method proved has been demonstrated to be an effective and versatile method of choice for extraction and has also been recently applied for the analysis of various xenobiotics and veterinary drugs in animal products [12].

The retention times of fipronil desulfinyl, fipronil and fipronil sulfone were 16.80, 21.25 and 24.45 min, respectively. The linearity of the analytical response across the studied range of concentrations (0.005-0.050 mg kg^{-1}) was excellent, obtaining correlation coefficients higher than 0.99 (Table 1).

Table 1. The linearity of the pesticide and residues at concentrations 0.005-0.05 mg kg^{-1}

Analyte	Regression equation	R^2
Fipronil desulfinyl	y=0.227182x + 3.86920	0.999147
Fipronil	y=0.281971x – 10.2428	0.997444
Fipronil sulfone	y=0.245241x – 9.58740	0.999139

The validation parameters are based on the Document SANTE/12682/2019 [13]. The average recoveries of the residues ranged from 75 to 106%, for fortification levels of 0.005, 0.01 and 0.05 mg kg-1 (Table 2). The precision values, expressed as RSD values, were less than 20% (Table 2). The LOQ is defined as the lowest validated spiked level (mean value for n=6) meeting the method performance acceptability criteria (mean recovery was in the range 70-120 %, with RSD ≤ 20%).

Table 2. Recoveries (%, n=5), RSD and LOQ of the pesticide and residues in chicken meat matrixes

Analyte	Spiked (mg kg-1)	Recovery (%)	RSD (%)	LOQ (mg kg-1)
Fipronil desulfinyl	0.005	85.63	11.15	0.003
	0.010	78.45	7.15	
	0.050	86.36	3.45	
Fipronil	0.005	75.06	7.12	0.003
	0.010	94.56	6.13	
	0.050	91.12	2.13	
Fipronil sulfone	0.005	103.52	6.94	0.003
	0.010	105.14	5.41	
	0.050	106.09	3.94	

Fipronil sulfone is the main metabolite of fipronil, deriving from the oxidation of the sulphinyl moiety. This metabolite showed an efficacy similar to fipronil against insect gamma-aminobutyric acid (GABA) receptors [14]. Practically, no clean-up method completely removes all the matrix components from a crude extract. Clean-up methods, where EMR-Lipid d-SPE extraction kits were used, gave the best reduction of co-extracted matrix compounds [15]. Figure 1 shows a chromatogram

in full scan where the presence of niacinamide, one of two forms of vitamin B3, can be seen at a retention time of 7.14 min. Niacinamide is found in many foods including yeast, meat, fish, milk, eggs, green vegetables, beans and cereal grains. The following fatty acids are also present in the extract: 9-octadecenoic acid (Z) - methyl ester (oleic acid); 9,12-octadecadienoic acid (Z, Z) (linoleic acid) and 5,8,11,14-eicosatetraenoic acid, methyl ester (arachidonic acid methyl ester) at the retention time 26.60 min, 31.12 min and 32.72 min, respectively.

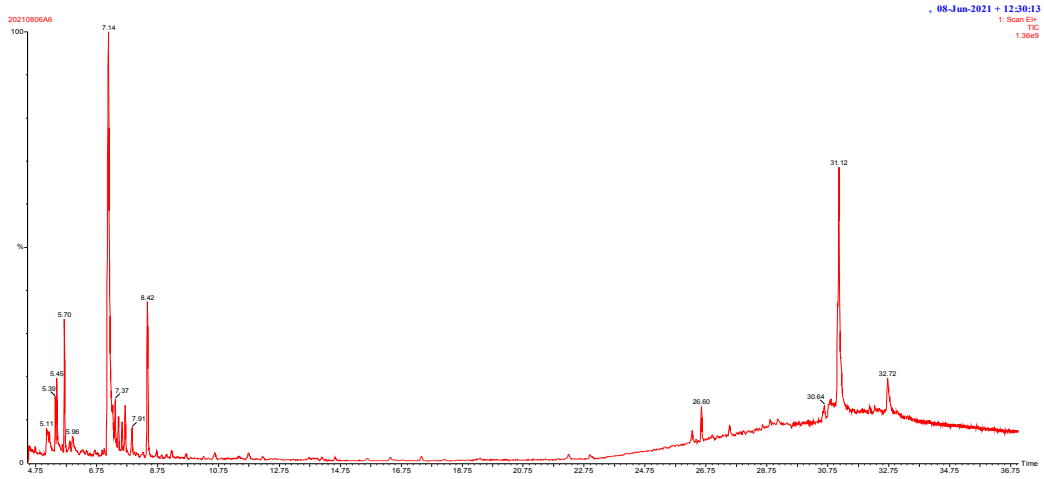

Figure 1. GC-MS chromatogram of chicken meat sample in the full scan

Development of certain methods for the detection of pesticides in food is fundamental for guaranteeing the nutritional quality and safety of food and the consumer health. In research, the results show the presence of fipronil in meat samples had a adverse effect on nutritional quality, causing a decrease in the amount of total and essential amino acids [16]. Using the QuEChERS method for the determination of fipronil and other pesticides, such as carbaryl, fenpyroximate, thiamethoxam, boscalid and difenoconazole, results were obtained that were in real samples at low concentrations and below acceptable limits [17]. During the extraction, water can also be used as an extraction agent, whereby no satisfactory recovery was obtained for the determination of fipronil in melon, while a satisfactory recovery was obtained for the determination of milk [18]. On the other hand, successful validation of fipronil by the QuEChERS method was achieved in beef meat using liquid chromatography coupled to mass spectrometry detection (LC-MS), while in the examined real samples, the amounts of pesticides were the result of LOQ [19]. Animal products are matrices characterized by high water, protein and lipid concentration that require the use of complex methods for cleanup and extraction. In published papers on the presence of pesticides in meat, quantified amounts in meat were determined for the pesticides carbaryl and chlorpyriphos ethyl [20].

The maximum residue limit (MRL) set for fipronil in eggs and chicken meat is 0.005 mg kg^{-1} ((EC) No 396/2005). This MRL value has been set for the sum of fipronil and its sulfone metabolite (expressed as fipronil). Fipronil used to prepare the formulations can contain fipronil sulfone at the time of formulation. In other words, it is possible that useful grades of fipronil have some amount of fipronil sulfone already present as a by-product of its preparation. Some grades of fipronil can have low levels of fipronil sulfone, even 0%. However, once fipronil has contacted oxidation agents, it is believed that fipronil sulfone could continue to form in solution. Further, it is significantly more costly to use fipronil that is completely free of fipronil sulfone. Using this validated method, the accuracy of the method was confirmed using the reference meat material from Fapas (13.9 ± 3.5 mg kg^{-1}).

In the tested real samples of chicken meat, the amount of fipronil and its metabolites was below the LOQ. The metabolite fipronil-desulfinyl was detected in four of the samples of chicken meat. The results of the analysis of fipronil-desulfinyl in the tested samples ranged from 0.004 to 0.006 mg kg^{-1}.

4.Conclusion

Successfully developed new method of analysis proved to be able to meet the requirements of modern methods in the field of quantification of fipronil in food of animal origin, i.e. meat. The validation data of chicken meat sample proved the compliance of the new analysis method with the legal requirements of the quality control criteria according to the SANTE 12682/2019 document. This confirmed that the results achieved by means of the newly developed analysis method are in accordance with the currently available sample preparation methods. Consequently, the presented analysis method is suitable for application for other matrices in routine analysis. Successful application of this method is relevant to monitoring the impact of fipronil contamination in the environment, primarily through monitoring the presence of fipronil in food, which will provide better insights into the present situation of the environment. Consequently, the successful investigation of the quality test materials proved the applicability of the presented analysis method as a confirmation method in routine analysis, quantifying the same amounts as were declared. Therefore, it is expected that new modifications to the QuEChERS methodology will continue to be developed to expedite the analysis of more veterinary drugs and other xenobiotics in animal products, and the methodology could become a basic choice for monitoring most contaminants in the future.

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The dry aged beef paradox: Why dry aging is sometimes not better than wet aging

The dry aged beef paradox: Why dry aging is sometimes not better than wet aging

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Abstract. An increasing consumer demand for a higher quality and eating experience has led to a revisit to the dry aging process. Therefore, research also focuses on the effects of different dry aging methods and aims to improve the dry aging process. However, an optimal process cannot be defined and, unfortunately, most of the dry aging results only hold true for the individual experiment. If one repeats a dry aging process in a different facility, the result might differ. The paradox is that the same dry aging process in two different ripening chambers does not inevitably contribute to equal tenderness and flavor. Since this paradox has still not been understood well, this review presents results of the most relevant dry aging studies by illustrating different process parameters and cuts. Some conclusions which may be useful to explain the paradox are derived from the literature in order to understand the crucial factors and commonalities in the dry aging process.

1. Introduction

Postmortem aging of beef is a necessary and common process to improve eating quality attributes (e.g. tenderness and flavor). Before the development of vacuum/sealing techniques in the 1970s, dry aging for storing unpackaged carcasses, primals or subprimals in cold environments (mainly in a cooler) was the most typical fresh preserving and yet value-adding process. The process known as wet or vacuum aging became increasingly widespread with the development of vacuum packaging [1].

1.1 The Paradox

Acquiring a clear picture of the effect of different processing factors on the dry aging result seems to be difficult, in particular in comparison to wet aging. Exemplarily, Oh et al. [2] described a positive effect of dry aging, whereas Oh et al. [3], using a very similar aging process (28 days aging at 0 – 3 °C, about 75 – 85 % relative humidity and 0 – 7 m/s air velocity), described no advantages of the dry aging in comparison to the wet aging process. The dry aged beef paradox is as follows: Why is dry aging sometimes no better than wet aging. This paradox has been said to depend on the multiple factors, exemplarily the aging process itself, but also the feed, the microflora, the fat oxidation and metabolite changes etc. Until now, and to the best of our knowledge, there is no comparison of the available dry

and wet aging literature with the aim to clarify the different outcomes. Our approach in this review is to compare just one factor (e.g. pH, water loss, breed) at a time and evaluate if this factor and the outcome in the respective studies can explain the paradox. Based on this comparison, we try to overcome the puzzling issue that tenderness and flavor is not always created with a specific dry aging process.

Additionally, progress in understanding this paradox is based on more literature and more indirect analytical measurements, such as the concentration of amino acids and the consideration of the microbiological flora. In that context, the concentration and type of amino acids and microorganisms can give insights into why tenderness and flavor differ. We give an overview of the different conclusions and give insights into this paradox. We will highlight some key factors that influence the dry aging results by laying out different studies regarding dry aging and comparing the influences on the aging result.

2. Factors Affecting the Dry Aging Result

2.1 Aging Time

Overall, dry aging time affects the dry aging result and is the most controllable process parameter. The influence on tenderness occurs in the first two weeks of aging in most studies. However, strong effects during longer aging were also reported in some studies. Flavor and tenderness development are continuously changing during the dry aging process in some studies. In conclusion and with regard to the paradox, the aging time used in the studies is important, since a different aging time is needed to create a good result with wet and dry aging. For example, prolonged wet aging (> 35 days) can result in a negative assessment by consumers, whereas dry aging for the same aging period may not be enough time to create a recognizable flavor-benefit.

2.2 Temperature, Relative Humidity and Air Velocity

Based on the literature, we can conclude that the parameters, air velocity, relative humidity and temperature, of dry aging define the dry aging result. These parameters are of major importance and even minor changes could affect the dry aging result. Regarding our paradox, we state that the dry aging time should be about 40 days at about 2 °C, 85 % relative humidity and 0.5 m/s air flow. However, these do not influence the dry aging result directly, but rather, for example, the microbial development, the fat oxidation or the water loss, which is specified in section 5. The air velocity was not described in half of the studies and was too high to achieve good aging results in some studies.

2.3 Breed, Muscle and Feed

An effect of raw material on the outcome of the dry aging is obvious. Parameters, such as bone-in or bone-out, species, muscle type, sex, age, and breed, affect the aging result but do not explain the paradox, since the same muscle was used.

3. Factors Affected by the Dry Aging Process

3.1 pH-value

Overall, the pH is influenced by the process (wet vs. dry) and dry aging parameters, and, respectively, by the microflora. Why this is not the case in all studies is not currently clear but could be when microbiota on the crust is analyzed. When comparing the studies which reported effects of pH with those reporting no effect of dry aging, neither a positive nor a negative correlation to the pH could be found. Moreover, the pH might be more influenced by the microbiota on and in the beef. In regard to the paradox, no differences in starting pH were observed in the studies with equal process parameters but varying aging results. Still, a study with the same set-up but a different starting pH in the beef would be of interest.

3.2 Water and Cooking Loss

Water loss during the dry aging process seems to have a minor effect, but the water content of the steak before consumption is at least of interest for the explanation of the paradox. Kim et al. [4], for example, aged beef with the dry and wet aging processes and reported a total higher weight loss (after cooking) for dry aging compared to the wet aged steaks that weighed about 10 % more, and higher ratings for flavor and liking, determined in a consumer test. It appears that the reduced water content of dry aged beef can sometimes be equalized by cooking and this sometimes equalizes the differences between the aging types and if not, the dry aged beef is rated higher. This conclusion about the effect of water is also in line with the explanation of Kim et al. (4), who reported that the main contributor to higher levels of many metabolites in the dry aged group is the evaporation of water during the dry aging.

Currently, it is not clear why the water content is only sometimes equalized. However, the reduced water content could affect other factors, such as microbial growth or oxidation, which will be discussed further.

3.3 Oxidation, Metabolite Changes & Amino Acid Changes

The aging method influences the flavor and aroma of the beef. As stated above, this is only the case if the aging parameters are in the right range to achieve a meaningful dry aging result. Furthermore, if changes in the flavor and aroma profile are pronounced, as in these studies, the sensory panel should and can find these differences, but only if the panel is well trained. This data is somehow missing, so training can be a part of the paradox.

3.4 Microbial attributes

The microorganisms on the surface impact the quality, and respectively, the success of the dry aging process, as the latter affects the growth and type of organism. Moreover, the air flow influences the microbiology, which influences the proteolysis, and this in turn affects the taste and tenderness.

4. Conclusion

When comparing the individual factors above, six explanations for the paradox why dry aging is sometimes not better than wet aging can be concluded:

The water loss during dry aging must be high enough that the difference is not equalized during the cooking of the beef.

The latter is a result of the aging parameters. The dry aging time should be about 40 days and the air flow (0.5m/s), temperature (2°C), and relative humidity should be low (< 85 %).

The latter affect the microbiota. A specific microbiota is needed to achieve a difference in flavor and aroma between dry and wet aged beef.

These differences can be detected by sensory analysis: The design needs to be defined properly for the purpose; for example, consumer groups should be as large as possible.

A round robin test between different facilities and countries applying the same process conditions on identical/same, beef would also be of great interest in the future. Moreover, there is a need to consider microbiota in the aging room, such as the meat scientists are doing for raw fermented sausages.

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Attitudes and beliefs of Eastern European meat consumers–a review

Attitudes and beliefs of Eastern European meat consumers – a review

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Abstract. Eastern European consumers are traditional meat eaters who are still not looking forward in order to substitute their traditional meat-based dishes with meat analogues or their substitutes. In general, vegetarianism is in its infancy if we do not take into account Lenten fasting, the period of the year when in Orthodox countries of Eastern Europe one can find food not containing meat, dairy and eggs, widely available. Chicken meat is the most frequently consumed type of meat, and it is preferred because it is easy to prepare. Eastern European meat consumers believe that meat from castrated pigs is of better quality, and one third of them is indifferent towards animal welfare issues. The highest proportion of Eastern European consumers (42.9%) believes that game meat has many health benefits, is rich in proteins and is more organic than other types of meat. However, two thirds of Eastern European consumers eat game meat less than once a year. Differences in attitudes and beliefs of Eastern European meat consumers compared to their Western European equivalents are evident.

1. Introduction

Nothing is the same as it was back in the 1990s when it comes to European meat consumption and production inclinations. We were able to observe the increase in production of pig and poultry meat [1] while acknowledging the decrease in lamb and beef production [2]. Most likely, the major reason that has chased away European consumers from beef meat is their apprehension towards environmental sustainability of its production, as is the case in Japan [3] and Brazil [4]. Health concerns are also an important issue for meat consumers [5], followed by the rising interest in societal benefits and in more "ethical" meat production methods [6-8]. Attitudes and beliefs of European meat consumers are important to all stakeholders in the food (meat) chain, and not surprisingly, they have already been investigated on numerous occasions and explored from different aspects in the past. What is surprising is the fact that, although European by name [9-11], these investigations have excluded (almost by default) meat consumers from Eastern European countries. Because of this, until recently, attitudes and beliefs of Eastern European meat consumers remained unknown to scientific readers, the research community and public in general. Therefore, the purpose of this review was to collect, categorize and

summarize all the recently published (relevant) data that will help to make a portrait of an average Eastern European meat consumer.

2. Chicken Meat Consumers

On average, only 2.6% of Eastern European consumers avoid consumption of chicken meat, while the majority (51.7%) and more than a half of them eat it on a fortnightly basis. One fifth (19.8%) eats chicken only once a week, while almost equal percentages of consumers do so once a month (13%) or on a daily basis (12.9%) (Table 1). Not surprisingly, and because Islam is today the largest religion in Bosnia and Herzegovina and is adhered to by half of the nation's population, Bosniaks are the most avid chicken meat consumers in the whole of Eastern Europe. More than a third of Bosniaks (34.4%) eat chicken on a daily basis. The second largest proportion of any country population (21.7%) that are daily chicken eaters was observed in Serbia, not because of religious but because of economic motives, since chicken meat is the most affordable type of meat available on Serbian meat markets. Overall, Hungarian consumers seem to be most reluctant chicken meat consumers, since 89.8% of them eat it only on a fortnightly or even monthly basis (Table 1). On the other hand, although Bulgarian consumers are not among the most frequent chicken meat consumers in Eastern Europe, they seem to have the highest average consumption of chicken meat in households per month, in the range of 6-9 kg, and chicken meat products in the range 3-6 kg [12].

The age group of 50 to 64-year-old Eastern European consumers eats the most chicken meat and chicken meat products, in the range of 3-6 kg per month. Monthly, female consumers seem to eat less than males, since the highest percentage of females consume chicken meat and chicken meat products in the range from 500 g to 3 kg, while the highest number of male respondents consume chicken meat in the range 3-6 kg and chicken meat products in the range 500 g to 3 kg [12].

The most important quality attribute of chicken meat for the Eastern European consumers is its visual appearance, namely freshness and colour as the leading quality attributes [13]. This is why researchers and industry are exploring novel analytical methods for the evaluation of chicken meat colour [14]. When it comes to retailing chicken meat, Eastern European consumers believe the three most important characteristics are temperature at point of sale (28.35%), shelf illumination (22.6%) and product placement (14.7%) [13].

Most of the Eastern European consumers (39.1%) prefer chicken meat because it is easy to prepare and since it can easily be accompanied by many side dishes. These consumers are mostly female (64.3%), and chicken meat consumption is perceived as a good way to show their cooking skills and as ideal for children’s diets, so chicken meat forms an important element of their family diet [12]. It was also revealed that the second largest proportion of Eastern European chicken meat consumers (34.5%) are very selective eaters who prefer particular chicken cuts and have particular healthy-diet attitudes [12]. This was best explained by the fact that they are predominantly the working population, which contains more selective and experienced consumers than the younger population, in general [15].

3. Pork Meat Consumers

As an exploratory approximation, we can assume that the largest proportion of the Eastern European consumers eat pork on fortnightly basis (43.3% on average and varying from 19.0% in Moldova to 58.5% in Croatia) with almost equal percentages of them eating pork on a weekly (21.1% on average and varying from 11.3% in Hungary to 29.4% in Poland) or daily (20.3% on average and varying from 8.0% in Hungary to 54.3% in Moldova) basis [16].

Table 1. Demographic profile of the sample and frequencies of meat consumption in Eastern Europe (adopted from [16])

Characteristic		Overall n (%)	B & H (324)	Bulgaria (n=352)	Czech R. (n=284)	Croatia (n=301)	Macedonia (n=285)	Hungary (n=400)	Moldova (n=300)	Poland (n=504)	Romania (n=557)	Serbia (n=678)	Slovakia (n=301)	Slovenia (n=246)	Ukraine (n=750)
Sex	Male	2457 (44.7)	166 (51.6)	144 (40.9)	197 (38.6)	119 (39.5)	137 (48.1)	224 (56.0)	94 (31.3)	245 (48.6)	277 (49.7)	323 (47.8)	120 (39.9)	109 (45.0)	302 (40.3)
	Female	3043 (55.3)	156 (48.4)	208 (59.1)	313 (61.4)	182 (60.5)	148 (51.9)	176 (44.0)	206 (68.7)	259 (51.4)	280 (50.3)	353 (52.2)	181 (60.1)	133 (55.0)	448 (59.7)
Age	Less than 26	1128 (20.5)	43 (13.3)	27 (7.7)	116 (22.7)	43 (14.3)	67 (23.5)	126 (31.5)	116 (38.7)	115 (22.8)	138 (24.8)	121 (17.9)	47 (15.6)	70 (30.0)	99 (13.2)
	26 – 40	1377 (25.1)	68 (21.0)	77 (21.9)	149 (29.2)	84 (27.9)	47 (16.5)	103 (25.8)	99 (33.0)	152 (30.2)	148 (26.6)	167 (24.7)	78 (25.9)	56 (24.0)	149 (19.9)
	41 – 60	2117 (38.5)	175 (54.0)	207 (58.8)	130 (25.5)	128 (42.5)	98 (34.4)	135 (33.8)	52 (17.3)	152 (30.2)	172 (30.9)	298 (44.0)	128 (42.5)	84 (36.1)	358 (47.7)
	Over 60	872 (15.9)	38 (11.7)	41 (11.6)	115 (22.5)	46 (15.3)	73 (25.6)	36 (9.0)	33 (11.0)	85 (16.9)	99 (17.8)	91 (13.4)	48 (15.9)	23 (9.9)	144 (19.2)
Education	Elementary	296 (5.4)	29 (9.2)	15 (4.3)	12 (2.4)	34 (11.3)	21 (7.4)	4 (1.0)	11 (3.7)	5 (1.0)	58 (10.4)	69 (10.3)	16 (5.3)	14 (5.8)	8 (1.1)
	Higher	2308 (42.1)	215 (68.5)	190 (54.0)	280 (54.9)	102 (33.9)	151 (53.2)	160 (40.0)	136 (45.5)	132 (26.2)	247 (44.3)	416 (62.4)	139 (46.3)	87 (36.3)	53 (7.1)
	University	2874 (52.5)	70 (22.3)	147 (41.8)	218 (42.7)	165 (54.8)	112 (39.4)	236 (59.0)	152 (50.8)	367 (72.8)	252 (45.2)	182 (27.3)	145 (48.3)	139 (57.9)	689 (91.9)
Household members	One	319 (5.9)	19 (6.1)	17 (4.9)	59 (11.6)	18 (6.0)	12 (4.2)	5 (1.3)	16 (5.4)	40 (7.9)	43 (7.7)	33 (5.1)	23 (7.7)	11 (4.6)	23 (3.2)
	Two – three	2611 (48.1)	157 (50.0)	240 (68.6)	283 (55.5)	132 (43.9)	69 (24.3)	124 (31.1)	119 (40.1)	179 (35.5)	337 (60.5)	298 (46.3)	145 (48.3)	96 (40.0)	432 (59.7)
	Four – five	2212 (40.8)	138 (43.9)	93 (26.6)	147 (28.8)	128 (42.5)	177 (62.3)	236 (59.1)	135 (45.5)	241 (47.8)	167 (30.0)	279 (43.4)	107 (35.7)	104 (43.3)	260 (35.9)
	Over five	281 (5.2)	0 (0.0)	0 (0.0)	21 (4.1)	23 (7.6)	26 (9.2)	34 (8.5)	27 (9.1)	44 (8.7)	10 (1.8)	33 (5.1)	25 (8.3)	29 (12.1)	9 (1.2)
Growing place	Rural	3279 (59.6)	313 (96.6)	120 (34.1)	295 (57.8)	186 (61.8)	122 (42.8)	241 (60.3)	171 (57.0)	306 (60.7)	290 (52.1)	545 (80.4)	171 (57.0)	196 (81.7)	323 (43.1)
	Urban	2221 (40.4)	11 (3.4)	232 (65.9)	215 (42.2)	115 (38.2)	163 (57.2)	159 (39.8)	129 (43.0)	198 (39.3)	267 (47.9)	133 (19.6)	129 (43.0)	44 (18.3)	426 (56.9)
Frequency of pork consum.	Never	280 (5.1)	23 (7.3)	26 (7.4)	24 (4.7)	7 (2.3)	12 (4.2)	7 (1.8)	13 (4.3)	43 (8.5)	41 (7.4)	54 (8.1)	10 (3.3)	9 (3.7)	11 (1.5)
	Daily	1110 (20.3)	115 (36.3)	42 (12.0)	74 (14.5)	36 (12.0)	43 (15.1)	32 (8.0)	163 (54.3)	128 (25.4)	100 (18.0)	199 (29.7)	43 (14.3)	32 (13.1)	103 (14.1)
	Weekly	1153 (21.1)	74 (23.3)	65 (18.6)	109 (21.4)	40 (13.3)	63 (22.1)	45 (11.3)	55 (18.3)	148 (29.4)	146 (26.2)	121 (18.1)	72 (24.0)	33 (13.5)	182 (24.9)
	Fortnightly	2368 (43.3)	83 (26.2)	200 (57.1)	286 (56.1)	176 (58.5)	131 (46.0)	220 (55.0)	57 (19.0)	165 (32.7)	235 (42.2)	179 (26.7)	150 (50.0)	139 (57.0)	347 (47.4)
	Monthly	559 (10.2)	22 (6.9)	17 (4.9)	17 (3.3)	42 (14.0)	36 (12.6)	96 (24.0)	12 (4.0)	20 (4.0)	35 (6.3)	117 (17.5)	25 (8.3)	31 (12.7)	89 (12.2)
Frequency of chicken consum.	Never	140 (2.6)	19 (6.1)	6 (1.7)	13 (2.6)	6 (2.0)	7 (2.5)	5 (1.3)	5 (1.7)	17 (3.4)	20 (3.6)	35 (5.3)	5 (1.7)	1 (0.4)	1 (0.1)
	Daily	704 (12.9)	107 (34.4)	40 (11.4)	54 (10.6)	14 (4.7)	30 (10.5)	15 (3.8)	52 (17.3)	70 (13.9)	56 (10.1)	144 (21.7)	20 (6.7)	24 (9.9)	78 (10.6)
	Weekly	1084 (19.8)	74 (23.8)	93 (26.4)	90 (17.7)	13 (4.3)	73 (25.6)	21 (5.3)	90 (30.0)	123 (24.4)	69 (12.4)	183 (27.5)	49 (16.3)	15 (6.2)	191 (25.8)
	Fortnightly	2828 (51.7)	82 (26.4)	152 (43.2)	334 (65.6)	223 (74.1)	147 (51.6)	270 (67.5)	110 (36.7)	189 (37.5)	304 (54.6)	228 (34.3)	204 (68.0)	190 (78.5)	395 (53.5)
	Monthly	709 (13)	29 (9.3)	61 (17.3)	18 (3.5)	45 (15.0)	28 (9.8)	89 (22.3)	43 (14.3)	105 (20.8)	108 (19.4)	75 (11.3)	22 (7.3)	12 (5.0)	74 (10.0)
Frequency of lamb consum.	Never	2490 (46.3)	61 (20.1)	109 (31.0)	329 (64.5)	95 (31.6)	118 (41.4)	192 (48.0)	133 (44.3)	252 (50.0)	239 (42.9)	228 (34.7)	187 (62.3)	148 (62.2)	399 (59)
	Daily	2134 (39.6)	101 (33.3)	205 (58.2)	168 (32.9)	163 (54.2)	113 (39.6)	186 (46.5)	148 (49.3)	168 (33.3)	243 (43.6)	231 (35.2)	94 (31.3)	84 (35.3)	230 (34)
	Weekly	420 (7.8)	59 (19.5)	37 (10.5)	8 (1.6)	22 (7.3)	34 (11.9)	15 (3.8)	13 (4.3)	57 (11.3)	41 (7.4)	94 (14.3)	16 (5.3)	5 (2.1)	19 (2.8)
	Fortnightly	235 (4.4)	39 (12.9)	1 (0.3)	5 (1.0)	21 (7.0)	14 (4.9)	7 (1.8)	3 (1.0)	24 (4.8)	30 (5.4)	61 (9.3)	2 (0.7)	1 (0.4)	27 (4.0)
	Monthly	104 (1.9)	43 (14.2)	0 (0.0)	0 (0.0)	0 (0.0)	6 (2.1)	0 (0.0)	3 (1.0)	3 (0.6)	4 (0.7)	43 (6.5)	1 (0.3)	0 (0.0)	1 (0.1)
Frequency of beef consum.	Never	943 (17.4)	56 (18.4)	79 (22.4)	42 (8.3)	35 (11.6)	57 (20.0)	47 (11.8)	73 (24.3)	102 (20.2)	130 (23.3)	138 (21.4)	31 (10.4)	7 (2.9)	146 (20.6)
	Daily	2460 (45.5)	134 (43.9)	208 (59.1)	211 (41.5)	125 (41.5)	75 (26.3)	266 (66.5)	172 (57.3)	203 (40.3)	217 (39.0)	289 (44.7)	160 (53.5)	45 (18.6)	355 (50.1)
	Weekly	1072 (19.8)	55 (18.0)	56 (15.9)	148 (29.1)	55 (18.3)	57 (20.0)	51 (12.8)	21 (7.0)	114 (22.6)	117 (21.0)	124 (19.2)	65 (21.7)	60 (24.8)	149 (21)
	Fortnightly	805 (14.9)	31 (10.2)	9 (2.6)	106 (20.8)	79 (26.2)	81 (28.4)	34 (8.5)	27 (9.0)	77 (15.3)	91 (16.3)	60 (9.3)	41 (13.7)	125 (51.7)	44 (6.2)
	Monthly	128 (2.4)	29 (9.5)	0 (0.0)	2 (0.4)	7 (2.3)	15 (5.3)	2 (0.5)	7 (2.3)	8 (1.6)	2 (0.4)	35 (5.4)	2 (0.7)	5 (2.1)	14 (2.0)

Eastern European pig meat consumers have positive to neutral preferences for meat from castrated pigs, and piglet castration is not considered an issue that needs to be dealt with in this part of Europe [16]. Reasons can be found in the belief that meat from castrated pigs is of better quality. At the same time, they are ambivalent about the statement that meat from castrated pigs is leaner, apart from the pig meat consumers from Bulgaria who think otherwise. On average, Eastern European consumers are indefinite about whether the meat from castrated pigs is more expensive than that from non-castrates, while they are more willing to pay for meat from physically castrated pigs [16] than an average Western European consumer [17].

Regardless of the geographical or demographical determinants, the colour of pork meat remains the most important quality attribute when it comes to the consumers and their preferences [18], and this consequently provoked research into finding novel techniques in pork and pork meat products' colour assessment [19].

4. Game Meat Consumers

Only one third of Eastern European consumers eat game meat at least once a year. The only exception is Bulgaria, where game meat is consumed at least once a month by 79.6% of the consumers [20]. One thing is also very certain, and it is that in this part of Europe women eat less game meat than do men. Similar trends are observed in other parts of the world like in the USA [21] or Norway [22]. The highest proportion of Eastern European consumers (42.9%) believes that game meat has many health benefits, is rich in proteins and is more organic than other types of meat [20]. This is in sharp contrast with the situation in Africa or Australia for example, where despite the great potential of wild game meat, consumers are ill-informed regarding the positive attributes of game meat [23, 24]. The second largest proportion of Eastern European consumers (32.8%) believe that game meat is low in fat and cholesterol, but that it is not easily available for purchase; it is, therefore, considered only as a seasonal commodity. Almost a quarter of Easter European consumers (24.3%), who are mostly youngsters below 24 years of age, believe that game meat is more expensive compared to other types of meat and that sensory attributes of game meat (taste, odour and colour) are associated with wild species [20].

Almost a half (49.2%) of Eastern European consumers eat game meat mainly on social occasions, and because it is easily accompanied by many side dishes. Nearly 40% of them, who are mainly females and youngsters below 24 years of age, eat game meat mainly because of the eating enjoyment and because it tastes great. Only a fraction above one tenth of the consumers (11.8%) consider game meat a part of their country's culinary tradition [20].

5. Animal Welfare Attitudes and Beliefs

In terms of animal welfare attitudes and beliefs, three distinctive, but almost identical in size, groups of Eastern European consumers can be identified. The first group (37%) consists of the consumers who are concerned about animal welfare, and they believe it is possible to achieve. However, the second group (32%) is the consumers who are indifferent towards animal welfare. The third group (31%) of Eastern European consumers is concerned about animal welfare, but they believe it is difficult to achieve.

6. Conclusions

Differences in attitudes and beliefs of Eastern European meat consumers compared to their Western European counterparts are evident. Meat analogues and substitutes are not even being considered yet, since the consumers are pretty satisfied eating their traditional meat-based dishes. Animal welfare, environmental and sustainability issues are less of a concern because of the lower incomes in the Eastern compared to the Western part of Europe. The health and wellness food market in Eastern Europe is under development and growing, but still passing through a period of tests and looking for some stabilization.

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Pesticide monitoring in food in Bosnia and Herzegovina in 2019

Pesticide monitoring in food in Bosnia and Herzegovina in 2019

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Abstract Pesticide residues in or on foods of plant/animal origin occur as a result of the use of chemical agents in plant protection, biocide preparations and veterinary medicine and may pose a risk to public health. For this reason, a comprehensive legal framework has been adopted in Bosnia and Herzegovina, defining rules for the approval of active substances used in plant protection products, the use of plant protection products and the regulation of maximum permitted quantities of pesticide residues in and on food. In 2019, 195 samples were analysed as part of pesticide monitoring. The control programme carried out the monitoring of residues/remains of 180 active substances in 155 products of plant origin, 30 products of animal origin and 10 products from the category of food for infants and young children. In total, of the 195 samples analysed, 141 samples did not contain pesticide residues at the quantification level, but 54 samples (27.7%) contained pesticide residues in/above the quantification level.

1. Introduction

Pesticide residues in or on foods of plant/animal origin occur as a result of the use of chemical agents in plant protection, biocide preparations and veterinary medicine and can pose a risk to public health. In order to ensure a high level of consumer protection, the permitted limits, the so-called maximum allowable quantity or in short, MRL, are prescribed by the Bosnia and Herzegovina Ordinance on maximum levels of pesticide residues in and on food and feed of plant and animal origi bn [1] in accordance with Regulation (EC) 396/2005 [2]. The regulation established a system that is in line with the EU legislation, prescribed MRLs for more than 500 pesticides and covered more than 370 food/food groups. Also, for pesticides not listed in the regulation, values of 0.01 mg/kg are applied. The provisions of Article 12 of the Ordinance, stipulates the implementation of a multi-year program for controlling pesticide residues. The Law on Phytopharmaceutical Products of BiH [3] regulates the basis for remnants of phytopharmaceutical products (hereinafter: FFS), as well as the proper use and registration of FFS. This Law assumes Regulation (EC) 1107/2009 [4] relating to the sale of plant protection products. The use of this Regulation should ensure that the industry reaches the point that the regulated products marketed for plant protection have no harmful impact on human and animal health or an unacceptable environmental impact. The Multiannual Programme of Control is prepared and coordinated by the Food Safety Agency of Bosnia and Herzegovina (hereinafter: The Agency) in cooperation with the Directorate of Bosnia and Herzegovina for Plant Health Protection and the Veterinary Office of Bosnia and Herzegovina. The programme will be updated annually, will be based on a risk assessment and will focus in particular on assessing consumer exposure and compliance with applicable legislation. The multiannual control programme will be in line with the coordinated multiannual control programme implemented in the Member States of the European Union for 2018-2020, i.e. in accordance with the Commission Implementing Regulation [5] on a coordinated multiannual Union control programme for 2018, 2019 and 2020 to ensure compliance with maximum levels of pesticide residues and assess consumer exposure to pesticide residues in and on foods of plant and animal origin. The multiannual pesticide residue control programme defined the food products and pesticides monitored in Bosnia and Herzegovina. The control programme is partly aligned with the EU-coordinated control programme relevant for the calendar year 2019, and also contains a proportion of national products selected on the

basis of the results so far of the implementation of pesticide residue control programmes in and on food, the importance of food consumption products, Rapid alert system for food and feed (RASFF) notifications and other parameters.

2. Results and Discussion

According to Commission Regulation **[6]** on a coordinated multiannual control programme implemented in the European Union member states for the period 2018-2020, which is the basis for the development of the control programe, a total of 13 different products have been selected: apple, strawberry, peach, lettuce, head cabbage, tomatoes, spinach, oats, barley beans, grape wine, cow's milk, pork fat and food for infants and young children. In addition to the above, eight national products (plum, raspberry, table grapes, pear, corn, cucumber and chicken eggs) were sampled, which were selected on the basis of the results of the monitoring of pesticide residues in and on food, the importance of products from the point of view of food consumption and RASFF notifications. A total of 195 samples were taken: 66 fruit samples, 55 vegetable samples, 24 cereal samples and 50 samples of other food categories (infant and young children's food, grape wine, cow's milk, chicken eggs, pork fat.
In the 195 samples analysed, 141 samples did not contain pesticide residues at quantification level (72.30%), but 54 samples (27.7%) contained pesticide residues in/above quantification levels. The values of the data were slightly higher than the average values in the European Union [7]. In 2019, in the European Union, in 53% of food samples pesticide content was lower than quantification levels. Unlike the number of samples in which the presence of pesticides was not recorded, a slightly higher percentage (45%) of the samples contained pesticides. In our study, six samples (3.07%) with residues of active substances above the MRLs were found, which is a higher frequency of non-compliant product than the average frequency of non-compliance recorded in the EU (1%), and excepting one sample of a head of cabage (1.54%) with pesticide content above the MRL, but within the limits of measurement uncertainty, which is considered appropriate. A detailed view of foods with pesticide residues above the MRLs is displayed in Table 1.

Table 1. Samples with residues pesticides above MRL, Bosnia and Herzegovina, 2019

rb	Active substances	Products	Results of analysis mg/kg	MRL mg/kg
1	Dimethoat	Peach	0.034 (±0.017)	0.01
2	Metrafenone	Head of cabbage *	0.019 (±0.009)	0.01
3	Carbendazim (RD)	Apple	0.45 (±0.225)	0.02
4	Cyazofamid	Lettuce	0.918 (±0.459)	0.01
5	Cyazofamid	Lettuce	1.446 (±0.723)	0.01
6	Chlorantraniliprole (DPX E-2Y45)	Corn	0.134 (±0.067)	0.02

* Sample above MRL, but within the limits of measurement uncertainty

Among the active substances analyzed in plant products, the following were quantified in more than 5% of the samples analyzed: boscalide (10.3%), carbendazim (RD) (7.27%). In food samples of animal origin (cow's milk, chicken eggs and pig fat), pesticide residues were not quantified in any sample. Five samples were inadequate (2.56%). These were samples of lettuce (2), peach (1), apple (1) and corn (1). The results of laboratory analyses showed that 14 products contained the remains of active substances that are not on the List of Active Substances permitted for use in phytopharmaceutical means in Bosnia

and Herzegovina. [8] Detected residues of active substances were (the number in bracket indicates how many products have been detected): aldicarb (1), carbendazim (RD) (12), chloropropham (1). The presence of active carbendazim can be explained to some extent by the fact that carbendazim is the main product of decomposition of the approved active substance thiofanate-methyl [9]. Products with remnants of illicit active substances originated from: Argentina (1); Bosnia and Herzegovina (7); Italy (1); Serbia (1); Egypt (1); Moldova (2) Macedonia (1). Out of a total of 195 samples taken from 21 food types, samples from five (23.8%) food types did not contain pesticide residues. These were the following types of foods: oat grain (8), pork fat (10), chicken eggs (10), infant and toddler food (10), cow's milk (10). In 16 types of food (76.2%), pesticide residues were found in or above quantification levels. These were the following types of foods (the number in parentheses indicates the total number of food samples/number of samples containing pesticide residues at the quantification level): apple (12/11); grapefruit (10/10); banana (10/9); pear (11/9); broccoli (10/8); centipede grapes (10/8); eggplant (10/7); raspberry (11/6); Cornishon (5/5); cucumber (5/2); plum (11/5); peppers (10/4); cultivated mushrooms (10/3); melon (10/3); wheat grain (10/1).

3. Conclusions

Out of a total of 195 samples analysed in 2019, 54 samples (27.7%) contained pesticide residues in/above quantification levels. In 2019, five samples (2.56%) were inadequate and contained pesticide residues above the MRLs. These were samples of lettuce (2), peach (1), apple (1) and corn (1). In one sample of head cabbage (1.54%), pesticide residue above the prescribed MRL was identified within the limits of measurement uncertainty, and so the sample was considered appropriate. Therefore, in 2019, there were a total of six samples (3.07%) with pesticide residues above the prescribed MRLs. A rapid risk assessment in one apple sample assessed exceedance of the identified toxicological benchmarks (ARfD) (243%) for the active material, carbendazim. The results of laboratory analyses showed that 14 products contained the residues of active substances that are not on the List of Active Substances permitted for use in phytopharmaceutical purposes in Bosnia and Herzegovina. The detected remnants of active substances are (the number in bracket indicates how many products have been detected): aldicarb (1), carbendazim (RD) (12), chloropropham (1). Products with remnants of illicit active substances originated from: Argentina (1); Bosnia and Herzegovina (7); Italy (1); Serbia (1); Egypt (1); Moldova (2) Macedonia (1). The presence of unapproved active substances could signal possible misuse (application) of unapproved active substances, but could also be the consequence of degradation of approved active substances (thiophanate-methyl → carbendazim). During official controls, competent inspection authorities must decide whether any residues detected are the result of the use of illicit phytopharmaceutical preparations, or are present as a result of metabolic processes. Also, some active substances and/or their residues can remain in the environment for years after their use.

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Mercury in female cattle livers and kidneys from Vojvodina, northern Serbia

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Mercury in female cattle livers and kidneys from Vojvodina, northern Serbia

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Abstract. Concentrations of mercury (Hg) were analysed in livers (n = 26) and kidneys (n = 26) of female cattle (412–2502 days old) from farms for milk production in the area of Vojvodina. Concentration of Hg was analysed by ICP-OES, after digestion by microwave. The Hg concentrations in the livers and kidneys ranged from below detection limits (LOD < 0.006 mg/kg) to 0.206 mg/kg wet weight and from below detection limits (LOD < 0.006 mg/kg) to 0.018 mg/kg wet weight, respectively.

1. Introduction

All types of cattle, whether their primary purpose is meat, milk, draft power or some combination of these, will be used ultimately in beef production [1]. In everyday diet, red meats are the best source of high biological value nutrients (vitamins: vitamin B_{12}, niacin and vitamin B_6, minerals: iron, zinc and phosphorus, proteins). Beside positive effects on diet and health, red meat could potentially be the source of some chemical substances with toxic effects [2-23].

In 2020, in the Republic of Serbia total cattle number was 886,127, and 259,527 of them were raised in the Autonomous Province of Vojvodina [24].

Variety meat, or edible offal, are also a kind of meat even though are not skeletal muscles. They are used as food, and mostly have higher contents of some micro-nutrients, especially vitamins and minerals, comparing to meat tissue [13,25-28]. According to Serbian legislation [29], the edible offal (organs and glands) that are separated in dressing butchered cattle include liver, kidney, spleen, heart, tongue, lungs, brain, thymus and testis. Liver is the most often used organ and is incorporate in recipes for number of different types of processed products of meat. These organs from older cattle animals are much more suited for processed meats, especially for liver paté and sausages, since they possess intensive aroma and are tough. On contrary, kidneys are not usually used in processed products of meat but are generally used as braised, grilled or sautéed either whole or sliced [30].

Mercury (Hg) is recognised as toxic pollutant at global level, which bioaccumulate and biomagnify within the food chain [31]. Hg toxic characteristics have a serious negative impact to both environment and living organisms [32]. This metal is present in soil, water and air, due to both anthropogenic and natural emission sources [33]. The living organisms can adopt mercury from contaminated soil, water and air. In this sources, mercury is transformed by microorganisms to more bioavailable organic forms [34], and is incorporated into next levels of the food chain [35-39]. For vertebrates, the diet is considered to be one of the main sources of exposure to contaminants from environmental [40]. Human population, being at the highest food chain level is exposed to Hg intake from different kinds of food, especially by consumption of fish [41-43], seafood and freshwater crustaceans [44-46], and food of animal origin [47].

The Regulation of European Union [48] only defined maximum levels for total Hg concentration for seafood and fish from 0.5 to 1.0 mg/kg wet weight. FAO/WHO Expert Committee on Food Additives (JECFA) established PTWI (provisional tolerable weekly intake) for methylmercury of 1.6 μg/kg body weight and of 4 μg/kg body weight for inorganic mercury. The PTWI for methylmercury of 1.6 μg/kg body weight/week corresponds to 0.112 mg/week for a person weighing 70 kg [49,50].

Studies aiming to determine concentration of Hg in animal tissues were conducted in number of countries and regions. The available literature indicate a large variability in the concentrations of Hg in muscle tissue and offal of cattle [51-61].

The concentrations of toxic (heavy) elements in food must be permanently monitor and control in order to protect or/and reduce their negative effects on human health. Considering the fact that there is a lack of information about Hg content in cattle tissues from Vojvodina, the goals of this paper were (i) to measure Hg levels in livers and kidneys from adult dairy cattle in the Autonomous Province of Vojvodina and (ii) to determine possible trends in bioaccumulation of Hg in cattle tissues.

2. Materials and methods

Samples (liver and kidney) were collected from 26 cattle slaughtered at the slaughterhouse in Novi Sad (Vojvodina, northern Serbia), during 26 consecutive weeks (i.e. samples from one animal per week were collected). All animals were slaughtered for human consumption. Slaughtered heifers and cows (female cattle) came from 26 different farms for milk production (i.e. one animal per farm was sampled) in Vojvodina (northern Serbia), so it can be stated that samples of liver and kidney were collected from the whole region. Information about animals (date of birth, sex and type of the animal) were received from farms with copy of the passport. The investigated cattle aged from 412 to 2502 days.

Samples (liver and kidney) were collected from the same cattle and minced in a stainless steel cutter. After homogenization, approximately 250 g of samples were taken for analysis. Samples were vacuum packed in polyethylene bags and stored at constant temperature (-80°C) until determination of Hg. Hg content was determined using ICP-OES (inductively coupled plasma-optical emission spectrometry, iCAP 6000 Series, Thermo Scientific, Cambridge, UK) method, after digestion by microwave (MWS-3+, Berghof, Germany). Hg contents are showed as mg/kg wet weight. Standard reference material (ERM-CE278, mussel tissue IRMM, Institute for Reference Materials and Measurements, Geel, Belgium) was used for quality control of the analytical measurement. The results obtained for the analytical quality control programme are showed in Table 1.

Table 1. The results obtained for analytical quality control programme (n = 5)

Analyte	Matrix	Certified concentration	Found concentration	LOD	LOQ
Hg (mg/kg)	ERM – CE278, Mussel tissue, IRMM, Geel, Belgium	0.196	0.191	0.006	0.015

3. Results and discussion

Concentrations of Hg in the analysed samples are showed in Table 2.

Table 2. Individual Hg concentration (mg/kg wet weight) in the edible offal of female cattle raised in Vojvodina

No	Liver	Kidney
1	0.206	0.010
2	0.121	0.015

3	0.057	0.018
4	0.069	< 0.006
5	0.063	< 0.006
6	< 0.006	0.011
7	0.043	< 0.006
8	0.021	0.014
9	0.019	0.013
10	0.015	< 0.006
11	0.009	0.012
12	0.009	0.012
13	0.013	0.015
14	0.010	0.006
15	0.009	0.008
16	< 0.006	< 0.006
17	0.012	0.008
18	< 0.006	0.006
19	< 0.006	0.011
20	0.008	< 0.006
21	< 0.006	0.011
22	< 0.006	< 0.006
23	< 0.006	< 0.006
24	< 0.006	< 0.006
25	< 0.006	< 0.006
26	< 0.006	< 0.006

The Hg concentrations of the female cattle liver and kidney were in the range from below detection limits (LOD < 0.006 mg/kg) to 0.206 mg/kg wet weight and from below detection limits (LOD < 0.006 mg/kg) to 0.018 mg/kg wet weight, respectively.

Reported Hg concentrations in foods, that is, red meat and edible offal of cattle, vary widely (Table 3).

Table 3. Mean concentrations (mg/kg wet weight) of Hg in red meat and edible offal of cattle from different countries

Meat	**Liver**	**Kidney**	**Country**	**Source**
0.005	0.006	0.010	Sweden	[51]

< LOD-0.0191 (interval)	< LOD-0.0938 (interval)	< LOD-0.0733 (interval)	Spain	[52]
0.011	0.012	0.015	Finland	[53]
0.0012	0.0042	0.011	Poland	[54]
	0.02 (median)	0.02 (median)	USA	[55]
		0.006 (geometric mean)	Croatia	[56]
		0.008	Ireland	[57]
	0.0003 (median)	0.004 (median)	Zambia	[58]
	< LOD-0.100	< LOD-0.481	Serbia	[59]
0.00391	0.00581	0.01014	Egypt	[60]
0.0210 0.0011	0.0243 0.0033	0.0510 0.0183	Czech Republic	[61]

4. Conclusion

The literature indicates a large variability of Hg concentration in red meat and edible offal (liver and kidney). The available data showed that considerable regional differences exist for the Hg concentration of animal edible tissues. Thus, content of Hg in edible offal (liver and kidney) can be used as relevant indicator of environmental contamination by Hg. Generally, monitoring and control of Hg in living organisms, i.e. red meat and edible offal, is necessary.

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Traditional meat preparations in the Balkans region

Traditional meat preparations in the Balkans region

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Abstract. The aim of this study was to determine the fatty acid (FA) profile and trans fatty acid (TFA) content of the Balkan's traditional meat preparations. Twenty-four meat preparations were examined: fresh sausages, ćevapčići, pljeskavica and hamburger. Gas chromatography of FA and TFA was used. Trans fat was higher than the recommended value of 0.5% in ćevapčići, pljeskavica and hamburger (0.54-0.62%) and lower in fresh sausages (0.26%). There were significant differences ($P < 0.05$) in the atherogenicity index (AI), which was lower in fresh sausages (0.58) and ćevapčići (0.64) and pljeskavica (0.64) and highest in hamburger (0.77). Concerning thrombogenicity indices (TI), there was a significant difference ($P < 0.05$), being the lowest in fresh sausages (1.42), ćevapčići (1.28) and pljeskavica (1.40) and the highest in hamburger (1.82). AI and TI of traditional meat preparations were higher than recommended indices, 0.5 and 1.0, respectively, and so are not desirable for health protection. The n-6/n-3 ratio was significantly ($P < 0.05$) lower in pljeskavica (10.76) and hamburger (7.30) compared with ćevapčići (22.25) and fresh sausages (28.41). Promotion of the Mediterranean diet requires changes in the food systems and public health policies to improve overall diet quality of individuals, communities, and populations.

1. Introduction

Fresh sausages are meat products sold fresh without prior heat treatment. In many countries, they are manufactured on request in butcher shops. The basic raw materials used in fresh sausage manufacture are pork and beef, including their trimmings. Fresh pork sausages are a very popular breakfast item in many European and American homes and restaurants and, throughout the years, have been a leading pork product [1, 2]. Technologically, hamburgers are typical fresh beef meat preparations that are not stuffed into casings. Different techniques and formulae are used in manufacturing fresh sausages in various countries. Fresh sausages are mostly stored and sold in chilled form, although some are sold frozen. The frozen product is bought in the shop or supermarket. Fresh sausages are produced according to different quality standards and, in large parts of the world, they are sold very cheaply. It is absolutely critical that the meat and fat materials processed have low microbiological counts. A bacteria count of 10^2–10^4/g of meat should be achieved; a maximum count of 10^3/g of meat is often seen as the standard in most processing companies [3].

Ćevapčići or ćevap is a grilled skinless Serbian product, found traditionally in the countries of southeastern Europe (the Balkans). They are considered a national dish in Bosnia and Herzegovina and Serbia, and are also common in Croatia, Montenegro and Slovenia, as well as in Albania, North Macedonia, Bulgaria and Romania. The typical portion consists of 5 pieces of small, rolled patties of mixed ground meats that are heavily seasoned, grilled and served on a plate or in a flatbread, usually with chopped onions, yogurt/sour cream, or potato salad [4, 5]. Pljeskavica is a popular meat patty dish, second only to ćevapčići. It is often served with milk cream, ajvar sauce of peppers and urnebes, a mixed spicy cheese spread. Other popular dishes in the Balkans include roasted sausage and hamburger.

Analysis of the Global Burden of Disease Study 2010 shows that dietary factors are the most important factors that undermine health and well-being in every Member State in the WHO European Region [6]. Excess body weight, excessive consumption of energy, saturated fats, *trans* fats, sugar and

salt, as well as low consumption of vegetables, fruits and whole grains are leading risk factors and priority concerns. Overweight and obesity are also highly prevalent among children and adolescents, particularly in southern European countries. The World Health Organization (WHO) defines four factors in the epidemiology of cardiovascular diseases, cancers, obesity and type 2 diabetes mellitus – poor diet, physical inactivity, tobacco and alcohol use – are of overwhelming importance to public health [6]. Childhood obesity, too, is a growing problem across the world, with physical inactivity a major factor. There are clear associations between physical activity and health and between diet and health, and the two relationships are often linked through obesity [6]. The Mediterranean diet abundant in minimally processed plant-based foods, rich in monounsaturated fat from olive oil, but lower in saturated fat, meats and dairy products, seems an ideal nutritional model for cardiovascular health [7].

Desirable total fat intake in the diet, according to most experts, should amount to only 25-30% of the total daily energy intake (E%) [8, 9], provided that 10% of E% in the form of saturated fatty acids (SFAs) would enable the body to completely fulfil its essential metabolic functions. On the one hand, an intake of 0.5 to 1-2% E% of trans fatty acids (TFA) was proposed [8]. However, there was agreement among the experts that in populations with inadequate total E%, such as is seen in many developing regions, dietary fats are an important macronutrient to increase E% to more appropriate levels. The study also showed a higher prevalence of overweight associated with lower socioeconomic status in some countries. There are not enough data on the fat quality of fresh sausages, ćevapčići, pljeskavica and hamburger. The final goal of these studies is to establish and expand food composition and trans fatty acid (TFA) databases as a priority. Therefore, the aim of this study was to determine the fatty acid (FA) and TFA profiles of the Balkan's traditional meat preparations, and to calculate the atherogenic index (AI) and thrombogenic index (TI) of the products.

2. Materials and Methods

2.1. Samples

Twenty-four traditional meat preparations were examined. Traditional meat preparations were purchased from meat companies from Serbia.

2.2 Fatty acid and trans fatty acids analysis by capillary gas chromatography

The total fat content was determined according to ISO standard method 1443:1973. FA and TFA were extracted from traditional meat preparations by accelerated solvent extraction (ASE 200, Dionex, Sunnyvale, CA). Further, fatty acid methyl esters (FAMEs) and TFA were prepared by transesterification using 0.25 M trimethylsulfonium hydroxide (TMSH) in methanol (EN ISO 5509:2000). Separation and quantification of the FAMEs and TFA was carried out using a gas chromatograph (Shimadzu 2010, Japan) equipped with a flame ionization detector and an automatic sample injector and using fused silica capillary column HP-88 (100m, 0.25 mm i.d., 0.2 μm film thickness). The chromatographic conditions were as follows: initial column temperature 125 °C and ending 280 ºC. The injector and detector were maintained at 250 and 280 °C, respectively. Nitrogen was used as the carrier gas at a constant flow-rate of 1.33 mL · min^{-1}.The split ratio was 1:50 and 1 μL of solution was injected. Individual FAMEs and TFA were identified by comparing their retention times with those of authenticated standards (Supelco 37 component FAME Mix, Supelco, Bellefonte, USA). Data regarding FAME composition were expressed in percentage according to the weight of the total identified FAMEs.

2.3. Calculation of daily intake of total fat, saturated fats and trans fat

The rules on labelling and advertising in Serbia [10] and in the US [11] recommend intakes of fats 70 g d^{-1}, saturated fats of 20 g d^{-1}, and trans fat of 40 g d^{-1}. These values are informative for consumers in interpreting nutritional values of food products.

2.4. Statistical analysis

Data obtained for the FA compositions were subjected to analysis of variance (ANOVA) with the Tukey-Kramer HSD test for the comparisons of means at the 5 % level of significance. Statistical analysis was performed using SAS Institute Inc. JMP 10 software.

3. Result and discussion

The FA compositions and TFA of different traditional meat preparations are presented in Table 1.

Table 1. FA composition (g in 100 g) of traditional meat preparations, AI, TI, fat content and TFA

FAs	Fresh sausage (n=6)	Ćevapčići (n=6)	Pljeskavica (n=6)	Hamburger (n=6)
C14:0	1.49 ± 0.16^{NS}	2.11 ± 0.11^{NS}	2.38 ± 0.02^{NS}	2.49 ± 0.09^{NS}
C15:0	0.11 ± 0.02^{B}	0.27 ± 0.02^{AB}	0.27 ± 0.01^{AB}	0.33 ± 0.01^{A}
C16:0	27.50 ± 0.28^{NS}	27.55 ± 0.20^{NS}	27.05 ± 0.02^{NS}	28.76 ± 0.22^{NS}
C16:1	2.49 ± 0.18^{B}	3.40 ± 0.24^{AB}	4.94 ± 0.03^{A}	3.08 ± 0.13^{B}
C17:0	0.41 ± 0.03^{C}	0.64 ± 0.02^{B}	0.66 ± 0.01^{AB}	0.83 ± 0.01^{A}
C18:0	13.39 ± 0.02^{C}	14.65 ± 0.05^{B}	12.38 ± 0.07^{D}	16.94 ± 0.07^{A}
C18:1cis-9	44.28 ± 0.19^{BC}	44.92 ± 0.10^{AB}	45.76 ± 0.08^{A}	43.61 ± 0.15^{C}
C18:1 trans-9	0.45 ± 0.07^{C}	0.96 ± 0.05^{BC}	1.36 ± 0.09^{B}	2.20 ± 0.07^{A}
C18:2n-6	10.13 ± 0.48^{A}	8.91 ± 0.79^{AB}	5.22 ± 0.07^{BC}	2.63 ± 0.06^{C}
C20:0	0.23 ± 0.01^{A}	0.23 ± 0.01^{A}	0.12 ± 0.01^{B}	0.15 ± 0.07^{B}
C18:3n-6	0.03±0.01	nd	nd	nd
C18:3n-3	0.43 ± 0.02^{NS}	0.36 ± 0.01^{NS}	0.44 ± 0.01^{NS}	0.34 ± 0.01^{NS}
C20:1	0.45 ± 0.03^{A}	0.49 ± 0.04^{A}	0.17 ± 0.01^{B}	nd
C20:2n-6	0.30 ± 0.03^{A}	0.19 ± 0.02^{AB}	0.04 ± 0.01^{B}	0.05 ± 0.01^{B}
C22:0	0.03±0.01	nd	nd	nd
C20:3n-6	0.04 ± 0.01^{NS}	0.03 ± 0.01^{NS}	0.05 ± 0.01^{NS}	0.04 ± 0.01^{NS}
C22:1n-9+C20:4n-6	0.10 ± 0.01^{A}	0.09 ± 0.01^{A}	0.04 ± 0.01^{B}	0.06 ± 0.01^{AB}
C20:5n-3	nd	nd	0.05 ± 0.01^{NS}	0.03 ± 0.01^{NS}
C22:5n-3	nd	0.09±0.01	nd	nd
SFA	43.21 ± 0.44^{B}	46.34 ± 0.50^{AB}	42.33 ± 0.35^{B}	49.08 ± 0.38^{A}
MUFA	46.53 ± 0.20^{C}	47.87 ± 0.04^{BC}	51.03 ± 0.14^{A}	48.27 ± 0.22^{B}
PUFA	11.66 ± 0.56^{A}	6.60 ± 0.36^{B}	5.55 ± 0.21^{B}	3.50 ± 0.20^{B}
n-3	0.43 ± 0.14^{NS}	0.36 ± 0.19^{NS}	0.49 ± 0.03^{NS}	0.49 ± 0.03^{NS}
n-6	10.49 ± 3.05^{A}	5.55 ± 2.58^{B}	5.31 ± 0.42^{B}	5.31 ± 0.41^{B}
n-6/n-3	28.41 ± 7.42^{A}	22.25 ± 8.83^{A}	10.76 ± 0.82^{B}	7.30 ± 1.40^{B}
AI	0.58 ± 0.10^{B}	0.65 ± 0.09^{B}	0.64 ± 0.05^{B}	0.77 ± 0.02^{A}
TI	1.42 ± 0.13^{B}	1.28 ± 0.51^{B}	1.40 ± 0.05^{B}	1.82 ± 0.03^{A}
Fat	23.28 ± 1.23^{A}	22.43 ± 0.02^{AB}	15.48 ± 0.45^{BC}	11.20 ± 0.34^{C}
TFA	0.45 ± 0.14^{C}	0.96 ± 0.13^{BC}	1.35 ± 0.46^{B}	2.30 ± 0.90^{A}

*n, number of samples; results are represented as mean ± SEM; nd = not detected <0.01%. Values in the same row with the same letter are not significantly different (P≥0.05); NS – not significant; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; AI – atherogenic index; TI – thrombogenic index; TFA – trans fatty acids

Generally, traditional meat preparations were characterized by higher contents of SFAs. The monounsaturated fatty acid (MUFA) content of the traditional meat preparations was the second most predominant fatty acid group, with oleic acid (C18:1n-9) being the most common MUFA. The most common n-6 polyunsaturated fatty acid (PUFA) was linoleic acid (C18:2n-6). The most common n-3 PUFA was α-linolenic acid (ALA, C18:3n-3), which occurred in lower amounts (Table 1). Fat E% was higher in fresh sausages (33%) and ćevapčići (32%) (Table 2) than the recommended value of 25-30% [8, 9]. Saturated fats E% (27-50%) was higher than the recommended value of 10% [8, 9]. Trans fat was also higher than the recommended value of 0.5% for ćevapčići, pljeskavica and hamburger (Table 2), and lower in fresh sausages [8, 9]. Pljeskavica (33%) had similar E% (33.90%) to fine minced pork cooked sausages [12]. Hamburger was a low fat meat with high levels of SFA E% (27%), which is similar to beef luncheon meat (23%), and with high levels of trans fat E% (0.62%) [13]. Fresh sausage (50%) was similar in SFA E% (53%) to pork liver pâté [13].

The lipid indices of AI and TI, calculated from the data on the FA composition, are shown in Table 1 according to equations of Ulbricht and Southgate [14]. These lipid indices indicate the suitability of food for prevention of cardiovascular disease in humans, and for any health benefit, they have to be low. In terms of human health, AI and TI less than 0.5 and 1.0, respectively, are recommended in the diet [15]. There were significant differences ($P < 0.05$) in the AI between traditional meat preparations we examined, being the lower in fresh sausages (0.58), ćevapčići (0.64) and pljeskavica (0.64) and the highest in hamburger (0.77). The AIs of these Balkan products were higher than in study of pork meat (0.51-0.54) [16] and pork meat reported by Kasprzyk et al. [17] (0.47) but lower than in rabbit (0.90) [18]. Concerning TI values, there was a significant difference between traditional meat preparations ($P < 0.05$) being the lowest in fresh sausages (1.42), ćevapčići (1.28) and pljeskavica (1.40) and highest in hamburger (1.82). The TI values we obtained are lower than were reported in lamb (0.87) [19], chicken (1.14) and turkey meat (0.91) [20, 21]. However, AI and TI of traditional meat preparations were higher than recommended indices [13] and are not desirable for health protection.

The n-6/n-3 fatty acid ratio in fresh sausages was above the recommended levels of 4:1 [22] (Table 1). The n-6/n-3 ratio was significantly ($P < 0.05$) lower in pljeskavica (10.76) and hamburger (7.30) compared with ćevapčići (22.25) and fresh sausages (28.41). However, the n-6/n-3 ratio in fresh sausages and ćevapčići were higher than in pâtés (4.01-21.86), cooked chicken sausages, chopped canned meats (8.55-14.98) [13], pork canned meat pieces (13.80) [12] and pork meat (19.62-20.88) [16]. The estimated percentage of fat daily intake, saturated fat daily intake and trans fat for products is presented in Table 2.

Table 2. Percentage of total fat, saturated fat derived and trans fat from traditional meat preparations in relation to the reference intake of 2,000 kcal per day

Daily intake	Fresh sausages	Ćevapčići	Pljeskavica	Hamburger
Fat E%	33	32	22	16
Saturated fat E%	50	52	33	27
Trans fat E%	0.26	0.54	0.53	0.62

Legend: E% - energy value/intake

By the linear discriminant analysis (LDA), the separation between traditional meat preparations might be improved. LDA clearly differentiates the sampled traditional meat preparations into four groups (Figure 1).

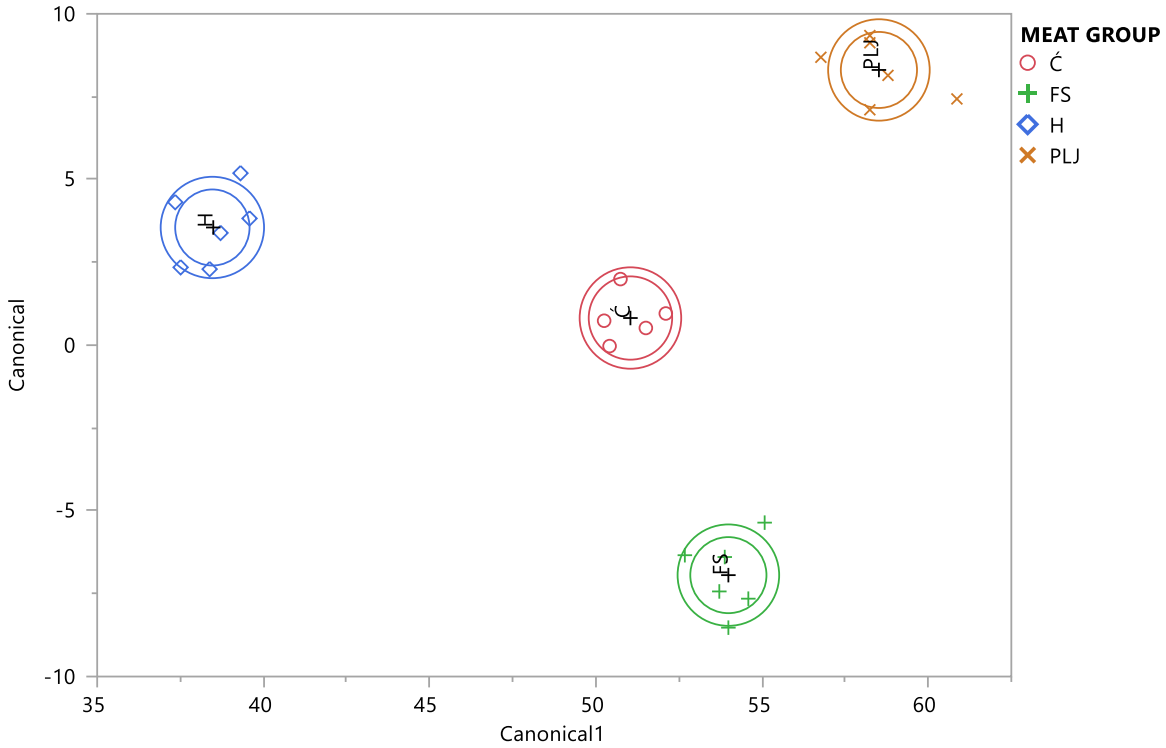

Figure 1. Linear discriminant analysis (canonical plot) of fatty acid profiles of traditional meat preparations
Legend: Ć – ćevapčići/ćevap, FS – fresh sausages, H – hamburger, PLJ –pljeskavica

The results of the classification are very satisfactory and allow 100% of traditional meat preparations to be correctly grouped. Out of 24 samples of traditional meat preparations, all 24 samples were classified according to the fresh meat origin. Data were expressed as discriminant scores along 2 eigenvectors, as a function of group provenance regarding FA content. All FAs herein considered had a direct influence on the differentiation of group origin. The first discriminant eigenvalue explained 61.3% of the total variance and the second eigenvalue explained 33.8% of the total variance. The established Wilks value was equal to 0.0005 ($P < 0.0001$). By canonical correlation, the first and the second discriminant functions were 0.993 and 0.987, respectively. The shortest distance between the points on the canonical plot in Figure 1 represents the smallest differences in the FA profiles of the samples. Pljeskavica was very distant from hamburger, ćevapčići and raw sausages and hamburger far from pljeskavica, ćevapčići and fresh sausages; these results correlate to the type of meat. Based on stepwise variable selection, the fat content of fresh meat did not influence the separation of samples ($P > 0.05$). Our results confirm the statement of other authors [23, 24]. They conclude that the region of South-Eastern Serbia has a population of lower socio-economic status, potentially coupled with malnutrition and poor dietary habits. The results obtained indicate a high intake of saturated fats from consumption of traditional meat preparations in the region. Although this dietary pattern does not appear hazardous, improving nutritional habits is desirable. Education and national/regional policies for advancement of dietary quality in Balkan regions should promote complex carbohydrates and relevant food choices such as whole grains, legumes, fruits and vegetables; as well as optimize n-3 balance through increased intake of fish, seafood, nuts and green vegetables.

4. Conclusion

People in the Balkan region want to eat fresh sausages, pljeskavica, ćevapčići and hamburger. Our research shows these traditional products are full of saturated fat. Although bad food does not really

exist, bad eating habits can impair health in the long-term. The Balkan region has a population of lower socio-economic status with poor dietary habits. There is a lot of strong evidence to support the benefits of the Mediterranean diet on cardiovascular health. Promotion of the Mediterranean diet requires changes in the food environment, the food systems, and public health policies to improve overall diet quality of individuals, communities, and populations.

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Surface adsorption and survival of SARS-CoV-2 on frozen meat

Surface adsorption and survival of SARS-CoV-2 on frozen meat

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Abstract. The first case of a severe acute respiratory syndrome caused by coronavirus-2 was reported in December 2019 in China. The disease spread globally quickly, causing the 2019–2021 COVID-19 pandemic. The meat industry became concerned over the possibility of transmitting the virus in the slaughterhouse environment. The level of air exchange strongly affects the distribution of SARS-CoV-2 aerosols within the slaughterhouses. The adsorption of the SARS-CoV-2 virus on the surface of the frozen meat is dictated mainly by the interplay of electrostatic forces between the virion and tissue (pH) and environmental conditions (temperature and humidity) in the vicinity of adsorption micro-location. Suppose the virus contaminates the meat surface, whereby pH is 5.5 or less. In that case, it firmly adsorbs due to bonds established by protonated amine group and a hydrogen bond between the COOH group of the viral protein and oxygen in hydroxyl groups present on meat surfaces. The meat surface, coated with a thin water film, interacts with the SARS-CoV-2 virions by establishing strong hydrogen bonds. Although there is no proof of COVID-19 contraction by food consumption, the strong surface adsorption and ability of SARS-CoV-2 to survive meat freezing indicate a potential risk of virus transmission by meat.

1. Introduction

One of the most severe varieties belonging to the β-CoV genus of coronavirus – the SARS-CoV-2 – has emerged in late November 2019 in Wuhan, China causing a global pandemic. The World Health Organization (WHO) officially announced this fact on March 11, 2020 [1]. Over the following months, the infection rate raised progressively, so the disease became a severe global threat to public health, the international economy, and the eudaemonia of individuals.

The SARS-CoV-2 virion particle measures 125 nm in diameter. The particle itself consists of the envelope (composed of lipids and proteins) and a single-stranded RNA molecule (29,903 nucleotides). Viral RNA contains a couple of genes encoding structural proteins and enzymes responsible for its reproduction and spreading.

To control a pandemic, one of the risk assessment factors was understanding the virus's spreading mechanism. The majority of scientific reports have claimed that the principal transmission route of the SARS-CoV-2 was to be from person to person, primarily through aerosols or droplets released from the respiratory tract during coughing or sneezing and then inhaled [2]. Also, several reports indicated plausible transmission of the virus via the gastrointestinal tract based on the successful isolation and

identification of SARS-CoV-2 RNA from feces [3]. Although respiratory RNA viruses possess a lipid envelope, their general structure is not robust. Single-stranded RNA is a chemically unstable molecule and readily undergoes hydrolysis. In particular, ribonucleases that are ubiquitous in the environment (mainly originating from the human skin) tend to denature viral RNA quickly. Outside of the human body, SARS-CoV-2 has a short life. Virions tend to degrade in dry conditions and during irradiation by ultraviolet light from sunlight or artificial sources. The harmful effect of UV reflects in cross-linking of the purine and pyrimidine bases in the viral RNA. With these facts in mind, it is clear that the only chance for the SARS-CoV-2 virus to survive is to nest itself in places where it is protected and appears in huge numbers.

Following the reports on COVID-19 outbreaks in major U.S. and German meat processing facilities [4,5], the meat industry became concerned over the possibility of transmitting the virus in the slaughterhouse environment. Working conditions in slaughterhouses and meat processing plants are aimed to maintain a low ambient temperature for food safety reasons. However, these conditions improve the survivability of SARS-CoV-2 at lower temperatures in comparison to room temperature [6]. Next, the coughing or sneezing of the infected workers in this environment is followed by airborne transmission of the aerosolized virus from the lungs. Duration of the aerosol infectivity is mainly dependent upon droplet size and air circulation intensity. Tiny virus-laden aerosol droplets evaporate quickly on the surfaces, and if the intensity of air circulation in a closed environment is high, one could consider that the virus does not vanish at all. The majority of slaughterhouses are equipped with a forced ventilation system. The air exchange inside these facilities consists of forceful moving of outdoor air and recirculating inside airflow. In some meat industries, operating procedures such as meat processing and packaging involve minimal and close contact between the workers during all-day shifts, so the level of air exchange strongly affects the distribution of SARS-CoV-2 aerosols indoors, presenting significant risk.

2. Meat surface adsorption of the SARS-CoV-2

The WHO report declared the possible transmission of SARS-CoV-2 by frozen food packages in February 2021 [7]. This report raised concerns if COVID-19 could be contracted by consumers coming into contact with contaminated frozen foods and packaging. Of course, this virus is not a foodborne pathogen, transmitted by a fecal-oral route such as Norovirus or Hepatitis A virus, diminishing the risk of COVID19 contraction by oral consumption to almost zero. However, β-CoV genus coronaviruses such as SARS-CoV and MERS-CoV substantially differ in transmissibility potential, which is reflected in a violent spreading rate, particularly noted in crowded environments [8]. The deposition of virus-contaminated droplets onto frozen meat and fomites significantly increases the risk of people being infected through daily contact with contaminated surfaces or objects (fomites).

The adsorption of the SARS-CoV-2 virus on the surface of the frozen meat is dictated mainly by the interplay of electrostatic forces' interactions between the virion and tissue (pH) and environmental conditions (temperature and humidity) in the vicinity of adsorption micro-location.

2.1. The influence of pH on SARS-CoV-2 adsorption to the meat surface

In a normal living muscle, the pH is approximately 7.2. At this pH, myofibrillar proteins have a net negative charge, otherwise responsible for water-holding capacity. Several hours after the slaughtering, during the *post mortem* changes, the pH value of the muscle tissue drops to pH=5.4-5.8 (pork), i.e., pH=5.4-5.7 (beef). Once the isoelectric point of myofibrillar proteins is reached, they possess a net neutral charge, decreasing their water-holding capacity.

The majority of SARS-CoV-2 virions carry a net negative charge at neutral pH since their isoelectric point is below pI 7 [9]. However, the size of SARS-CoV-2 particles is relatively large (compared to other RNA viruses). This results in extreme heterogeneity of its outer surface proteins that contain multiple "patches" of positive and negative charge in the pH range rendering viruses stable [10, 11]. The isoelectric point of the SARS-CoV-2 "spike" glycoprotein is pI=6.2 [12]. Suppose a SARS-CoV-2 virus is transmitted on the surface of the muscular tissue when the meat pH is approximately 5.5. In that case,

the net charge of the SARS-CoV-2 particle will become positive due to the protonation of both the carboxylate and amine groups. Protonated amine group (NH_3^+) will electrostatically bind to the electron-rich meat matrix. At the same time, a hydrogen bond will be established between the COOH group of the viral protein and oxygen in hydroxyl ($^-$OH) groups present on meat surfaces. Both processes will promote strong virus adsorption.

2.2. The effect of humidity and temperature molecules on SARS-CoV-2 adsorption to the meat surface

The capacity of SARS-CoV-2 to survive and continue to be infectious is also governed by the humidity and temperature [13]. The complex interaction between the virus and meat surfaces is primarily governed by the surface energy of the water molecules [12].

In the liquid phase, present water molecules tend to shrink from their vapor phase on a surface between the outer edge of the viral envelope and meat matrix. Subsequently, the shrinking of water molecules forms liquid "bonds" of a curved shape [14]. The hydrophilic meat surface, coated with a thin capillary water film, interacts with the SARS-CoV-2 virions by establishing strong hydrogen bonds between water molecules and proteins protruding through the virus envelope. Furthermore, suppose there is a gap between two adjacent virus particles whose distance is smaller than the distance between the virus and meat surface. In that case, the water molecules can quickly fill that gap, creating an active centre for further aggregation of SARS-CoV-2 particles.

Once the temperature in the environment rises higher than 12°C, the thin water engulfing virions into complexes tends to vaporize, leading to molecular instability, significantly lesser water bridge-linking, and a lower quantity of SARS-CoV-2 particles that potentially could adsorb onto frozen meat surface. The fact indicates that in a slaughterhouse environment, at temperatures of 4-7°C, water molecules (meat matrix, condensation, washing) play an essential role in keeping virus-laden droplets infectious long enough.

3. SARS-CoV-2 survival in frozen meat

Currently, there is no scientific proof to support the hypothesis that handling or consumption of food is associated with the contraction of COVID-19. Current scientific opinion is that the probability of exposure of consumers to SARS-CoV-2 via food is very low with high uncertainty [15]. However, the uncertainty associated with this estimate is high as there is still no evidence to confirm or refute the hypothesis that people can be infected by ingesting SARS-CoV-2 in food.

Several published papers have dealt with the topic. A research study by Han et al. [16] investigated a series of findings involving frozen food and storage environment as carriers of SARS-CoV-2, discussing the likelihood of contamination in "cold chain." In the same study, the authors hypothesized that low temperatures could generate a favorable condition for SARS-CoV-2 to maintain its viability during more extended exposure. Next, Dhakal et al. [17] assessed for survivability of herpes simplex virus 1 and SARS-CoV-2 in chicken and seafood. In this study, these two viruses were held at 4°C and at 0 h, 1 h, and 24 h after inoculation. At all three time points, recovery of SARS-CoV-2 was similar from chicken, salmon, shrimp, and spinach, ranging from 3.4 to 4.3 log PFU/mL. However, the rate of virus recovery from apples and mushrooms at T0 was significantly lower compared to poultry and seafood. In the end, they discovered that direct comparison of infectious virus titers with viral genome copies using the common RT-qPCR method could (at best) indicate only the presence of SARS-CoV-2 RNA. The result, in no way, correlates with the number of infectious viruses. Moreover, Harbourt et al. [18] reported that SARS-CoV-2 remains stable on porcine skin for 96 h at 22°C, 8 h at 37°C, and 14 days at 4°C In their opinion, these findings indicate a substantial risk of infection and virus shedding by meat handling. However, no published data exist on the long-term survival and infectivity of SARS-CoV-2 in essential commodities such as beef and pork.

4. Conclusions

In meat processing facilities where close-proximity working procedures are often encountered, the aerosols from the respiratory tract of the infected workers act as a primary source for meat contamination. The poor filtration of recirculated air, high relative humidity, and a slightly acidic pH favour electrostatic adsorption of SARS-CoV-2 to the surface of frozen meat. Once adsorbed, SARS-CoV-2 is capable of surviving chilling and most probably freezing temperatures. Although there is no proof of COVID-19 contraction by food consumption, the food business operators should be aware of potential risks of virus transmission by meat. They should also implement more stringent antimicrobial measures during this pandemic since the SARS-CoV-2 is way more resistant than bacterial flora commonly found in food processing facilities.

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Organic agriculture – importance and development

Organic agriculture – importance and development

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Abstract. Agriculture, as the most important strategic industry, is tasked with providing sufficient quantities of quality and safe food. Intensive and often excessive, uncontrolled, and unskilled use of the means for protection and nutrition of plants, as well as means for the prevention or treatment of animals, are carried out to increase yields in conventional production. This approach in food production has contributed to the increasingly common endangerment of the health of plants, animals and humans, as well as significant environmental endangerment. Unlike conventional production, organic food production is now increasingly attracting interest from modern consumers. However, organic agriculture involves not only producing without artificial fertilizers and other agrochemicals, but without antibiotics and hormones too. It is more of a holistic production system that functions as a sustainable unit, and unites interconnected and conditioned actors: plants, animals, microorganisms, insects, organic and mineral soil matter, and humans. In Serbia, organic agriculture has been developing for the last thirty years. However, the intensive development of organic agriculture has only happened in the last decade, with plant organic production being more developed than animal organic production.

1. What is organic agriculture and organic food?

An estimated 1.8 billion people worldwide are involved in agriculture, with the majority of the population using conventional principles of production. Conventional, predominant food production is based on the requirement to meet the growing needs for large amounts of food, which is conditioned primarily by population increase and society's unstoppable imperative to increase available capital [1]. The quality of these foods has long been questioned, primarily due to the extensive and often uncontrolled or unregulated use of agrochemicals, antibiotics, and hormones. In 2019, the agrochemical market worldwide was worth approximately US$234.2 billion. This is expected to increase to more than US$300 billion in 2025 [2].

Organic food production developed as a spontaneous and natural solution to this existing problem. Organic production as part of ecological, sustainable development includes food production (primary agriculture, processing to distribution), but also some other products of plant and animal origin (fabric, leather). According to the National Organic Standards Board, it is "an ecological production management system that promotes and enhances biodiversity, biological cycles and soil biological

activity; it is based on minimal use of off-farm inputs and on management practices that restore, maintain, and enhance ecological harmony" [3].

Based on these characteristics and combining the best elements of tradition, innovation and science, the overall health of land, ecosystems and humans is achieved. In addition to the above and the most comprehensive definition of organic production, there is a large terminological patchwork in the world and in our country, which often confuses the public. Namely, only in Serbian and related languages, there is talk of "organic production/agriculture", "ecological production/agriculture", "biological production/agriculture", "alternative production/agriculture", "sustainable production/agriculture", "biodynamic production", etc. On the other hand, in English literature, one can meet terms such as "organic agriculture", "organic production", "organic farming" (U.S), whereby the author's tendency is to highlight the difference between this agricultural practice and "sustainable agriculture". In addition to the above, terms "biological agriculture" (Germany, Denmark, Western European countries) "produzione biologico", "agricoltura biologica" (Italy), "production/agriculture biologique" (France) are in use [4]. However, regardless of the term used, organic agriculture indicates activities aimed at introducing an ecological dimension into agricultural production, i.e., management of agriculture with respect to the principles of environmental preservation [5, 6].

Describing organic food, some authors tie it to principles of "biological" or "natural production" [7], others associate it with "green concepts" and "environmental friendliness" [8], while some authors identify it with the absence of the use of agrochemicals and veterinary medicines in food production [9]. In any case, organic food is "a result of the organic philosophy practices and principles" [10].

Organic agriculture is based on the application of the principles of agroecology, which includes a prohibition on the use of artificial mineral fertilizers and pesticides, as well as genetically modified organisms [8]. Similarly, related to animal production, organic production regulations prohibit the use of hormones, growth promotors etc., while the use of antibiotics is severely restricted (they are allowed only in special cases). It also prohibits the use of many other synthetic compounds that, as food additives, are used in the conventional food chain (e.g., preservatives, colouring agents) [11, 12]. In organic animal production, all actions are directed towards the health and well-being of domestic animals.

However, organic agriculture is not only producing without artificial fertilizers, other agrochemicals, antibiotics and hormones. It is more of a holistic production system that functions as a sustainable unit, and unites interconnected and conditioned actors: plants, animals, microorganisms, insects, organic and mineral soil matter and humans [13]. In other words, the role of organic agriculture is to sustain and improve the health of the ecosystem as a whole, as well as all present organisms, from the smallest ones found in the soil, through plants and animals, to humans. On this basis, the International Federation of Movements for Organic Agriculture [12] has defined the basic principles of organic agriculture development, the primary goal of which is to produce high quality healthy food, with preventive environmental action and well-being: the principle of health, the principle of ecology, the principle of fairness and the principle of nurturing and aging.

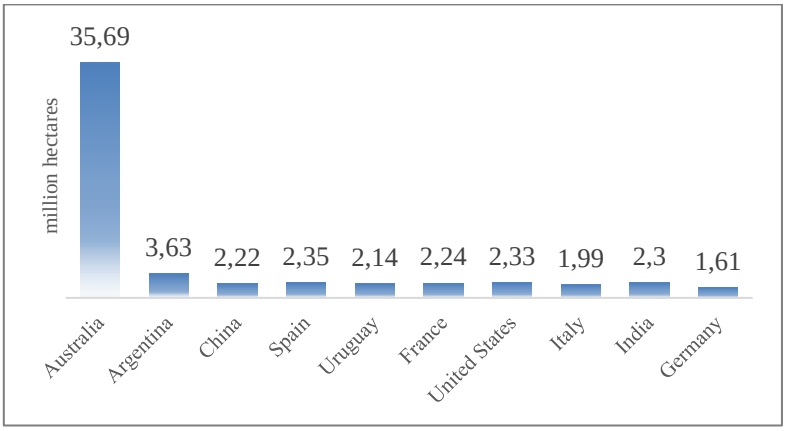
Definitely, organic food is now increasingly attracting interest from modern consumers, and we can see it as food that is grown with the utmost consideration for the environment and animals, and which is safe and of suitable quality [11, 14].

2. Organic production in the world

According to the Food and Agriculture Organization (FAO), organic agriculture is gaining popularity among farmers and consumers around the world [15]. In the last 20 years, the area under organic cultivation has nearly doubled, from 15 million hectares in 2000 to an estimated 71.5 million hectares in 2018. Figures from 2019 show that India was the world's leading producer of organic food (1.4 million organic food producers, which is higher than the number of all organic food producers in the other nine leading countries in the world combined) [16]. The biggest consumers of organic food by population are Denmark and Switzerland (where people spend about €344 and €338 per capita annually, respectively, on organic food). Of the ten leading countries with the highest organic food consumption per capita, the top eight are in Europe. In ninth and 10th place are the United States and Canada,

respectively. On the other hand, although India has the largest number of organic food producers, Australia has by far the largest share of organic agricultural land (approximately half of the world's total organic agricultural land). Behind Australia are Argentina and China with 5.08% and 4.39%, respectively, of world organic agricultural land (Table 1).

Table 1. Organic agricultural land area worldwide in 2019, by leading country (in million hectares) [16]



3. Organic production in the Republic of Serbia

The first organized organic food production in Serbia and its exports to the EU market are tied to the period three decades ago. That is when the Den Juro Organic company from Brus started developing organic fruit production in southern Serbia (around Blace). Today, after 30 years of development, exports of organic products from Serbia (2020) reached €37 million [17]. This is further encouraged by the fact that over 80% of the land in Serbia is uncontaminated, which provides significant preconditions for the development of this type of production [18]. Also, in parallel with the development and institutional organization of the organic sector, public (consumer) awareness is developing, as well as new markets in the country and the world.

During the past ten years, agricultural production in Serbia showed a growing trend (annual growth was approximately 10%). Out of the total agricultural area (5,734,000 ha, of which 4,867,000 ha is arable), about 0.60% is under organic production, in contrast to European countries where it is about 6%. In 2018 and 2019, we saw a significant increase in areas under meadows and pastures due to the development of livestock production, which is developing more intensively in regions with hills and mountains.

During 2019, organic plant production took place on an area of 13,725 ha (arable land – 9,880 ha, meadows, and pastures – 3,845 ha), while an additional 7,539 ha is in the conversion period. In addition to great interest from modern consumers, the government directly supports the development of organic food production by implementing subsidies.

In Serbia, fruit, cereals, industrial and fodder plants are mostly grown according to the principles of organic production. In fact, organic arable production (industrial plants, cereals, fodder, medicinal and aromatic plants) is the most prevalent (it accounts for 57% of organic production), followed by fruit production (33.5%), while vegetable production is very underrepresented (1.15%). Other crops make up 8.35% of organic production according to production area [17, 19].

Organic plant production by regions in Serbia is shown in Figure 1.

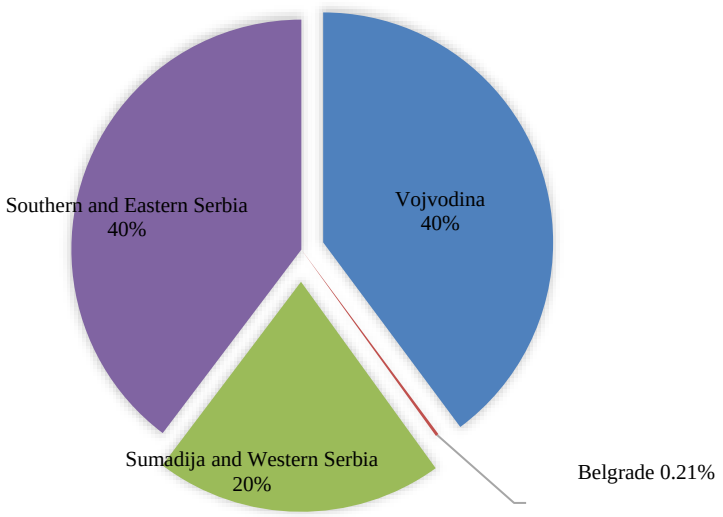


Figure 1. Organic plant production in Serbia by regions

Organic livestock production in Serbia is still poorly developed. This can be explained by the fact that this type of production is significantly more demanding than organic plant production. Namely, the Law on Organic Production defined special construction, technical and sanitary conditions for the facilities in which these animals are bred; their procurement procedures from other farms are strictly controlled, while the manner of nutrition and treatment is tailored to the principles of ecology and safety. At the same time, special conditions are prescribed for facilities in which organic products of animal origin are produced, stored, and transported [18]. Most of all, poultry, bee societies, sheep and pigs are bred this way [17, 19].

The basis for the development of organic production was accompanied by the development of domestic legislation in this area (Law on Organic Production, Official Gazette of RS, No. 30/10, Rulebook on Control and Certification in Organic Production and Methods of Organic Production, Official Gazette of RS, No. 95/20, Rulebook on Documentation submitted to an authorized control organization for issuing of certificate, as well as on the conditions and manner of sale of organic products, Official Gazette of RS, No. 88/16), harmonized with the legal achievements of the European Union, Codex Alimentarius (FAO/WHO), ISO documents, Council Regulation (EC) No. 834/2007, Commission Regulation (EC) No. 889/2008.

4. Conclusions

The growing education level of modern consumers, accompanied by their expressed awareness of the need for healthy food and healthy lifestyles, has contributed to increased interest and demand for organic products. This legislated form of food production is particularly pronounced in highly developed countries, where we have significantly higher demand relative to supply, which provides great opportunities for the development of this agriculture sector. Organic agricultural production combines the best models from nature with a high respect for environmental principles and the preservation of natural resources, as an imperative to implement natural procedures and substances, while limiting or completely eliminating synthetic chemical substances.

Today in Serbia, organic agriculture is increasingly popular and has a tendency to grow. Thanks to its potential, which is primarily reflected in small properties, and on land that is not contaminated with harmful substances, this type of agriculture can contribute significantly to the development of rural areas, and thus, in general, to agriculture in the country.

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Seasonal overview of beef meat quality in a small-scale slaughterhouse

Seasonal overview of beef meat quality in a small-scale slaughterhouse

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Abstract. The objective of this study was to investigate a possible relationship between blood parameters related to animal welfare and defined beef meat quality characteristics during winter and summer seasons in one small-scale slaughterhouse. At exsanguination, blood samples were collected, and serum concentrations for total proteins (TP), albumin and C-reactive protein (CRP) were evaluated. After 24 h of chilling, ultimate pH was measured and meat samples were used for drip loss and cooking loss determination. Dehydration was not observed during seasons, while elevated concentrations of TP accompanied by higher CRP values pointed to summer as a more stressful season. Analysing the meat quality parameters, it was observed that during the two seasons, ultimate pH values were in the range for normal meat acidification, but values for drip and cooking loss were significantly increased during the summer season. In conclusion, CRP could be used as potential biomarker for beef meat quality estimation, in the first instance drip loss and ultimate pH.

1. Introduction

Beef meat quality has increasingly become an important production trait worldwide, both from the aspect of customer satisfaction and of the economic profit of the industry [1-2]. Conditions and procedures with animals, starting from the farm, during transportation and in the lairage period are just some of the elements that could greatly affect the quality of meat [3]. Improper conditions at farm level lead to animals' adoption of the negative experience and inability to acclimatize to the new environment [4-5]. Inadequate handling during transportation, dehydration, limited space in trucks, climatic conditions, mixing of animals in the lairage, and social dominance are some of the disturbed procedures that impair animal well-being and affect the level of the individual's stress response [6-7]. Challenges, especially before slaughter, activate an autonomic response, and as a product, glucocorticoids are released, which perturb metabolism homeostasis, particularly at muscle level [8]. It has been reported that in term of returning the homeostasis balance, synthesis of acute phase proteins, depending on the type, is increased or decreased in order to gain protective effects against acute stressors [9-10]. Unlike pigs, humans and dogs, in cattle, C-reactive protein (CRP) is not considered as prototypical acute phase protein which could be used for diagnostic herd health assessment [11]. Despite its synthesis during acute infection, it has been observed that during severe stressful reactions, a large amount of CRP is

released in the blood with the purpose of achieving immediate protection [12]. All the above mentioned adverse conditions of the animal production cycle can alter meat quality parameters, particularly due to impaired pH, affecting water holding capacity, meat tenderness and colour [13-14].

The objective of this study was to investigate a possible relationship between blood parameters related to animal welfare and defined beef meat quality characteristics during winter and summer seasons in one small-scale slaughterhouse.

2. Materials and methods

The study was conducted on 44 Simmental crossbreed bulls, 22 per season with an average live weight of 550±20 kg. The transport distance was short, about 70 km, and animals were rested for 1 h at lairage. The sampling was performed during February and July, referred as winter and summer season, respectively. During exsanguination, a plastic cup was used to collect the blood samples which were immediately transferred to the tube with potassium oxalate. Within 4 h, the samples were centrifuged at 2500 rpm, and the serum was separated and immediately frozen for further analysis. The serum samples were analysed for total protein (TP), albumin and CRP concentrations using an automated analyser (Architect c8000, Abbott, Wiesbaden, Germany).

Ultimate pH of *Musculus Longissimus dorsi* was measured after 24 h of chilling between the 10th and 12th ribs using a portable pH meter (Testo 205, Testo AG, Lenzkirch, Germany). From the same anatomical position, 2.54 cm thickness meat samples were taken for water holding capacity determination. Evaluation of water holding capacity was done using two methods: drip loss and cooking loss. Drip loss was tested using a bag method [15] by measuring the initial weight of each individual meat sample and weighing it again after 48 h of storage period at 4 °C. Differences of two weights gave drip loss percentage. Thereafter, the samples were put in plastic zip bags, boiled until reaching 75 ºC of internal temperature, cooled under chilled conditions (1-4 ºC) and then re-weighed [15]. Cooking loss was expressed as a percentage of initial weight.

Before any statistical analysis, the obtained data were checked for normality using Shapiro-Wilk test ($P>0.05$) and outliers were rejected. Differences between winter and summer seasons in terms of meat quality parameters (pH, drip loss, cooking loss) and animal-related factors (TP, albumin, CRP) were evaluated using Student's t test. Pearson's test was also used for estimation if there was any correlation between and within meat quality and animal-related characteristics. Statistical analysis of the results was performed using SPSS 21 software package.

3. Results and discussion

Concentrations of TP and CRP taken from the bulls during summer season were significantly increased while albumin concentration as an indicator of dehydration was slightly increased, but not significant (Table 1). Dehydration, as a negative state of transportation and lairage conditions, particularly with long journeys was not manifested in this case, because it was a short transport with relatively short animal retention at abattoir level [16].

Table 1. Evaluation of animal-related parameters in bulls during two seasons

	Winter	Summer
TP g/L	66.09±1.08[a]	70.86±0.95[b]
Albumin g/L	30.32±0.49[a]	31.05±0.48[a]
CRP g/L	2.49±0.13[a]	3.41±0.27[b]

[1]Values are shown as arithmetic mean ± standard error of the mean.
Values with different lowercase letters (a-b) in the same line differ significantly ($P<0.05$)

However, concentration of total serum proteins in the summer season may be an indicator of increased globulin fraction in the blood, including acute phase proteins, which was accompanied by elevated CRP concentration. Evaluating the concentration of CRP, it could be stated that summer as a season was more stressful for bulls, which is not in agreement with a study conducted in Spain where the winter season was significantly more stressful [17].

Analysing the meat quality parameters, it was observed that during the two seasons, ultimate pH values were in the range for normal meat acidification, but water holding capacity was significantly decreased during the summer season (Table 2). When the ultimate pH approaches the isoelectric point, the ability of muscle proteins to bind water is diminished, resulting in greater water release [18]. Within the complexity of water retention at protein level, the elevated stressful reaction, with possible heat stress and decreased ultimate pH, additional analyses are required for better understanding the glycolytic potential of the meat and the development of rigor after slaughter [19].

Table 2. Examination of meat quality parameters during two seasons

	Winter	Summer
pH ^{24h}	5.77±0.04^{a}	5.61±0.03^{b}
Drip loss %	1.68±0.21^{a}	2.99±0.27^{b}
Cooking loss %	26.73±1.36^{a}	32.71±0.60^{b}

[1]Values are shown as arithmetic mean ± standard error of the mean.
Values with different lowercase letters (a-b) in the same line differ significantly (P<0.05)

Correlation coefficients among investigated variables are shown in Table 3. Ultimate pH had an intermediate negative correlation coefficient with drip loss and cooking loss, which was also confirmed in a Hungarian Simmental bull study [20]. It indicates that as pH increased, drip and cooking loss decreased, and vice versa. On the other hand, CRP was negatively correlated with ultimate pH and positively with drip loss, at intermediate level. Practically, this means as CRP concentration increased, ultimate pH decreased and drip loss increased. Based on above, ultimate pH could be used as prediction indicator for water holding capacity *post mortem*, and CRP for ultimate pH and drip loss *pre mortem*. More studies are needed to confirm these facts, especially within the occurrence of dark, firm and dry meat.

Table 3. Correlation coefficients among investigated variables

	pH ^{24h}	Drip loss %	Cooking loss %	TP g/L	Albumin g/L
CRP g/L	-0.40**	0.48**	0.25	0.24	0.49**
Albumin g/L	-0.26	0.22	0.13	0.42**	
TP g/L	-0.32*	-0.13	0.29		
Cooking loss %	-0.55**	0.43*			
Drip loss %	-0.57**				

* P<0.05
** P<0.01

4. Conclusion

Based on the results of this study, CRP could be used as potential biomarker for beef meat quality estimation, in the first instance for drip loss and ultimate pH. Recognition and identification of indicators, important in terms of animal well-being, stress and meat quality and their incorporation in animal production cycle is necessary, with the purpose of achieving higher customer satisfaction and profitability of producers.

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The significance of *Enterobacteriaceae* as a process hygiene criterion in yogurt production

The significance of *Enterobacteriaceae* as a process hygiene criterion in yogurt production

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Abstract. Yogurt is one of the most popular fermented dairy products with a worldwide acceptance. There are many types of yogurt differing in flavor, physical and chemical properties. Yogurt is produced by adding bacterial culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* to milk and cream products. During the period from January 2017 to December 2020, a total of 202 yogurts from different small and medium sized dairy plants were analyzed as part of HACCP self-control programs. The determination of *Enterobacteriaceae* was performed as an alternative indicator of good hygiene practice. The results showed that 21.29% of analyzed yogurts contained more than 10 CFU/g *Enterobacteriaceae*, which is the evidence of poor hygiene or inadequate processing, process failure and post-process contamination. Generally, dairy products are potential vehicles for microorganisms from the *Enterobacteriaceae* family. Good manufacturing practices and good hygiene practices must be followed throughout the production line thoroughly. The absence of classic foodborne pathogens does not indicate that the yogurt is fit for consumption, since other potentially pathogenic bacteria of the *Enterobacteriaceae* family could be present. Thus, rather than pathogen testing, using *Enterobacteriaceae* to monitor the effectiveness of implemented preventive prerequisite measures could offer a better view of the quality, sanitary conditions, and safety of yogurt products.

1. Introduction

Yogurt is one of the most popular fermented dairy products with a worldwide acceptance [1]. There are no available records regarding the origin of yogurt, but it is believed that milk fermentation dates to 10,000-15,000 years ago, together with beginnings of animal domestications. Ancient civilizations such as the Sumerian, Babylonian, Pharonic, Indian, Greek and Roman used to make yogurt in order to preserve milk and extend its shelf-life [2, 3].

At present there are many types of yogurt produced worldwide, which differ in flavor, physical and chemical properties [4]. According to the current requirements of the Serbian Rulebook on milk products [5], yogurt is defined as a fermented dairy product produced by adding characteristic bacterial cultures, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* to milk and cream products.

This easily digestible dairy product is highly nutritious. Although the nutritional composition of yogurt can vary, depending on type of milk used, animal species the milk is obtained from, strains of starter culture used in the fermentation, adding and length of fermentation, it is, generally, a rich source of proteins, carbohydrate, minerals such as calcium and phosphorous, and vitamins such as riboflavin (B2), thiamin (B1), cobalamin (B12), folate (B9), niacin (B3) and vitamin A [6]. However, highly nutritious yogurt is suitable for bacterial growth. During its manufacturing, processing, storage, distribution, and marketing, this dairy product can be subject to inadequate hygiene conditions, which can promote spoilage and contamination with pathogenic microorganisms, including *Enterobacteriaceae* [7].

The *Enterobacteriaceae* family comprises a large group of Gram-negative non-spore-forming bacteria. These facultative anaerobes include some harmless commensal species as well as important human and animal pathogens. Their ubiquitous distribution means that it is inevitable that some members of the *Enterobacteriaceae* will enter the food chain. This family includes a large number of organisms (*Escherichia*, *Salmonella*, *Shigella*, *Yersinia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus*, *Edwardsiella*, *Erwinia*, *Morganella* and *Providencia*) [8, 9]. So, as an alternative indicator of good hygiene practice in the yogurt production line, *Enterobacteriaceae* can be used.

The purpose of this paper was to present the occurrence of *Enterobacteriaceae* in yogurt in small and medium sized dairy plants from Vojvodina, Serbia and to emphasize the importance of good hygiene practices along the yogurt production line.

2. Materials and methods

During the period from January 2017 to December 2020, a total of 202 yogurts from different small and medium sized dairy plants were collected. All samples were part of HACCP self-control programs. The yogurt samples were transported from the dairy plants to the laboratory of the Scientific Veterinary Institute Novi Sad in cooling transport boxes at ≤ 4 °C in their original packages, and analyzed for the presence of the *Enterobacteriaceae* following the standard method ISO 21528-2 [10, 11] within 24 hours. After incubation on violet red bile glucose agar (VRBG) (Biokar Diagnostics, France), characteristic pink to red or purple colonies were selected for biochemical confirmation tests. For oxidase reaction, commercially available disks were used (Himedia, India). All oxidase negative colonies were further analyzed for glucose (Biokar Diagnostics, France) fermentation. Results of the microbiological analyses were expressed as number of bacteria per milliliter (CFU/mL).

3. Results and discussion

The *Enterobacteriaceae* incidence in yogurt is shown in Table 1. In total, 21.29% of analyzed yogurts contained more than 10 CFU/mL *Enterobacteriaceae*. The Serbian Rulebook on food hygiene requirements [12] limits the number of *Enterobacteriaceae* in pasteurized milk and other pasteurized liquid dairy products to no more than 10 CFU/mL throughout the shelf-life. The popularity of yogurt as a fermented dairy product recommended for both children and adults has led many microbiologists to focus on its quality and safety.

Table 1. The number of *Enterobacteriaceae* (CFU/mL) detected in yogurt samples

Enterobacteriaceae (CFU/mL)	No of yogurt samples	% of yogurt samples
< 10	159	78.71
11 – 50	15	7.43
51 – 100	8	3.96
101 – 300	9	4.45
> 300	11	5.45
Total	**202**	**100.00**

Generally, *Enterobacteriaceae* are considered as process hygiene indicators. In the study conducted by N'Guessan et al. [13] similar results were presented, where the *Enterobacteriaceae* was above the limit in 21% of yogurt samples. In our study, 5.45% of yogurt samples contained > 300 CFU/mL of *Enterobacteriaceae*. In a study conducted in Albania 34.58% of collected samples counted > 300 CFU/mL of *Enterobacteriaceae* [14].

Enterobacteriaceae in yogurt indicate evidence of poor hygiene or inadequate processing (especially heat-treatment), process failure and post-process contamination. The failure to respect hygiene rules can take place in different parts of the production line. The need to improve microbiology, biochemistry and food engineering has yogurt production a complex activity. The generalized process of yogurt manufacture is comprised of standardization of milk, homogenization, pasteurization, cooling to incubation temperature, fermentation, cooling, packaging and storage [15].

The first step in safe fermented dairy products surely is the primary production of milk. Namely, dairy farms can be important reservoir of *Enterobacteriaceae*. They are ubiquitous in the environment from which they contaminate the cow, equipment, water, milkers' hands and milking machines. Contamination of milk can be due to excretion from the udder of an infected animal during the milking process [16]. These bacteria can also appear during collection and transportation of milk. Further manipulation of milk is equally important. The heat treatment of milk has a number of beneficial effects. One of them is to reduce the number of microorganisms present in milk [1]. Various heat treatments can be applied. Usually, the milk mixture is pasteurized at 85 °C for 30 minutes or at 95 °C for 10 minutes. A high heat treatment is used to denature the whey proteins and allows the proteins to form a more stable gel [17]. The high temperature further reduces the number of microorganisms in the milk to provide a better environment for the starter cultures to grow. Milk intended for yogurt production is pasteurized before the starter cultures are added to ensure that the cultures remain active in the yogurt after fermentation to act as probiotics. However, bacteria from water and air in the filling equipment or immediate surroundings can recontaminate the product [18]. Post-pasteurization contaminations of yogurt are mainly due to the presence of biofilms on contact surfaces of filling machines [19]. Biofilms are matrix-enclosed bacterial populations [20]. Due to their resistance, they are difficult to eradicate with conventional cleaning and disinfection regimens [19]. Yogurt packaging ensures its hygienic condition is maintained and protected during distribution.

For years, coliform testing has been used to indicate the hygienic condition of dairy products. Studies between 2001 and 2010 have shown post-processing contamination with coliforms in 7.6–26.6% of tested U.S. fluid milk samples [21]. Generally, coliforms are Gram-negative, aerobic or facultative anaerobic, non-spore-forming rod shaped bacteria. Coliforms are capable of fermenting lactose, resulting in gas and acid production [22]. A majority of the yogurt samples collected in Cameroon from 2012 to 2013 had coliform counts higher than 10^2 CFU/mL [23]. Thereof, from 72 bacterial isolates previously considered as coliforms, 21 *Enterobacteriaceae* species were identified. The carbohydrate specified in the coliform test is lactose. Lactose is not fermented by *Salmonella*, *Shigella*, or *Yersinia*, so their presence would not be detected by the test. However, substituting glucose for the lactose in the test would allow detection of all members of the *Enterobacteriaceae*, including the pathogens. *Enterobacteriaceae* have greater resistance to environmental conditions than the coliforms, so they may be better indicators of sanitation [24].

Also, older starter culture can be a source of contamination. Survival of *Salmonella* spp. was noticed in 4% of commercial collected yogurt samples in the study conducted by Motawee et al. [25]. With testing of 211 bacterial isolates, Hervert et al. [26] demonstrated that testing for *Enterobacteriaceae* offers a more comprehensive indicator for the hygienic status of dairy products and processing environments when compared with coliform organisms.

4. Conclusion

Dairy products are potential vehicles for microorganisms from the *Enterobacteriaceae* family. Good manufacturing practices and good hygiene practices must be followed thoroughly throughout the yogurt production line. The absence of classic foodborne pathogens does not indicate that the yogurt is fit for

consumption, since other potentially pathogenic bacteria of the *Enterobacteriaceae* family could be present in the food. Thus, using *Enterobacteriaceae* to monitor the effectiveness of implemented preventive prerequisite measures could offer a better view of the quality, sanitary conditions, and safety of yogurt products. Testing for *Enterobacteriaceae* is useful to verify that the hygiene measures in a manufacturing facility are working as intended.

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Strategy for the study of the proteome in animal muscle tissue

Strategy for the study of the proteome in animal muscle tissue

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Abstract. The existing approaches to the methodology of using proteomic and mass-selective methods for the analysis of the component composition of meat product proteins are systematized, formalized and modified. The scientific and practical foundations of a systemic proteomic strategy for identifying the protein composition of meat raw materials and the authenticity of meat products have been developed. A scheme for constructing proteomic maps and spectra of identified proteins and peptides of meat and meat products using bioinformatic data processing has been developed, and a scheme for choosing a research methodology as a tool for identifying and confirming the composition of meat products has been formed.

1. Introduction

One of the main problems of modern production of meat products is the quality of raw meat, which depends on various factors, including genetic components, conditions of transportation, production and processing. The most significant components of meat are proteins, the total content, structure and functional state of which is constantly changing as part of this complex biological system, with a large number of interacting components. Recently, there has been an intensification of research on the patterns and mechanisms of transformations of biomolecules, in particular proteins, under the influence of endogenous and exogenous factors. The science of meat is developing especially intensively in this direction. In particular, study is intensified on the influence of the protein phenotype, that is, the individual combination of external and internal factors on the change in protein expression and, as a result, the change in the composition and properties of animal tissues. Mammalian tissue samples usually contain from 10,000 to 30,000 different types of proteins, so it is necessary to use a wide range of methods in order to simultaneously prepare, separate and quantify the results of the synthesis of these thousands of proteins. The study of changes in proteins during autolysis, or under the influence of heat treatment, and the possibility of quantitative and qualitative determination of the composition of raw meat or finished meat product and its components determine the ways the acquired knowledge is practically used. These aspects, together with the confirmation of food safety, form the scientific basis for monitoring raw materials and food products, which is becoming an increasingly urgent task worldwide. Proteomics provides identification and quantitative determination of all proteins in the sample, and most importantly, monitoring of their changes.

Proteomics should become an effective tool for solving this problem. Proteomics is the main direction of functional genomics, within which it is possible to conduct a highly reliable analysis of meat products. With the help of this methodology, impressive results have already been obtained in almost all areas of biomedical research. Currently widely used methods of studying the proteome – two-dimensional electrophoresis with subsequent identification of the detected proteins by mass spectrometry and interpretation of data using bioinformatics, require refinement and adaptation for the study of animal tissues and meat products. All these methods have their

own individual advantages and limitations, and none of them is equally well suited for analyzing all the types of proteins present in a complex tissue sample. The complexity of adapting the methods to complex meat products is explained by (1) high-temperature heat treatment leading to protein denaturation; (2) the multicomponent composition of the meat product, in which muscle, milk, egg and vegetable proteins can be present simultaneously, while being from several species of animals, plants or poultry; (3) the need to identify proteins with one molecular weight or one pI.

Therefore, the use of bioanalytical methods for proteomic studies of meat product samples is an actual and modern approach. Such studies are conducted in order to establish the functional role of each of the identified proteins. Depending on the type of organism and the metabolic state of the cells, the proteome can contain from several thousand to hundreds of thousands of proteins. Many of them undergo gene expression, the nature of which is often impossible to predict [1].

One of the most promising areas of research on changes in proteins under the influence of technological processing is the identification, study and determination of the possibility of practical application of biomarkers of these changes.

It is obvious that over the past five years, the number of proteomic studies aimed at studying meat proteins has increased significantly, which opens up the possibility of their routine use in control and analytical laboratories engaged in the analysis of the quality and safety of meat products in the future. In the database of the V. M. Gorbatov Federal Research Center for Food Systems of the Russian Academy of Sciences, work was carried out to develop a scientific and practical model for identifying tissue- and species-specific substances of protein nature in meat products, on the basis of which tools were developed to confirm the authenticity of meat products and a product passport developed according to generally accepted regulatory documentation.

Of undoubted scientific interest is the potential: of using the proteomic strategy as a tool for studying the protein profile of objects of plant and animal origin; of identifying biomarkers of technological processes, for qualitative and quantitative identification of the composition of raw materials and finished meat and meat-growing products and; for studying the mechanisms of proteome changes for the directed formation of the specified characteristics of animal raw materials.

2. Materials and Methods

Research on the identification and identification of muscle proteins of meat raw materials and processed products included the study of the protein composition of muscle tissue of various types of animals and poultry and meat products produced from them. Experimental studies were carried out in accordance with the tasks set at the V. M. Gorbatov Food Systems Research Center of the Russian Academy of Sciences in the Research and Testing Center. Some stages of the work were performed on the basis of the FITC of Biotechnology of the Russian Academy of Sciences.

In accordance with the purpose of the work, the objects of research were:

- m. longissimus dorsi: pork, beef, horse meat, camel meat;
- pectoral muscles of poultry (chicken, turkey);
- model minced meat from m. longissimus dorsi animals and poultry pectoral muscles in a 1:1 combination;

Experimental batches of meat products were manufactured in industrial conditions of the meat processing plant, in accordance with GOST R 52196-2011 and GOST 33673-2015, and also were taken from the retail network (various manufacturers), including those developed according to technical specifications.

When performing the work, a set of generally accepted, standard and modified proteomic methods was used, including descriptive, analytical, inductive and deductive methods in general domestic and foreign literature, which were used to ascertain the existing methodologies for identifying tissue-and species-specific substances of protein-peptide nature in meat products.

The research was based on an extraction-fractional approach to conducting proteomic studies, with further mass spectrometric identification using the bottom-up methodology and bioinformatic interpretation of the results obtained, characteristic of meat products [2-6].

The identification of protein fractions on two-dimensional electrophoregrams (2DE) was carried out after trypsinolysis by MALDI-TOF MS and MS/MS mass spectrometry on a MALDI-time-of-flight Ultraflex mass spectrometer (Bruker, Germany) in the mass range of 500-8000 Da. The analysis of the obtained mass spectra of tryptic peptides was performed using the Mascot program (Matrix Science, USA), with an accuracy of determining the mass of MN+ equal to 0.01%, searching the databases of the National Center for Biotechnological Information of the USA (NCBI) [7]. Individual proteins and peptides were determined by manual processing by comparing amino acid sequences during protein sequencing. In the comparative analysis of the proteomic profiles of the presented samples, the information modules "Cow skeletal muscle proteins (*Bos taurus*)", "Pig skeletal muscle proteins (*Sus scrofa*)", "Horse skeletal muscle proteins (*Equus caballus*)" and "Camel skeletal muscle proteins (*Camelus bactrianus*)" of the multilevel database "Proteomics of muscle organs" were used (http://mp.inbi.ras.ru). The poultry were additionally identified using NCBI databases.

3. Results and Discussion

As a result of the research conducted to modify classical methodologies for fractionation and identification of the composition of muscle proteins from farm animals and poultry, special approaches were developed [8-10]. They reflect the use of proteomic methods for the analysis of muscle proteins in the study of meat raw materials, and the identification and identification of tissue- and species-specific substances of protein nature in muscle tissue, which laid the scientific and practical foundations to create a systematic proteomic strategy for identifying the protein composition of meat products. The hierarchical scheme of the practical implementation of the strategy for studying the muscle proteome of farm animals and poultry is shown in Figure 1.

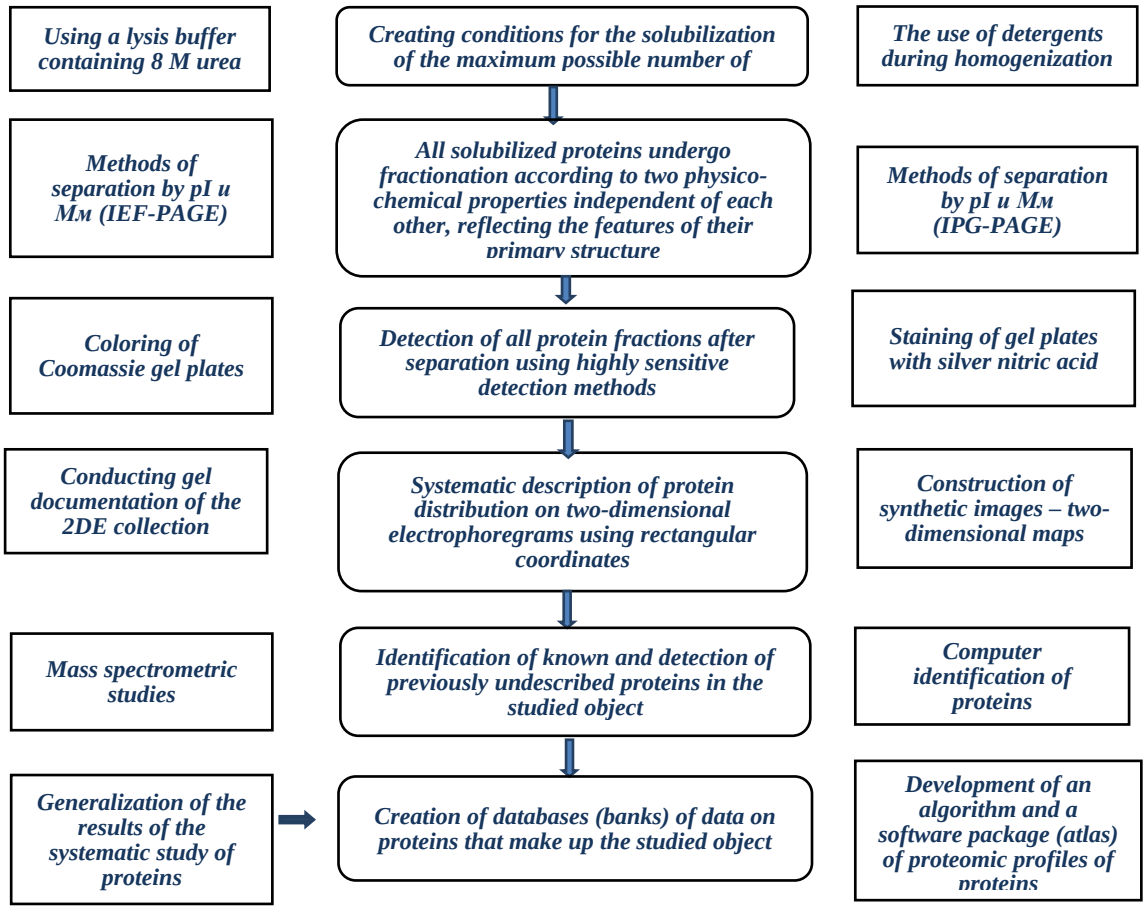

Figure 1. The hierarchical scheme showing practical implementation of the research strategy for the muscle proteome

The construction of proteomic maps of proteins in raw materials and meat products is based on the complex application of individual monomethodologies such as:

- electrophoresis (1D, 2D) – obtaining a protein pattern from a sample
- time-of-flight mass spectrometry – obtaining the spectrum of detected proteins and peptides from a sample;
- bioinformatic data processing – comparison of the amino acid sequence of proteins with the database of decoded genomes of similar species and identification by the corresponding transcripts (in the absence of information in the database), to identify each protein from the resulting pattern;
- analysis and archiving of the received information.

Bioinformatic data processing was used not only as an evidence base for the identification of proteins and peptides from samples, but also for the quantitative identification of the identified fractions.

The bioinformatic interpretation of the results obtained made it possible to formulate and significantly expand approaches to the identification and quantitative determination of protein markers that signify the quality, functionality and safety of meat raw materials (detection of falsification, determination of the presence of allergens) in finished meat products.

According to the obtained data, the information is systematized using bioinformatics methods, which is successfully integrated into the proteomic strategy used in the system of multi-level control of the origin of raw materials in meat products. It seemed interesting to form an assessment system for the control of the composition of meat products, aimed at identifying cases of violation

of established recipes by a set of methods for a two-level system of screening and arbitration. Figure 2 shows a multi-level system for the selection of methods previously evaluated for the identification of the composition of meat products.

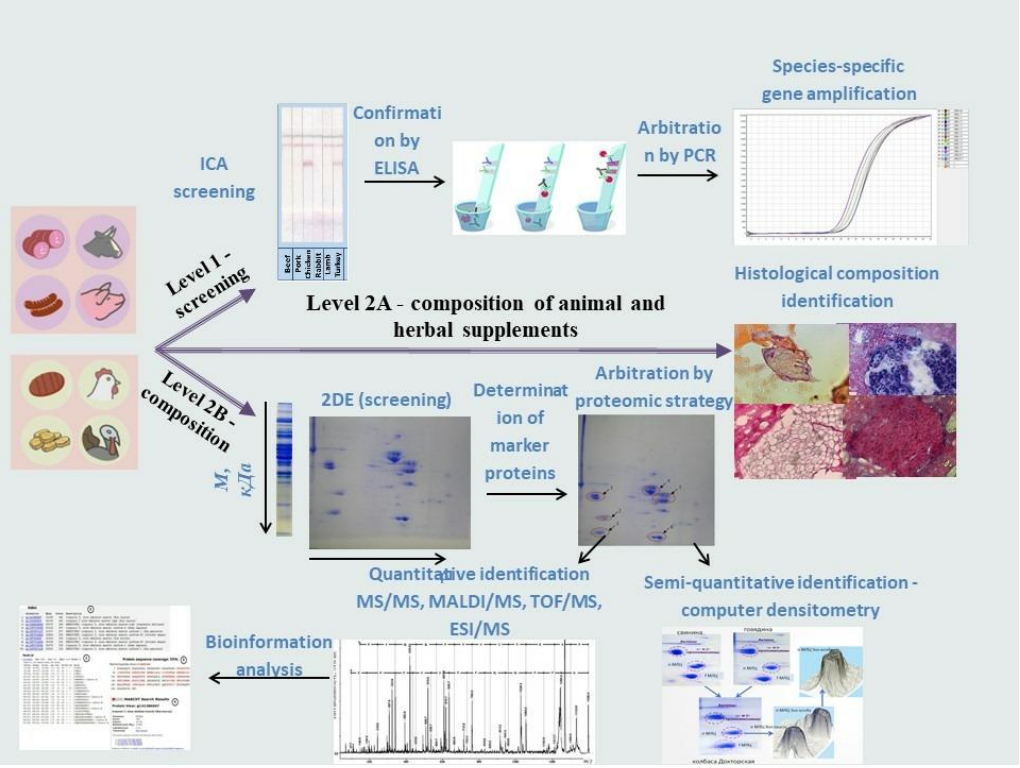

Figure 2. A two-level control system for the composition of meat products

Thus, a review of existing methodological approaches and their experimental confirmation revealed the absence of one specific method that would solve such an urgent problem as the quantitative determination of undeclared components in meat products. In accordance with the prospects for inclusion in the system of multi-level control of the composition of meat products, ICA and/or ELISA (inexpensive methods, with a high level of reliability) is recommended as a screening method, and LC-MS as an arbitration (confirming) identification method ($p < 0.05$). When analyzing the complementarity of the considered methods, it is worth using two or three methods together:

- IFA/IHA: within the framework of production control, in the context of rapid decision-making;
- IFA/MS: within the framework of confirmatory (arbitration) control, as the most highly reliable method;
- Identification of the ICA/2D/MS biomarker: within the framework of confirmatory (contradictory) control, as the most indicative confirmation.

4. Conclusion

Modern analytical technologies based on a systematic approach to analysis are required to study interspecific and intraspecific features of meat proteins and their transformation during maturation and technological processing. Proteomics opens up wide opportunities in this direction as a methodology for studying proteins in a certain system and at a certain time, which allows identification of complex patterns between the state of proteins, and the functional and technological properties of raw materials and processing methods. Proteomics also enables

development of accurate analytical methods for searching for biomarkers and identifying unfair practices. Therefore, the introduction of proteomics should be considered as an important step towards achieving higher quality of raw materials and sustainable production development.

As a result of the conducted research, a comprehensive approach has been developed to identify species-and tissue-specific substances of a protein nature when controlling a meat product. The proposed proteomic strategy in the study of the introduction of qualitative indicators in meat raw materials will combine the identification of the protein composition of meat products with a scheme for the identification and determination of the composition of protein and non-protein components in products, as well as additives of vegetable or animal origin.

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Nitrite content in meat products from the Serbian market and estimated intake

Nitrite content in meat products from the Serbian market and estimated intake

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Abstract: The aim of this study was to determine levels of nitrites in some meat products sold on the Serbian market over a period of 3 years (2018-2020) and to compare results with maximum residue levels as well to discuss dietary exposure of the Serbian adult population to nitrites.A total of 1291 meat product samples, produced by the Serbian meat industry or imported (509 dry fermented sausages, 37 semi-dry fermented sausages, 451 finely minced cooked sausages and 294 coarsely minced cooked sausages), were obtained from the Serbian retail market during 2018-2020.Higher mean levels of nitrite content, expressed as $NaNO_2$, were found in cooked sausages (40.35 mg kg^{-1}, finely minced and 33.75 mg kg^{-1}, coarsely minced) compared to fermented sausages (1.86 mg kg^{-1} dry fermented and 1.83 mg kg^{-1}, semi-dry fermented).The average dietary exposure to nitrites, expressed as nitrite ion, for the Serbian adult population varies from 0.001 to 0.015 mg kg^{-1}body weight (BW) day^{-1} and was far below the European acceptable daily intake (0.07 mg kg^{-1} BW day^{-1}). In conclusion, the concentrations of nitrite in all meat products were below established maximum permitted levels (national and European), indicating that the use of nitrite as a food additive in Serbia is generally in line with existing regulations.

1. Introduction

Protecting consumer health through improving food safety and quality has been an increased focus for both food processors and researchers. Consumers today are much more demanding in terms of food quality and safety, and product labelling, while producers are determined to implement necessary standards in food production, etc. [1]. The main goal of the meat industry is reducing economic losses and increasing the shelf life and storage stability of meat products while maintaining consumer health.

The use of curing agents (nitrite, nitrate) in meat processing has been a controversial issue since the 1970s. The mechanism of curing colour formation is well known and has been summarised by Honikel [2]. Nitrates and nitrites, in the form of the sodium and potassium salts, are widely used as preservatives in meat production. From the technological point of view, the main reason for adding nitrites and/or nitrates in the processing of meat products is to improve the quality (formation the unique pink colour, texture and flavour), [2, 3, 4], durability, due to its antioxidant action against lipid oxidation [5] and increase the safety of products [6, 7]. Taken together, because of their antimicrobial properties, they inhibit the growth and reproduction of bacteria *Staphylococcus aureus* and *Clostridium botulinum*. As an unstable ion, nitrite undergoes a series of reactions as soon as it is added to food. In an acid environment, nitrite is converted into nitric acid, which decomposes into nitric oxide. Nitric oxide, being

an important product from the standpoint of colour fixation in cured meat, reacts with myoglobin to produce a red pigment-nitrosomyoglobin [8]. The intake of nitrite is normally low and not an acutely toxic dose, but nitrite in food is considered primarily to cause health problems because its presence both in food and in the body could lead to the formation of carcinogenic nitrosamines [9, 10] and the clinical symptom of methemoglobinaemia [11, 12]. Time, temperature, pH and additives have an important effect on the depletion of nitrite in cured meat [8]. The contribution of dietary intake is given by exogenous nitrite via consumption of vegetables, fruits, water and meat. Cured meat products are the major source of the nitrites and N-nitrosamines in human dietary intake. Many studies have suggested that high dietary nitrate and nitrite intake is an aetiological factor in the development of certain cancers [13]. It is now well understood that endogenously acquired nitrate/nitrite can be converted to carcinogenic N-nitroso compounds upon digestion [14, 15]. Large amounts of these compounds in the body contribute to the development of cancer in gastrointestinal tract organs, the most commonly affected being colon, rectum, liver, thyroid and stomach [16]. The use of nitrite could lead to the formation some acute and chronic toxicity such as methemoglobinaemia, thyroid disorders and increased risks of gastric, oesophageal, nasopharyngeal and bladder cancers [17, 18, 19]. Nevertheless, some authors revised this risk downwards and claimed that nitrate and nitrite could have potential beneficial effects on human health by reducing hypertension and cardiovascular diseases [20, 21]. Another aspect to consider is the fact that there is no direct link between the in-going and the residual amount of nitrite, but the degradation of nitrite added to meat is influenced by several factors like pH, storage temperature, heat treatment of meat and the presence of reducing substances. Added ascorbate especially will increase the rate of degradation of nitrite [22].

The current Serbian legislation has restricted the concentration of residual $NaNO_2$ in processed meat to 100 and 150 mg kg^{-1} depending of the type of product [23, 24] whereas regulation in Europe permits concentrations up to 100 mg kg^{-1} [25].The Joint Expert Committee on Food Additives (JECFA) established an acceptable daily intake (ADI) for nitrite of 0-0.07 mg kg^{-1} body weight, expressed as nitrite ion on the basis of a no effect level (NOEL) of 6.7 mg kg^{-1} body weight per day for effects on heart and lung in a 2-year study in rats and a safety factor of 100 [19, 26, 27].

The aims of this study were to determine the levels of nitrites in some meat products sold on the Serbian market over a period of 3 years (2018-2020) and to compare the results obtained with maximum permitted levels (MPL) [23], as well as to estimate and discuss dietary exposure of the Serbian adult population to nitrites.

2. Materials and Methods

2.1. Meat products and sample preparation

A total of 1291 meat product samples, produced by the Serbian meat industry or imported (509 dry fermented sausages, 37 semi-dry fermented sausages, 451 finely minced cooked sausages and 294 coarsely minced cooked sausages) were obtained from different regions from the Serbian retail market during 2018-2020. In the most of the meat products, all parameters of quality defined by the legislation were examined, and in a smaller number, analyses were carried out as per client's request. The content of nitrite in examined meat products was determined according to the standard ISO procedure [28].

2.2. Exposure Assessment and risk characterization

The estimated daily intake (EDI) of nitrite from processed meat by consumers was calculated based on the individual food consumption data [29], body weight [30] and the analytical results obtained in the present study for the collected meat samples.

2.3. Statistical analysis

For statistical evaluation on data, Minitab 17 Ink statistical software was used ((Minitab Ink., Coventry, UK).

3. Results and discussion

The results of the determination of nitrite content in each type of processed meat products, expressed as minimum, maximum, mean and median as well as number of tested samples and MPL are presented in Table 1 and Figure 1. The data of the EDI for nitrite in the examined processed meat products are shown in Table 2. None of the analysed meat products exceeded the maximum permitted nitrite level of 150 mg kg^{-1}, according to the Serbian legislation on food additives [23].

Table 1. Mean levels and ranges of nitrite content expressed as $NaNO_2$ (mg kg^{-1}) in examined processed meat products; limit of quantification =0.03 mg kg^{-1}

Meat product	N	n (%)	Mean ±SD ($mgkg^{-1}$)[a]	Mean ±SD ($mgkg^{-1}$)[b]	Median ($mgkg^{-1}$)[b]	Min–Max ($mgkg^{-1}$)	MPL ($mgkg^{-1}$)
Dryfermented sausages	509	332 (65.2)	1.86±2.68	2.85±2.87	1.905	0.05-24.88	150
Semi-dryfermented sausages	37	28 (75.6)	1.83±2.48	2.42±2.60	1.36	0.1-10.02	
Finely mincedcooked sausages	451	451 (100)	40.35±19.93	40.35±19.93	40.30	0.09-94.57	
Coarsely minced cooked sausages	294	292 (99)	33.75±23.40	33.75±23.40	31.66	0.05-113.51	
Total	**1291**	**1103 (85.4)**	**22.57±24.22**	**26.41±24.18**	**23.26**	**0.05-113.51**	

N–total number of analysed samples; n–number of samples that contained nitrite (%); [a] mean nitrite content in the total number of analysed samples; [b] mean/median nitrite content in the number of samples that contained nitrite; MPL–maximum permitted level [23].

Table 2. Estimated dietary intake and risk characterization of nitrite ion (NO_2^-) intake.

	Mean ±SD ($mgkg^{-1}$)	ADC ($gday^{-1}$)	EDI ($mgkg^{-1}$ $BWday^{-1}$)	Contribution to ADI (%)	ADI ($mgkg^{-1}BWday^{-1}$)
Dryfermented sausages	1.24±1.80	38.3	0.001	1.42	0.07
Semi-dry fermented sausages	1.22±1.65		0.001	1.42	
Finely mincedcooked sausages	26.90±13.29		0.015	21.42	
Coarsely minced cooked sausages	22.50±15.60		0.012	17.58	
Average	**12.96±20.50**		**0.007**	**10.46**	

Nitrite ion content (66.65% of $NaNO_2$); ADC–average daily consumption of meat products [29]; EDI–estimated daily intake; BW –default body weight value for adults was 70 kg [30]; ADI–acceptable daily intake [19, 26].

The levels of residual nitrite and nitrate in processed meat products are variable depending on the time and temperature used during processing and storing, the initial addition of nitrite and nitrate, the composition of the meat, pH, addition of antioxidant components such as ascorbate and the presence of microorganisms [2, 31]. Honikel [2] estimated that the decline in nitrite levels due to heating during manufacturing is about 35% of the added level, and thereafter, there is a continuing decrease in nitrite levels during storage. Higher mean nitrite contents, expressed as $NaNO_2$, were found in cooked sausages (40.35 mg kg^{-1}, finely mincedand 33.75 mg kg^{-1}, coarsely minced) compared to fermented sausages

(1.86 mg kg^{-1}, dry fermented and 1.83 mg kg^{-1}, semi-dry fermented). In 65.2% (332 samples) of examined dry fermented sausages and 75.6% (28 samples) of semi-dry fermented sausages, nitrites were detected, while in the rest of these samples, in 34.8% (177 samples) and in 24.4% (9 samples) respectively, nitrites were not detected, i.e. nitrite contents were under the limit of quantification (<0.03 mg kg^{-1}) (Table 1, Figure 1A and 1B). The results obtained show distributions of nitrites differed in the four product types (Figure 1), which is related to stability of the nitrite content in the different meat products, i.e. is the consequence of their differing preparation and processing methods [32]. In fermented sausages, the content of nitrite decreased during the ripening of sausages as a result of the process that takes place in the sausage, i.e. reduction of nitrite content is significant where main process is nitrite conversion into nitrates in the weak acid environment. In fermented sausages, the presence of nitrite becomes latent, because the process is reversible and nitrates, under certain conditions, can revert into nitrites [33].

Our results were similar to previously reported nitrite contents in dry fermented and cooked sausages, 0.65 mg kg^{-1} and 36.60 mg kg^{-1}, respectively from the Serbian market in the period of 2016-2018 [32], and were lower than mean nitrite content in dry fermented sausages (7 mg kg^{-1}) from Croatia [34].

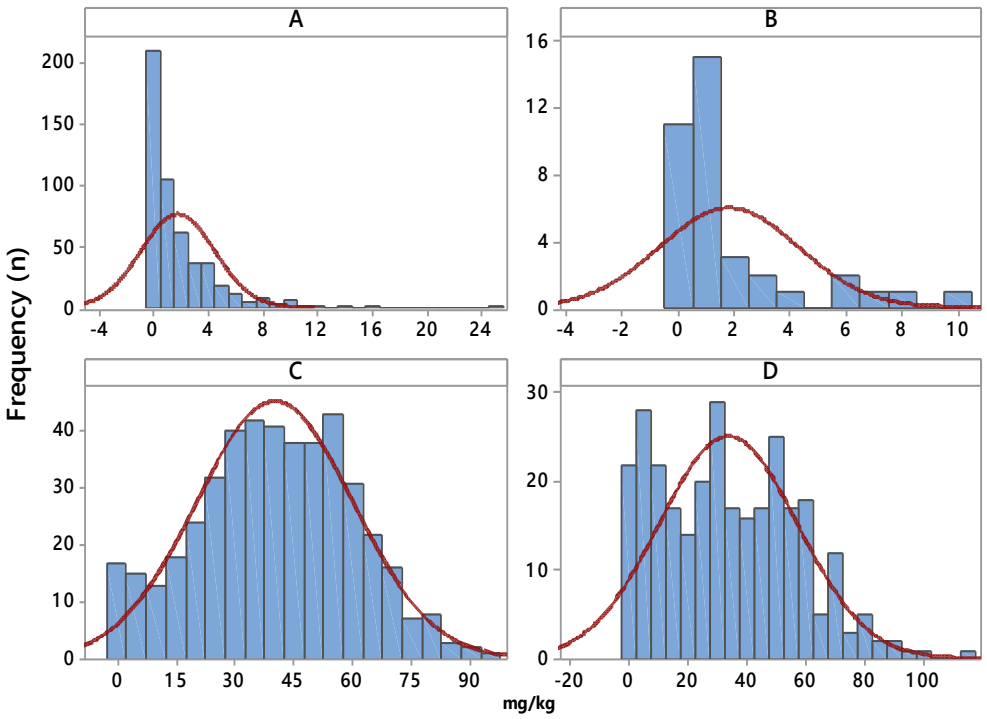

Figure 1. Distribution of $NaNO_2$ content (mg kg^{-1}) in dry fermented sausages (A), semi-dry fermented sausages (B), finely mincedcooked sausages (C), coarsely minced cooked sausages (D).

Many papers report the results of the analysis of the content of nitrite in different types of meat products, demonstrating a great variability in their concentrations [35]. According to the results of studies [36, 37, 38], the mean concentrations of nitrite in cooked sausages were 32 mg kg^{-1}; 26mg kg^{-1} and 30.5 mg kg^{-1}, respectively. In [39], sausages had a higher mean nitrite content 51.8±14.5 mg kg^{-1}, compared to our results. Yalcin [40] measured the residual nitrite content of dry fermented sausages and sausage samples from Istanbul and the reported average nitrite contents were 42.8 mg kg^{-1} in dry

fermented sausage (n=65) and 102.8 mg kg^{-1} in sausage (n=60), which were higher than our mean nitrite contents in these types of sausages.

The average dietary exposure to nitrites, expressed as nitrite ion, for the Serbian adult population varied from 0.001 to 0.015 mg kg^{-1} body weight (BW) day^{-1}, according to the product type (Table 2) and was far below the European ADI (0.07 mg kg^{-1} BW day^{-1}). Our results showed that cooked sausages, especially finely minced ones, were the main sources of nitrite intake, and they made up 21.42% of the ADI (Table 2). The lowest sources of nitrite intake among the examined meat product were dry fermented and semi- dry fermented sausages (both 1.42% of ADI).

The dietary exposure to nitrite was calculated in this study as accurately as possible. However, limitations were the impossibility of calculating relevant exposures for children and heavy consumers due to lack of data, and data paucity on consumer intake of nitrates, which contributes to the nitrite exposure because they are converted to nitrite during metabolism; nitrate intake was not included in the current intake estimates. Also, the consumption of processed meat per capita was estimated based on the family consumption divided by the number of family members, and assuming that the children consume the same amount as adults. Thus, the approach taken can be considered an initial approximation for the intake assessment.

4. Conclusion

In conclusion, the concentrations of nitrite in all 1291 processed meat samplesin the period of three years were below established MPLs (national and European), indicating that the use of nitrite as a food additive in Serbia is generally in line with existing regulations. The overall situation is controllable and safe. However, if other sources of nitrite exposure (vegetables, cereals, dairy products and drinking water) were taken into account, the EDI would be higher. To provide adequate consumer protection, further research and additional dietary studies are needed: updating adult and child dietary habits; especially collecting data for children's exposure and; measuring the nitrite content in other food sources. All together, the results of this study suggest need to investigate the complex effects of various reduced levels of nitrite and potential alternative compounds and/or technologies that can substitute nitrite in meat products. Considering the toxicity of nitrites and the possibility of their transformation to carcinogenic N-nitrosamines, the importance of the information of daily intake by children and adults is clearly indicated.

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Effects of using chia *(Salvia hispanica L.)* mucilage and different cooking procedures on quality parameters of beef patties

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Effects of using chia (*Salvia hispanica L.)* mucilage and different cooking procedures on quality parameters of beef patties

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Abstract. This study was carried out to investigate the effects of chia (*Salvia hispanica L.)* mucilage (CM) as a fat replacer in grilled or pan-fried beef patties. For this purpose, beef fat was replaced by CM at levels of 0, 25, 50, and 75%. The use of CM and cooking method affected cooking-related parameters. Cooking yield was lower in pan-fried patties, while the addition CM increased the cooking yields. Moisture retention, shrinkage, changes in diameter, and thickness of grilled patties were improved compared to the pan-fried samples. The addition of CM increased moisture retention, while shrinkage values decreased. Results of our investigation revealed that CM retarded oxidative changes in pan-fried patties. Textural parameters and sensory properties of samples were not negatively affected by the addition of CM.

1. Introduction

In recent years rising attention has been paid to specific types of healthy and beneficial food ingredients since consumers are becoming more health-conscious about the food in their diet [1]. Although meat and meat products occupy an important role in human nutrition as they provide valuable nutrients, such as proteins, vitamins, and minerals, they also contain saturated fat and cholesterol that are risky for cardiovascular diseases and some cancer types. From this point of view, the meat industry has attempted to develop low-fat meat products with desired properties by utilizing non-meat proteins, hydrocolloids, and replacing animal fat with plant/seed oils [1, 2].

Chia seeds contain significant amounts of dietary fiber (18-30%), protein (15-25%), natural antioxidants (tocopherol and polyphenols), vitamins, and minerals, and also, the seeds are good sources of omega-3 and omega-6 [3]. Moreover they increase the satiety index and have protective effects against cardiovascular diseases, diabetes, and cancer. A variety of chia forms such as seed [3, 4, 5] and flour [6, 7, 8] have been used as fat replacers, binders and extenders in comminuted meat products. The incorporation of chia seeds into various meat products retarded the progression of lipid oxidation [3, 4, 5]. It has been also observed that water holding capacity, cooking yield, and the amount of polyunsaturated fatty acids increased by the addition of chia flour to chicken nuggets [8] and fish burger [9]. However, some studies have shown that chia seed and flour caused undesirable sensory quality. The increasing amount of the chia flour/seeds decreased sensory acceptability in terms of texture, internal appearance, and flavor [4, 5, 6, 7]. When chia seeds are soaked in water, transparent and clear mucilaginous substances rapidly exude around the seeds. Mucilaginous seeds constitute mainly xylose and glucose together with uranic acid, glucuronic acid, galacturonic acid, arabinose, and galactose [10].

Chia mucilage (CM) is known to have both emulsifying agents and water-retaining properties. It has therefore become a promising fat substitute in bakery products [11]. However, to the best of our knowledge, limited research has been done on using CM in gel and powder forms as a pork fat substitute in model system pork emulsion, in which researchers reported that CM in powder form increased hardness and decreased elasticity; however, in gel form, it enhanced the textural properties [10]. Therefore, the aim of this study was to investigate the effects of utilization CM on chemical composition, lipid oxidation, technological and sensory quality characteristics of pan-fried and grilled beef patties.

2. Materials and methods

Post-rigor beef as boneless rounds and beef fat was kindly donated by MIGROS TRADE INC. Non-damaged whole chia seeds were purchased from a local market. Chia mucilage was prepared according to methods developed by Câmara et al. [10] and Brütsch et al. [12]. Chia seeds were downscaled and mixed with distilled water (1:10) and mixed on a magnetic stirrer at 45°C for 15-20 minutes to achieve full hydration. The viscous solution was centrifuged at 4100 rpm for 15 minutes to remove the excessive water. The water phase accumulated in the upper layer of the tubes was separated to obtain the CM to use in beef patty formulation as a fat substitute. CM was added to the patty formulation by replacing 0%, 25%, 50%, and 75% beef fat, and the following ingredients were added per kg meat mixture (Table 1); salt and spice mix (cumin, black pepper, and onion powder). The minced meat was mixed with beef fat and/or CM and other ingredients in a kneading machine (Mateka, Turkey) until a homogeneous mixture was obtained, then the patties were shaped by using a round metal mold (d:80 mm, h:1 cm). Subsequently, shaped patties were cooked by using two different cooking methods.

Table 1. Formulation of patties

Treatments		Beef meat (%)	Beef fat (%)	Chia mucilage (%)	Salt (%)	Spice mix (%)
Grilled	CG0	76	20	-	2	2
	CG25	76	15	5	2	2
	CG50	76	10	10	2	2
	CG75	76	-	20	2	2
Pan-fried	CF0	76	20	-	2	2
	CF25	76	15	5	2	2
	CF50	76	10	10	2	2
	CF75	76	-	20	2	2

Moisture contents of the patties were determined by the AOAC method [13] and fat content was calculated by a method stated by Flynn and Bramblett [14]. All proximate analysis was performed in triplicate. The cooking yield, moisture retention [15] and fat retention were calculated by weight differences for patties before and after cooking [16] and calculated according to these equations below:

$$Cooking\ yield(\%) = (Cooked\ patty\ weight)/(uncooked\ patty\ weight)x100$$

$$Moisture\ retention(\%) = (\%\ Yield\ x\ \%Moisture\ in\ cooked\ patty)/100$$

$$Fat\ retention(\%) = \frac{(Cooked\ weight)x\ (\%\ Fat\ in\ cooked\ patty)}{(Raw\ weight)x\ (\%\ Fat\ in\ raw\ patty)}x100$$

Reduction of patty diameter (measurements were taken using calibers) and change in thickness were calculated as:

$$Reduction\ of\ diameter(\%) = \frac{(Uncooked\ patty\ diameter\ -\ cooked\ patty\ diameter)}{(uncooked\ patty\ diameter)}x100$$

$$Change\ in\ thickness(\%) = \frac{(Uncooked\ patty\ thickness - Cooked\ patty\ thickness)}{(Uncooked\ patty\ thickness)}x100$$

Beef patty dimensional shrinkage was calculated according to following equation:

$$Shrinkage(\%) = \frac{(Raw\ thickness - Cooked\ thickness) + (Raw\ diameter - Cooked\ diameter)}{(Raw\ thickness + Raw\ diameter)} x100$$

Texture profile analysis (TPA) was performed five times for each treatment using a texture analyzer (TA-XT2, Stable Micro Systems, UK). Oxidative stability of beef patties was analyzed by determining 2-thiobarbituric acid reactive substances (TBARS) [17]. Sensory properties of patties were evaluated in terms of appearance, color, texture, juiciness, oiliness, flavor and general acceptability (1: not like, 9: extremely like). Data was analyzed by ANOVA and Duncan's Post-Hoc tests using SPSS 23 software.

3. Results and discussion

Moisture and fat contents of samples are given in Figure 1. For both cooking methods, moisture content increased and fat content decreased with the addition of CM ($p<0.05$). While there were no differences between 25%, 50%, and 75% substitution of beef fat with CM in grilled patties, using more than 50% CM in pan-fried patties significantly reduced the fat content ($p<0.05$).

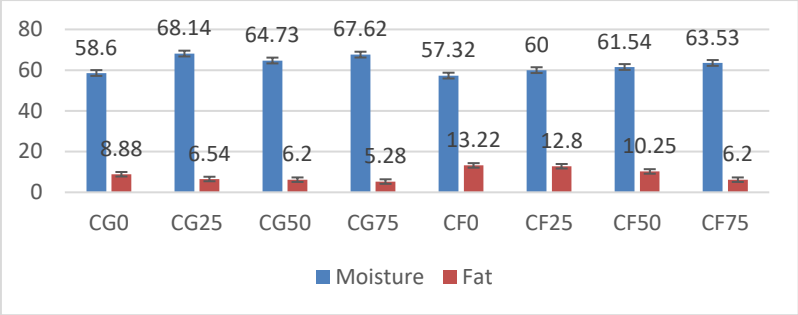

Figure 1. Moisture and fat content of patties

Cooking characteristics such as cooking yield, moisture, and fat retention, diameter reduction and shrinkage are some of the most important factors for the meat industry to predict the behavior of burger-type meat products during cooking. Cooking characteristics are given in Table 2. Interaction between cooking method and CM level significantly affected cooking yield and moisture retention ($p<0.05$). In grilled patties, cooking yield and moisture retention were improved by the effect of CM, and therefore, CG25 and CG75 treatments had the highest cooking yield and moisture retention. The incorporation of more than 50% CM made it possible to hold more water in the meat matrix. The lowest cooking yield in samples with 100% beef fat incorporated might be attributed to the excessive fat separation and water release during cooking. This result showed that cooking yield of patties increased by the addition of CM due to its high dietary fiber (34.4%) and protein (16-20%) content. According to Herrero et al. [7], in frankfurters, chia flour gel emulsion can increase moisture and fat binding properties. Patties formulated with 50% CM showed the highest thickness change, CG50 and CF50 patties showed similar effects on diameter reduction, but nevertheless, in CG75 and CF75, the cooking method was found to be significant. Swelling of chia in meat protein matrix resulted in patties swelling up during the cooking process, so the flat shape of patties changed to ridged up. Researchers found no effects of cornflour levels in meatball diameter changes [18]. Denaturation of meat proteins with the release of water and fat means the patties tend to shrink during the cooking process. CM levels and cooking methods affected shrinkage, as increasing the CM level significantly decreased the patty shrinkage ($p<0.05$). In grilled patties, shrinkage values at all CM levels were lower than the control treatment. Pan-frying resulted in similar shrinkage values in CF0 and CF25 treatments, but shrinkage values decreased with 50% and 75% replacement levels.

Textural parameters of the patties are set out in Table 3. The texture profile of reformulated meat products is a substantial analysis that should be considered due to the various fat sources in the

formulation [19]. In this study, hydrophilic protein and soluble fibers in chia mucilage hold the water and, therefore, caused a soft structure, so in grilled patties with added CM, hardness values decreased ($p<0.05$). Another probable explanation for this textural behavior could be the emulsifying and gelling properties of CM. However, in pan-fried patties, the same trend was observed up to 75% replacement level ($p<0.05$). Our results are in line with Fernandez et al. [20], who reported softening in texture when chia flour was added to frankfurter formulation. Interaction between the cooking method and CM significantly affected springiness and cohesiveness values, which confirmed the same similar strength of internal bonds in these samples [21]. In grilled patties, addition of CM had no effect on springiness and cohesiveness, but in pan-fried samples, replacing fat with CM at levels of 50% and 75% increased springiness and cohesiveness. On increasing levels of both in grilled and pan-fried patties, gumminess values showed a general declined trend in treatment patties when compared to controls. The highest chewiness scores were recorded in pan-fried control patties and CF25 samples. Câmara et al. [10] reported similar results for hardness and chewiness in pork emulsions.

Table 2. Cooking parameters of patties

Variance	Factor	Cooking yield (%)	Moisture retention (%)	Fat retention (%)	Change in thickness (%)	Reduction of diameter (%)	Shrinkage (%)
Cooking method (A)	Grill	84.29±5.47	54.85±6.68	37.94±6.65	10.00±2.32	5.00±3.28	4.69±2.81
	Pan-fried	78.84±4.11	47.85±4.17	55.50±7.29	35.40±15.76	10.53±2.57	6.51±3.09
	F value	48.77	175.57	1209.18	953.72	25.75	10.86
	P value	0.00	0.00	0.00	0.00	0.00	0.00
Lipid source (B)	0% 25% 50% 75%	76.10^{c}±2.21 81.46^{b}±7.37 82.54^{b}±3.58 86.15^{a}±2.14	44.10^{a}±1.29 52.60^{b}±8.52 52.15^{b}±3.48 56.53^{c}±3.33	49.85^{a}±17.76 44.01^{d}±14.47 45.69^{c}±6.06 47.35^{b}±0.41	14.76^{a}±1.99 17.38^{b}±9.57 31.22^{d}±25.26 27.45^{c}±19.37	9.23±4.32 8.36±5.36 7.74±3.66 5.73±2.50	8.84^{a}±1.72 6.08^{b}±3.84 4.50^{bc}±1.29 2.96^{c}±0.65
	F value	28.37	97.20	24.30	91.91	1.86	20.48
	P value	0.00	0.00	0.00	0.00	0.18	0.00
A x B	*Grill-0%*	77.93±1.24^{d}	44.54±1.86^{d}	33.65±0.88^{f}	13.12±1.04^{ef}		8.42±2.19^{a}
	Grill-25%	86.86±1.10^{a}	60.31±1.56^{a}	30.91±2.98^{g}	17.09±1.61^{e}		2.77±1.59^{b}
	Grill-50%	84.28±0.99^{b}	55.17±1.55^{b}	40.19±0.78^{e}	24.23±4.04^{d}		4.98±1.58^{b}
	Grill-75%	87.78±0.86^{a}	59.36±1.56^{a}	47.03±0.07^{d}	33.59±3.01^{c}		2.56±0.49^{b}
	Pan fried-0%	76.18±0.39^{de}	43.66±0.32^{d}	66.04±0.95^{a}	9.80±1.14^{f}		9.25±1.42^{a}
	Pan fried -25%	74.81±0.83^{e}	44.88±0.89^{d}	57.10±0.82^{b}	25.98±2.38^{d}		9.40±1.13^{a}
	Pan fried-50%	80.43±1.60^{c}	49.11±0.69^{c}	51.18±0.82^{c}	54.14±3.84^{a}		4.03±0.98^{b}
	Pan fried -75%	84.52±1.65^{b}	53.70±1.11^{b}	47.68±0.32^{d}	45.09±1.67^{b}		3.35±0.59^{b}
	F value	28.37	33.12	203.86	132.19	1.76	8.88
	P value	0.00	0.00	0.00	0.00	0.20	0.00

Table 3. Textural properties of patties

Variance	Factor	Texture properties				
		Hardness (N)	Springiness (mm)	Cohesiveness	Gumminess (N)	Chewiness (N x mm)
Cooking method (A)	Grill	9.16±2.83	0.17±0.00	0.19±0.00	1.85±0.42	0.31±0.09
	Pan-fried	10.52±4.40	0.15±0.02	0.18±0.02	2.02±0.58	0.30±0.12
	F value	25.14	17.67	12.35	29.25	2.19
	P value	0.00	0.00	0.00	0.00	0.15
Lipid source (B)	0% 25% 50% 75%	14.47^{a}±2.88 11.37^{bc}±1.15 6.82^{e}±0.54 6.69^{e}±1.03	0.16^{ab}±0.02 0.14^{b}±0.02 0.17^{a}±0.01 0.17^{a}±0.01	0.18^{ab}±0.02 0.17^{c}±0.02 0.20^{a}±0.01 0.19^{bc}±0.01	2.67^{a}±0.31 1.93^{b}±0.14 1.53^{c}±0.06 1.61^{c}±0.25	0.43^{a}±0.08 0.33^{b}±0.10 0.22^{c}±0.03 0.23^{c}±0.04
	F value	191.38	5.39	7.33	254.73	193.26
	P value	0.00	0.00	0.00	0.00	0.00
A x B	*Grill-0%*	11.87±0.34^{b}	0.18±0.00^{a}	0.20±0.01^{ab}	2.39±0.11^{b}	0.36±0.01^{c}
	Grill-25%	11.72±0.09^{b}	0.17±0.01^{a}	0.19±0.01^{ab}	2.04±0.09^{c}	0.42±0.01^{b}
	Grill-50%	7.26±0.19^{cd}	0.17±0.00^{a}	0.19±0.00^{ab}	1.55±0.06^{c}	0.25±0.01^{d}
	Grill-75%	5.74±0.12^{e}	0.17±0.00^{a}	0.20±0.01^{ab}	1.39±0.06^{f}	0.19±0.00^{e}
	Pan fried-0%	17.07±0.60^{a}	0.14±0.02^{b}	0.16±0.00^{c}	2.94±0.05^{a}	0.50±0.02^{a}
	Pan fried -25%	11.02±1.71^{b}	0.12±0.00^{b}	0.15±0.00^{c}	1.81±0.04^{d}	0.23±0.01^{d}
	Pan fried-50%	6.37±0.32^{de}	0.17±0.01^{a}	0.21±0.17^{a}	1.50±0.07^{ef}	0.19±0.02^{e}
	Pan fried -75%	7.63±0.06^{c}	0.16±0.01^{a}	0.18±0.01^{b}	1.83±0.10^{d}	0.26±0.01^{d}
	F value	27.16	5.09	8.05	32.70	107.63
	P value	0.00	0.01	0.00	0.00	0.00

TBARS values of patties are given in Fig. 2. No differences were recorded in TBARS values of pan-fried patties on the first day of storage. Although some fluctuations were recorded during storage, at the end of the storage, the highest and the lowest TBARS vales were recorded for control (0.76 mg MA/kg) and CF25 (0.40 mg MA/kg) samples respectively ($p<0.05$). CM showed antioxidant effects in pan-fried patties due to its bioactive compounds such as mrycetin, quercetin, rosmarinic acid, caffeic acid etc. [3, 9]. Interaction between the cooking method and CM was significant ($p<0.05$). TBARS values increased with the addition of CM in grilled patties. The increment in lipid oxidation probably resulted from the high concentration of PUFAs in mucilage. Similar to our results, TBARS values of pork burgers increased with the utilization of chia oil hydro gelled emulsion as a pork fat substitute [22]. However, Zaki [3] reported that TBARS values of samples formulated with chia seed were lower than controls formulated with pork fat.

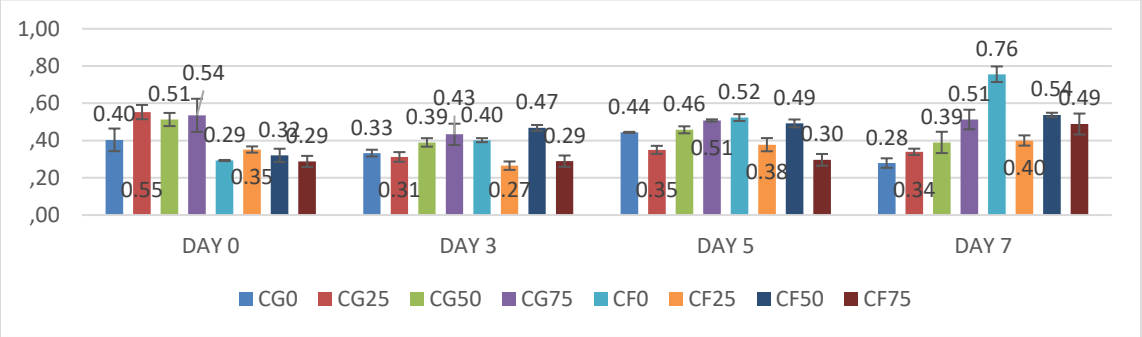

Figure 2. TBARS values of patties (mg MA/kg)

One of the limiting factors for fat-reducing strategies is sensory properties due to the functions of fat in meat products. The purchase intention of consumers depends on not only the safety but also some organoleptic features such as appearance, taste, texture. The sensory scores of the patties are shown in Fig. 3. In general, the cooking method was the most effective factor affecting the sensory properties except juiciness scores ($p<0.05$). Pan-fried patties had higher scores than grilled ones for all the sensory properties. CM had no effect on sensory properties juiciness ($p>0.05$). Concerning juiciness, the addition of CM increased juiciness scores. Sensory scores of all experimental groups were above 5. Thus, it could be concluded that all patties were preferred by the consumer, and in fact, that patties formulated with lower beef fat were preferred even more. Even though in some research chia had been reported to decrease sensorial quality [7, 23, 24], in our study, CM had no adverse effect on quality, but rather, improved juiciness.

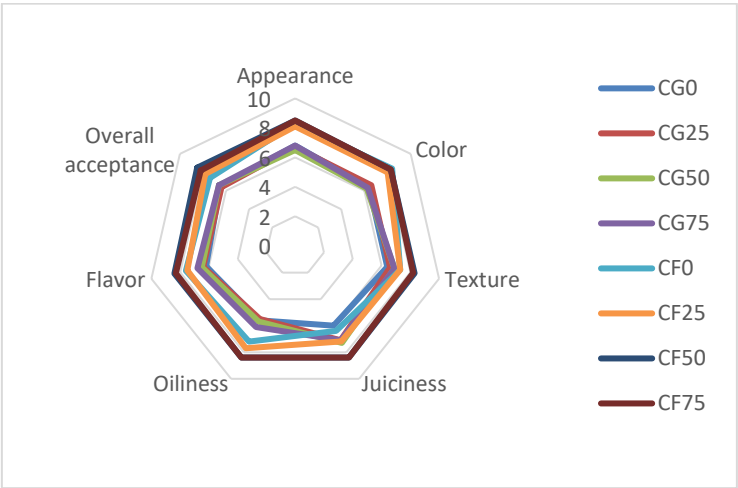

Fig. 3. Sensory scores of beef patties

4. Conclusion

The results of our investigation revealed that utilization of CM as beef fat replacer improved cooking characteristics. The addition of CM had no negative effects on sensory properties. In general, TBARS values were lower in pan-fried patties than in grilled patties. The results also indicated that CM, as a natural antioxidant, presents the opportunity to prevent oxidation in pan-fried patties, so addition of CM could be a good strategy, both improving technological values and retarding oxidative changes.

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Prevalence, serovar, and antimicrobial resistance of *Salmonella* isolated from meat and minced meat used for production smoked sausage

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Prevalence, serovar, and antimicrobial resistance of *Salmonella* isolated from meat and minced meat used for production smoked sausage

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Abstract. The objective of this study was to research the prevalence, serovars, and antimicrobial resistance profiles of *Salmonella* isolated from meat and minced meat used for the production of fermented sausage. A total of 116 samples were tested, and among them, 20 (17.2%) were positive. *Salmonella* was detected in 3 (10.3%) beef samples, 5 (19.2%) pork samples, and 6 (20.7%) poultry samples. In minced meat, the *Salmonella* prevalence was 18.8%. *Salmonella enterica* serovar Agama (5.2%) was the most commonly identified serovar, followed by *S.* Enteritidis (4.3%), *S.* Typhimurium (3.4%), *S.* Infantis (2.6%), and *S.* Lindenburg (1.7%). Most of the serovars identified in the present study are recognized as frequent causes of human salmonellosis. Thus, the presence of these serovars means foods with these meats are a likely source of human infections. We found the *Salmonella* isolates exhibited high rates of resistance to antimicrobials tetracycline, ampicillin, streptomycin, and ciprofloxacin. The highest level of resistance was to tetracycline (75%), followed by resistance to ampicillin (50%), streptomycin (30%), ciprofloxacin (20%), gentamicin (20%), and neomycin (10%). The high-level resistance observed for some of the serovars calls for concern. *Salmonella* with multidrug resistance in meat used to produce fermented sausages is considered a high additional risk for human health.

1. Introduction

Fermented sausages are high-value traditional products. Fermented sausages and air-dried meats are at additional risk of containing bacterial hazards, as in most cases, they are not subject to the heating process. Fermented meat products result from a complex microbiological activity that mainly consists of lactic fermentation and several characteristic biochemical changes triggered by lactic acid bacteria. These bacteria play a crucial role in this fermentation: acidification, while they can also have proteolytic and lipolytic activities [1].

Meat used for the production of fermented sausages can contain residual levels of antimicrobial compounds [2]. The presence of high levels of antibiotic residues in raw meat used to produce raw smoked sausage is a call for concern. Research by Kjeldgaard et al. shows that even minimal residual amounts of antibiotics at the level legally allowed in raw materials negatively affect starter cultures [2]. Fermented sausages prepared from meat with residual concentrations of antibiotics at or close to deemed acceptable levels can lead to full or partial fermentation failures, and therefore to the production of an unsafe product. Outbreaks of severe, sausage-borne gastrointestinal infections caused by verocytotoxic (Shiga toxigenic) *Escherichia coli* (VTEC/STEC), *Salmonella*, and *Listeria monocytogenes* occur

regularly [3]. Food-borne diseases caused by antibiotic-resistant bacteria are an important public health problem worldwide. The use of antimicrobial drugs in agriculture or the food industry can contribute to the formation of resistance in strains (including genotypic resistance), which leads to a relative increase in the number of resistant bacteria in microbial communities. *Salmonella* with multiple antibiotic resistance (AR) presents a considerable threat to public health and food safety [4].

This work aimed to detect *Salmonella* contamination of raw and minced meat used in the production of fermented sausages and determine the bacterial isolates' antimicrobial susceptibility.

2. Materials and Methods

2.1 Samples

The objects researched were meat (pork, beef, poultry) and minced meat used to produce raw smoked fermented sausages obtained from a meat processing enterprise in the central region of Russia. Samples were collected from June 2020 to December 2020.

2.2. Isolation and identification of Salmonella

The sample analyses were carried out according to the modified standard ISO 6579 [5]. Pre-enrichment was done using Buffered Peptone Water (BPW) (Merck, Germany). A homogenized sample (25 g) was added to 225 ml of BPW. After homogenization with a Stomacher (AES, France), the bag was then closed and incubated for 18 h at 37°C. Then, 0.1 ml aliquots of cultures were incubated in 10 ml of Rappaport-Vassiliadis *Salmonella* Enrichment Broth (RVS) (Merck, Germany) at 41.5°C and 10 ml Muller-Kauffmann Tetrathionate Novobiocin Broth (MKTT) (Merck, Germany) at 37°C for 24 h. Loopfuls of RVS and MKTT cultures were streaked onto Xylose-Lysine-Tergitol 4 (XLT4) selective agar plates (Merck, Germany) and chromogenic Brilliance *Salmonella* agar (Oxoid, United Kingdom), then incubated at 37°C for 24 h. Presumptive colonies were subjected to further analysis using biochemical tests. Biochemical identification was performed using a RapID ONE System (Remel, USA).

2.3 Serological identification

The *Salmonella* isolates were serotyped by slide agglutination using polyvalent O and H antisera (Petsal, Russia) following the Kauffmann-White scheme [6].

2.4 Antibiotic susceptibility testing

Six different antibiotics were used in this study, namely, ciprofloxacin (5 μg), streptomycin (300 μg), gentamicin (10 μg), amoxicillin (20 μg), neomycin (30 μg), and tetracycline (30 μg) (Oxoid, United Kingdom). Antimicrobial susceptibility tests were performed on Mueller-Hinton agar (Oxoid, UK) using the disc diffusion technique. The surface of the Mueller-Hinton agar plate was evenly inoculated with the culture using a sterile cotton swab. The antibiotic discs were applied from a disc dispenser (Oxoid, UK) onto the surface of the inoculated agar. After 18-24 h of incubation, the diameter of growth inhibition around the discs was measured and interpreted as sensitive or resistant according to the Clinical and Laboratory Standards Institute [7].

3. Result and Discussion

3.1 Salmonella contamination of raw meat and minced used for the production of raw smoked sausage

In the current study, 116 samples of meat and minced meat used to produce raw smoked sausage were assessed for contamination by *Salmonella*, and the overall prevalence was 16.7 %. *Salmonella* was detected in 3 (10.3 %) beef samples, 5 (19.2%) pork samples, and 6 (20.7 %) poultry samples. The prevalence of minced meat contaminated with *Salmonella* was 18.8%. A summary of the prevalence of *Salmonella* from meat samples and minced meat is presented in Figure 1.

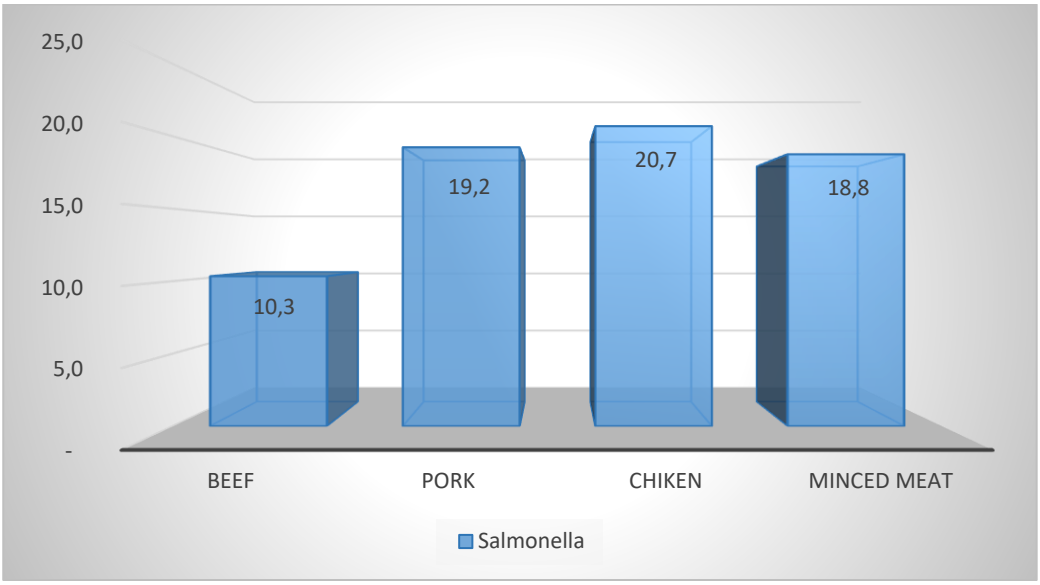

Figure 1. Prevalence of *Salmonella* in meat and minced meat used for the production of raw smoked sausage [%]

Salmonellosis is the most common and challenging to eradicate the zoonotic bacterial infection in the world that affects both animals and humans. According to the Federal Service Russian for Surveillance on Consumer Rights Protection and Human Wellbeing, by the end of 2018, more than 50 outbreaks of group morbidity associated with food consumption in public catering and trade were registered in Russia [8].

The presence of *Salmonella* in raw smoked sausage is a significant risk to consumer health. Sausage manufacturers commonly inoculate sausage meat with lactic-acid-producing bacteria to control the fermentation process. The fermentation induces pH reduction, and the reduction in water activity during maturation contributes to the inactivation of *Salmonella* spp. in the raw ingredient mix.

However, antibiotics used as growth promoters or to treat disease in livestock can eventually end up in meat, and starter cultures sensitive to antibiotics will not acidify the sausage meat effectively [2]. The complete or partial fermentation process failures allow survival of pathogens capable of causing severe food-borne infections, including *Salmonella*.

The prevalence of *Salmonella* in the minced meat for the production of fermented sausages was 18.8%. In a similar study, Piras et al. reported that *Salmonella* prevalence was 24% in ground meat for fermented sausage and products at the end of acidification. The pathogen was also detected in a sausage sample at the end of ripening (2%) [9]. Our result on *Salmonella* prevalence in samples of raw minced meat (18.8%) was higher than those reported by other authors who observed prevalences between 0.3 and 4.3 % [10,11].

Contamination of minced meat with *Salmonella* is still considered a significant problem in food hygiene. Despite substantial improvements in technology and hygienic practices employed in all stages of meat production, salmonellosis remains an intransigent threat to human health. Therefore, the resulting data will be hugely beneficial for future risk assessment in the production of fermented sausages.

3.2 Serological identification of Salmonella

From all samples, five different serovars were identified among 20 *S. enterica* isolates (Table 1). *S. Agama* (5.2%) was the most commonly identified serovar, followed by *S.* Enteritidis (4.3%), *S.* Typhimurium (3.4%), *S.* Infantis (2.6%) and *S.* Lindenburg (2 %).

The identified serovars from beef were *S.* Enteritidis, *S.* Typhimurium, and *S.* Agama. *Salmonella* isolates from pork were *S.* Typhimurium (7.7%), *S.* Infantis (7.7%), and *S.* Lindenburg (3.8%). The isolated *Salmonella* serovars from chicken were *S.* Agama and *S.* Enteritidis (6.7% each), *S.* Typhimurium (3.3%), *S.* Infantis (3.8%).

The identified *Salmonella* serovars from minced meat were *S.* Agama (9.3%), *S.* Enteritidis (6.3%), and *S.* Lindenburg (3.1%).

Table 1. Prevalence of *Salmonella* serovars in samples of raw meats and minced meat used for production raw smoked sausage

Serovars (antigenic formula)	Beef (n=29)	Pork (n=26)	Poultry (n=29)	Minced meat (n=32)	Total (n=116)
S. Enteritidis	1 (3.4)	0	2 (6.9)	2 (6.3)	5 (4.3)
S. Typhimurium	1 (3.4)	2 (7.7)	1 (3.4)	0	4 (3.4)
S. Lindenburg	0	1 (3.8)	0	1 (3.1)	2 (1.7)
S. Agama	1(3.4)	0	2 (6.9)	3 (9.4)	6 (5.2)
S. Infantis	0	2 (7.7)	1 (3.4)	0	3 (2.6)
Total *Salmonella* isolates, no (%)	3 (10.3)	5 (19.2)	6 (20.7)	6 (18.8)	20 (17.2)

S. Agama, *S.* Enteritidis, and *S.* Typhimurium were the most prevalent serovars identified in the current study. The prevalence of different *Salmonella* serovars in meat products has been investigated in many countries. *Salmonella enterica* ser. 6.7:d:- (29%), *S.* Agama (28%), and *S.* Typhimurium (16%) were the three most prevalent serovars in retail meat and meat products in China [12].

S. Derby, *S.* Typhimurium, and *S.* Enteritidis are frequently identified worldwide, and the latter is one of the most common serovars associated with human salmonellosis [13,14]. This serovar also accounted for 4.3 % of the isolates recovered from meat and minced meat in the current study.

S. Agama was isolated from all the meat types except pork in this study. *S. enterica* Agama was initially isolated and named after the rainbow lizard (*Agama Agama*) in West Africa. A study by Ahmed et al. showed that *Salmonella* Agama was obtained from the poultry environment (feed and water), dead birds (liver, spleen, and ovarian follicle), and seemingly healthy birds (cloaca swabs). Also, one of the predominant serovars in the study by Ahmed et al. was *S.* Agama - 28%. It was isolated from all the poultry farms in North Central Nigeria [15]. In our study, *S.* Agama was also detected in poultry but in beef too. The presence of *S.* Agama in the samples of minced meat is most likely from samples of poultry and beef.

S. Infantis has been one of the most frequent serovars in many countries. *S.* Infantis has been isolated from humans, animals, and vegetables, meats (e.g., broiler and chicken) [16,17].

Most of the *Salmonella* serovars identified in the present study are recognized as frequent causes of human salmonellosis. Thus, these serovars in meat products show these foods are likely sources of human infections.

3.3 Antibiotic susceptibility testing

In general, a high percentage of resistance to the tested antimicrobials was observed across all the serovars. Even though all isolates of *S.* Enteritidis were susceptible to gentamicin, streptomycin, and neomycin, they were resistant to tetracycline 60%, ampicillin (40%), and ciprofloxacin 20%. Around 25% of *S.* Typhimurium strains were resistant to gentamicin and neomycin, 50% were resistant to streptomycin, and 100% were resistant to tetracycline. The increase in antibiotic-resistant *Salmonella* is a significant concern worldwide.

Table 2. Antimicrobial resistance of *Salmonella* serovars isolated from raw meats and minced meat used for production fermentation sausage (AMC – ampicillin, GEN – gentamicin, TE – tetracycline, CIP – ciprofloxacin, STR – streptomycin, NEO – neomycin)

	AMC no (%)	GEN, no (%)	TE no (%)	CIP no (%)	STR no (%)	NEO no (%)
S. Enteritidis	2 (40)	0	3 (60)	1(20)	0	0
S. Typhimurium	3 (75)	1(25)	4(100)	0	2(50)	1(25)
S. Lindenburg	1 (50)	0	2(100)	0	1(50)	0
S. Agama	3 (50)	2 (33)	4(67)	3(50)	2(33)	1(17)
S. Infantis	1 (33)	1(33)	2(67)	1(33)	1(33)	0
Total	10(50)	4(20)	15(75)	4(20)	6(30)	2(10)

All the isolates showed high resistance to ciprofloxacin (20-50%) except for *S.* Typhimurium and *Salmonella* Lindenburg, which were susceptible to ciprofloxacin.

Resistance to two and more antibiotics was common in most isolates from samples in the current study. *S.* Agama was one of the most prevalent serovars in this study and showed a high level of resistance to most of the commonly used antimicrobials.

In a similar study, a total of 62% of *Salmonella* isolates exhibited resistance to at least one antimicrobial drug. In another study, resistance to sulfamethoxazole (38%), ampicillin (24%), nalidixic acid (24%), ciprofloxacin (24%), and tetracycline (19%) was identified the most frequently [18]. Notably, in our study, the highest level of resistance against most antibiotics was shown among *S.* Agama, which exhibited increased resistance to tetracycline (67%), ampicillin (50%), ciprofloxacin (50%), streptomycin (33%), gentamicin (33%) and neomycin (17%). The high level of resistance to most of the antimicrobials tested in this study, especially ciprofloxacin, is problematic because fluoroquinolones are used strategically to treat salmonellosis. This resistance can be caused by indiscriminate use of antimicrobials at recommended doses or at subtherapeutic doses in feed as growth promoters and chemotherapeutic agents to control epizootics on the farms [15].

The widespread overuse and misuse of antimicrobial agents in food animal production have contributed to the development of antimicrobial-resistant pathogens such as *Salmonella* that has emerged as a major health problem worldwide.

4. Conclusion

Residual antibiotics in meat can reduce the proliferation of starter culture in raw sausage fermentation and thus disrupt the fermentation process for raw smoked sausage. Still, these concentrations do not affect the survival or even multiplication of antibiotic-resistant pathogens. Therefore, the presence of pathogenic bacteria, such as *Salmonella*, in meat and minced meat used to produce smoked sausages pose a threat to consumers. Our study found *S.* Agama, *S.* Typhimurium, *S.* Lindenburg, *S.* Enteritidis, and *S.* Infantis in meat and minced meat. Worryingly, most *Salmonella* serovars identified in the present study are recognized as frequent causes of human salmonellosis. In cases of improperly fermented meat products, for example, due to the presence of antibiotics in meat, these serovars can survive and cause human disease. Also, in the present study, we found that *Salmonella* isolates exhibited high rates of resistance to antimicrobials tetracycline, ampicillin, streptomycin, and ciprofloxacin. The high-level resistance observed for some of the *Salmonella* serovars isolated in this study calls for concern. Our findings indicate that it is imperative to continue monitoring *Salmonella*'s prevalence and researching antimicrobial resistance in meat and minced meat used to produce raw smoked sausage.

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Preface

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Preface

It is our pleasure to welcome you to the 61st International Meat Industry Conference (MEATCON2021) to be held at Mt. Zlatibor, Serbia, on September 26-29th 2021, traditionally hosted by the Institute of Meat Hygiene and Technology, Belgrade, Serbia.

For many years, this Conference has been the converging point for food/feed scientists, experts from the industry and legislation sphere, members of the professional associations, and other entities striving towards a common goal: **healthy food for the present and future**. The rapid development of scientific and technological research in the areas of food/feed technology, safety, quality, regulatory, environmental, and numerous other fields imposed the necessity for a synthetic approach to food-related challenges. Such an approach is primarily governed by understanding the complexity of food science that is taking place within the scientific community for the past two decades. Hence our commitment to the multidisciplinary nature of the Conference.

This three-day gathering will try to maintain its holistic character, presenting the latest research in food and feed production and technology, quality and safety issues, risk-assessment, consumer-related concerns, governmental actions, and strategies – with 111 contributions submitted for publication. Each paper has been peer-reviewed through a rigorous process that included conference committee members and international reviewers, and an English language editorial service. We are excited to publish all the contributions to the Conference in this volume, hoping that its contents shall be helpful to Conference participants and other interested readers.

The 61st International Meat Industry Conference also provides the opportunity for fellow scientists to meet, exchange experiences, and perhaps agree on future joint scientific endeavours. Carefully selected speakers from Serbia and abroad will present their latest research through plenary lectures, invited lectures on specific topics, and poster presentations. Formats such as round tables and workshops are also available to disseminate knowledge and experience.

The Conference has been supported by: Ministry of Education, Science and Technological Development of the Republic of Serbia, Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia – Veterinary Directorate and Chamber of Commerce and Industry of Serbia. Co-organizers of the Conference are the Faculty of Veterinary Medicine, University of Belgrade, Faculty of Agriculture, University of Belgrade, and Institute of Food Technology, University of Novi Sad.

Target audience:

- Food scientists and researchers

- Food technologists

- Scientists and researchers in the area of food quality and safety

- Food professionals from manufacturing, retail, foodservice industry…

- Governmental officials and policymakers in the area of food quality and security

On behalf of the Programme Committee, we would like to thank all contributing authors, reviewers, speakers, participants, Organizing Committee, and sponsors who contributed to the success of the MEATCON2021.

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- **Conference submission management system: MachForm, Appnitro Software**
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Effects of composition and storage duration of mechanically deboned poultry meat on sensory properties of frankfurters

Effects of composition and storage duration of mechanically deboned poultry meat on sensory properties of frankfurters

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Abstract. This research aimed to study the influence of differences in the composition and storage length of mechanically deboned poultry meat (MDPM) on the sensory properties of frankfurters. Three variants of frankfurters were produced from three respective alternatives of MDPM that differed solely in proportions of meat from broiler backs and necks. Similarly, a commercially available and freshly produced MDPM of unknown composition was used as the control. All the four variants of MDPM were stored at -18 °C for 1, 45 and 90 days. Sensory profiling of the frankfurters was performed by 8 panellists using a quantitative-descriptive analysis (QDA). Two-factorial ANOVA and principal component analysis (PCA) of the sensory evaluation results revealed significant ($p < 0.05$) effects of the storage time of the MDPM variants on sensory characteristics of the frankfurters, regardless of their composition.

1. Introduction

Mechanically deboned poultry meat (MDPM) is produced by mechanical separation of soft tissues from bones after manual deboning. It is the main raw material for production of thermally processed poultry sausages, such as frankfurters. Due to a large number of consumers of frankfurters in Bosnia and Herzegovina (B&H), those sausages have a dominant place in the production portfolio of most of B&H meat processing companies. To meet the high expectations of the consumers and improve their own market competitiveness, it is essential for each of the companies to be devoted to continuously improve the sensory properties of its products.

Current B&H regulation on MDPM [1] requires, among other things, that it can be stored at a minimum of -18 °C for up to three months. Thus, it is implied that the storage duration of MDPM has a key impact on the overall quality of poultry sausages, in which their sensory quality attracts particular attention of the consumers. There are many approaches to sensory evaluation of sausages, among which the quantitative-descriptive analysis (QDA) is one of the most widely used and reliable sensory profiling methods [2].

The goal of the study was to evaluate the effects of the composition and the storage duration of MDPM on sensory characteristics of experimentally produced frankfurters.

2. Materials and methods

To assess the effects of composition and storage duration of MDPM on sensory properties of frankfurters, experimental production of frankfurters was performed as a two-factorial completely randomized design with a 4 × 3 factorial structure [3]. The first factor was the MDPM composition (variant) at four levels: MDPM1, MDPM2, MDPM3 and MDPMC, while the second factor was the MDPM storage duration (D) at three levels: D1, D2 and D3. All coded factors, levels and experimental treatments are displayed in Table 1.

Table 1. Two-factor completely randomized design for experimental production of frankfurters using MDPM[a] of different composition (variants) and storage time

Frankfurters (F)	MDPM variant	MDPM storage duration (D)		
		D1(1 day)	D2 (45 days)	D3 (90 days)
F1[b]	MDPM1	MDPM1×D1	MDPM1×D2	MDPM1×D3
F2[c]	MDPM2	MDPM2×D1	MDPM2×D2	MDPM2×D3
F3[d]	MDPM3	MDPM3×D1	MDPM3×D2	MDPM3×D3
Fc[e]	MDPMC	MDPMc×D1	MDPMc×D2	MDPMc×D3

[a] MDPM, mechanically deboned poultry meat
[b] F1, frankfurters produced from MDPM1 (50% of deboned back meat + 50% of deboned neck meat) after D1, D2 and D3 storage durations at -18 °C
[c] F2, frankfurters produced from MDPM2 (70% of deboned back meat + 30% of deboned neck meat) after D1, D2 and D3 storage durations at -18 °C
[d] F3, frankfurters produced from MDPM3 (30% of deboned back meat + 70% of deboned neck meat) after D1, D2 and D3 storage durations at -18 °C
[e] Fc, frankfurters produced from MDPMC (commercially available MDPM with unknown composition) after D1, D2 and D3 storage durations at -18 °C

Four variants of MDPM were used for production of the frankfurters. Three of them (MDPM1, MDPM2 and MDPM3) were experimentally produced with different proportions of broiler back and neck meat, while commercially available MDPMC with unknown composition was used as the fourth variant (Table 1). The three experimental variants of MDPM were produced in a commercial processing plant in B&H. After the commercial cutting of the carcasses, broiler backs (pelvic and thoracic portions) and necks were stored overnight at 2 °C before deboning. The deboning was done by AM2C SM 210 separator (AM2C SAS, Quimper, France) adjusted to yield about 50% ± 5% MDPM, and connected to a Wolf AW 160 grinder (K+G Wetter GmbH, Germany). Every batch of the MDPM was vacuum packed in Cryovac BB405 bags (Cryovac A/S, Oslo, Norway) as 10-kg blocks, which were frozen at -30 °C and stored at -18 °C. The MDPM blocks were delivered next day under cold chain conditions to Menprom Meat Industry, where experimental production of frankfurters was carried out. In the same way, the MDPMC was freshly produced and delivered from another common supplier. All the four MDPM variants were stored at -18 °C until the formulation of the sausages. On the same day of delivery, the first third of each of the four MDPM variants was used to produce the frankfurters (D1 storage duration), while the second and the third parts of the variants were used after 45 days (D2 storage duration) and 90 days (D3 storage duration), respectively.

The four variants of frankfurters (F1, F2, F3 and FC; Table 1) were produced according to the same recipe and technological procedure, which was routinely used at Menprom Meat Industry, i.e. they differed only in respective MDPM variants. Mechanically deboned poultry meat was thawed for 24 h at 4 °C before use. Each batch of the frankfurter variants was prepared with 25.7 kg of MDPM, 10.9 kg of chicken skin fat emulsion, 3.4 kg of water (ice), 0.58 kg of nitrite salt, 0.85 kg of corn starch, 0.55 kg of soya proteins and other ingredients (commercial spice and additive mixtures). The frankfurters were initially heated at 55 °C for 20 min, then smoked at 65 °C for 10 min, and finally processed at 80 °C for

25 min to reach a core-temperature of 79 °C. Immediately after the heating process, the sausages were rinsed in cool water (7 °C) for approximately 1 h and cooled at 4 °C for 2 h. After the cooling step, cellulose casings were automatically peeled off, the frankfurters were vacuum packed in pouches (4 sausages each) and stored at 4 °C (±1 °C) for 2 weeks until sensory analysis.

To evaluate the compliance of chemical composition of the four MDPM variants (Table 1) with legal norms [1], a 300 g sample of each of the variants was taken in triplicate before commencement of the sausage formulation. The samples were immediately transported under cold chain conditions to the Department of Food Hygiene and Technology, Veterinary Faculty, University of Sarajevo, where the contents of moisture [4], total fat [5], total proteins [6] and calcium [7] were analysed.

To perform sensory evaluation, the sausages were transported under cold chain conditions to the Faculty of Agriculture and Food Sciences, University of Sarajevo and stored at 4 °C (±1 °C) until the evaluation. Spatial conditions for the evaluation were arranged according to the international standard [8], and the sensory profiling method was inspired by the previously described QDA methods [2, 9, 10]. The sensory panellists were selected after four sessions of group discussions and individual rating. To avoid bias, the training sessions were performed by a passive panel coordinator, who was informed of the objective of the sensory analysis. Since the panellists had already been trained in the evaluation of meat products, the training primarily focused on the definition and respective intensity of sensory characteristics of the frankfurters, as well as on the application of the evaluation method. During the training, commercially available frankfurters from different producers were used, as well as samples of the experimental sausages. However, the assessors were not aware of the content and the origin of the sausages. Finally, the set of 10 sensory characteristics of the frankfurters included appearance, colour, palpation consistency, oral (first bite) consistency, chewiness, juiciness, surface odour, taste, flavour and overall acceptability (Table 3). Selection of the panellists was done based on calculation of the coefficient of variation (CV) of their testing results, and the panellists who showed CV > 30% were excluded. In this way, the sensory profiling panel was selected among 20 invited assessors, which finally consisted of eight (three female and five male) trained assessors aged between 32 and 64 and with from 5 to 20 years of experience in the sensory profiling of sausages and other meat products.

Prior to the final evaluation, the vacuum pouches were unpacked, the frankfurters were boiled in water for 5 min and each assessor was served with a hot sausage sliced into 4-5 cm long pieces on a warm plastic plate marked with a 3-digit random number and covered by foil. Samples of the each treatment (Table 1) were served in triplicates to the assessors in their own time, and the serving order was randomised with regard to sample, replicate and assessor, with short breaks through the assessment to reduce the sensory fatigue effect. The panellists were served with water and flat bread between samples for cleansing the palate. Sensory evaluation of the sausages was carried out on a scorecard using a 70 mm unstructured line scale for each of the 10 sensory characteristics, with an anchor of 10 mm from each side marked as “low” and “high” intensity of the sensory characteristic. The panellists scored each of the 10 sensory characteristics by placing a vertical mark across the interval line at the point which best reflected the magnitude of his or her perceived intensity of the sensory property. The evaluation results were converted into numerical scores by measuring the distance from the left end of the scale to the point marked by the panellists. The evaluation score of each panellist was calculated as an average grade of the three replicates of the each treatment (Table 1).

Normality of the sensory evaluation data was checked by Kolmogorov-Smirnoff test. Two-factorial ANOVA was used to test simultaneous effects between the two factors and their levels (the MDPM composition with four levels/variants and the MDPM storage time with 3 levels; Table 1). When the effect of each of the analysed factors and their interactions was significant, the means were separated using Tukey’s test. Similarly, one-way ANOVA with Tukey’s test was used to analyse the data of chemical analyses. All of the analyses were performed by using the IBM SPSS Statistics v. 20 software (IBM Inc., USA). P-values less than 0.05 were considered statistically significant. Principal component analysis (PCA) was applied to identify clusters of mutually independent variables and to visualise effects of the two factors on the sensory characteristics of sausages. PCA was done by using the statistical package StatBoX 6.7 (Grimmersoft, Paris, France).

3. Results and discussion

Results of chemical analyses of the four MDPM variants are shown in Table 2. All of the MDPM variants showed desirable protein contents, since they had more than the legally required minimum of 12% of proteins [1]. In addition, all the MDPM variants displayed a higher protein content than those used in studies of Trinidade *et al* [11] (9.3%-14.5%) and Botka-Petrak *et al* [12] (13.46%-15.57%). Similarly, Trinidade *et al* [11] reported lower moisture contents (63.4-66.6%) than those of MDPM1, MDPM2 and MDPM3, but higher than the moisture content in the MDPMC. In contrast, Botka-Petrak *et al* [12] estimated moisture content in MDPM made of whole carcasses at 69.14%, which is in agreement with the moisture content in MDPM1, MDPM2 and MDPM3. The range of the total fat content reported by Trinidade *et al* [11] for MDPM made from chicken backs and necks (14.45%-27.7%) includes the average fat content of the MDPM2, while the MDPM1 and the MDPM3 had lower average fat contents. The MDPMC variant displayed significantly higher ($p < 0.001$) fat content than the other experimental MDPM variants, which should be considered when designing the recipes for poultry products. None of the MDPM variants met the compulsory maximum of 0.1% for the content of calcium [1]. Similar findings on the calcium content were reported [12], where only the MDPM made of whole broiler carcasses displayed the desirable content of 0.06% of calcium, while the MDMP variants made of the deboned meat of backs, wings and necks displayed an average calcium contents of 19.50%, 29.36% and 21.60%, respectively, being much higher than those found in this study. Such findings surely indicate the need to check and modify the deboning process, as it is known that the mechanical deboning leads to extraction and oxidation of the lipids from the bone marrow, and, consequently, to undesirable taste and odour of the final products [13].

Table 2. Chemical content of MDPM[a] variants (mean±standard deviation of three measurements)

Parameter (%)	MDPM variants [b]				*p*
	MDPM1	MDPM2	MDPM3	MDPMC	
Moisture	69.60±0.46a	68.92±0.14a	69.93±0.71a	62.72±0.86b	< 0.001
Total proteins	17.04±0.47ab	16.86±0.59ab	17.57±0.37a	16.07±0.23b	0.018
Total fat	13.64±0.79a	14.70±0.51a	11.55±0.27b	20.21±0.43c	< 0.001
Calcium	0.20±0.02a	0.19±0.04a	0.23±0.02a	0.32±0.05b	< 0.001

[a] MDPM, mechanically deboned poultry meat
[b] MDPM variants differed in content of deboned back meat and deboned neck meat (see Table 1 for details)
Different letters (a-c) in the same row denote significant differences ($p < 0.05$) (ANOVA with Tukey's test)

Results of the sensory evaluation of the experimentally produced frankfurters showed that differences in the sensory attributes primarily occurred due to the effect of the MDPM storage duration. The two-factorial ANOVA (Table 3) resulted in highly significant ($p < 0.001$) effects of the MDPM storage duration (factor D) on all the 10 assessed sensorial characteristics, as well as a significant ($p < 0.05$) impact of the variants of frankfurters (factor F) on six of the sensory properties (oral consistency, juiciness, surface odour, taste, flavour and overall acceptability). Additionally, the interactions of the factors (D*F) also triggered a significant ($p < 0.05$) impact on chewiness of the sausages.

Table 3. Analysis of variance of effects of the MDPM[a] storage duration and the variants of frankfurters on sensory characteristics of the frankfurters (*F*-values for independent variables and interactions)

Characteristics	MDPM storage duration (D)[b]			Variants of frankfurters (F)[c]				Sources of variations		
	D1	D2	D3	F1	F2	F3	FC	D	F	D*F
Appearance	4.51a	3.77b	3.12c	3.73	3.82	3.86	3.80	371.09***	1.83 ^{NS}	1.53 ^{NS}
Colour	4.56a	3.86b	3.17c	3.82	3.85	3.88	3.90	479.65***	0.98 ^{NS}	0.69 ^{NS}
Consistency (palpation)	4.50a	4.14b	3.27c	3.89	3.96	4.05	3.98	283.80***	2.25 ^{NS}	0.65 ^{NS}
Consistency (oral)	3.70b	3.99a	3.31c	3.66ab	3.66ab	3.75a	3.61b	278.60***	5.94**	7.40***
Chewiness	3.77b	4.01a	3.32c	3.74	3.70	3.72	3.65	169.31***	1.61^{NS}	2.88*
Juiciness	4.21a	4.03b	3.29c	3.79	3.88	3.91	3.78	278.78***	3.62*	3.01*
Surface odour	4.32a	3.89b	3.13c	3.75ab	3.85a	3.86a	3.67b	528.42***	8.80***	5.38***
Taste	4.31a	3.85b	3.14c	3.75ab	3.82a	3.83a	3.67b	417.47***	4.51*	3.81**
Flavour	3.57b	3.71a	2.96c	3.49a	3.46a	3.46a	3.24b	161.30***	9.99***	5.77***
Overall acceptability	4.26a	3.96b	3.19c	3.80a	3.88a	3.87a	3.67b	428.95***	9.68***	2.93*

[a] MDPM, mechanically deboned poultry meat

[b] D1, D2 and D3 – mean intensity panel (n=8) scores of the sensory characteristics (using an unstructured line scale from 0 to 70 mm) of the frankfurters produced from the MDPM variants (see Table 1 for details) stored at -18 °C for 1, 45 and 90 days , respectively.

[c] F1, F2, F3 and FC – mean intensity panel (n=8) scores of the sensory characteristics (using an unstructured line scale from 0 to 70 mm) of the frankfurters produced from MDPM1, MDPM2, MDPM3 and MDPM, respectively (see Table 1 for details).

Different letters (a-c) in the same row denote significant differences ($p < 0.05$) (Two-factorial ANOVA with Tukey's test); NS, non-significant; * - significant at $p < 0.05$; ** - significant at $p < 0.01$; *** - significant at $p < 0.001$.

The dependence of the sensory properties of frankfurters on the MDPM storage duration is also displayed by the corresponding results of their PCA analysis (Figure 1), which explained 98.10% of variability of all results of the sensory evaluation of frankfurters. The PCA analysis determined that all frankfurters produced from all the four variants of MDPM after 1 day of storage (D1, blue coloured cluster), regardless of their composition, showed significantly better ($p < 0.05$; Table 3) colour, appearance, surface odour, taste, palpation consistency, juiciness and overall acceptability when compared to the frankfurters produced from the MDPM variants stored for 45 days (D2, red coloured cluster). On the other hand, D2 frankfurters displayed significantly higher ($p < 0.05$; Table 3) sensory scores for oral consistency, chewiness and flavour than all the other frankfurters, which indicates their favourable sensory properties. In contrary, the frankfurters produced from MDPM variants stored for 90 days (D3, green coloured cluster) exhibited significantly inferior ($p < 0.05$; Table 3) sensory characteristics than the sausages from MDPMs stored for 1 and 45 days. In support of this result, Froning *et al* [14] described a substantial decrease of sensory properties of frankfurters produced from frozen MDPM stored for 90 days. Also, it is notable that the poorest sensory properties in all the three clusters were obtained by frankfurters prepared from commercially available MDPMC (FCD1, FCD2 and FCD3). Such finding may be explained by significantly higher ($p<0.001$) content of total fat in MDPMC (20.06%; Table 2) compared to the other MDPM variants. A similar finding was described by Biswas *et al* [14], who reported significant differences ($p<0.05$) in some sensory properties of chicken sausages with increase of the fat content due to higher proportion of chicken skin and fat in the composition of the sausages.

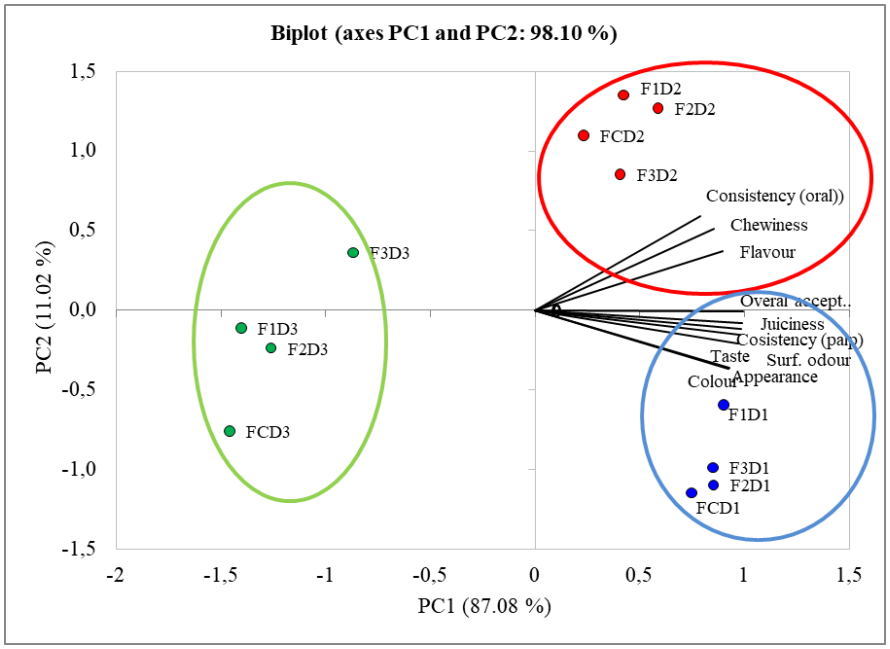

Figure 1. Principal component analysis **(**PCA) bi-plot of distribution of samples of the variants of frankfurters (F1, F2, F3 and FC – see Table 1 for details) and the different storage durations of mechanically deboned poultry meat (MDPM) at -18 °C (D1, D2 and D3 for 1, 45 and 90 days, respectively) with regard to sensory attributes of the frankfurters.

4. Conclusions

The findings of the study clearly indicate that the composition and storage duration of MDPM significantly affect sensory quality of frankfurters, where the intensity of the sensory properties of frankfurters significantly deteriorates with increasing the duration MDPM is stored at -18 °C.

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The radioactivity parameters in the food chain - legislation, control and critical points

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The radioactivity parameters in the food chain – legislation, control and critical points

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Abstract. Radioactivity, whether natural or artificial, today constitutes a significant segment in the process of protecting human and animal health. Natural radioactivity is an integral part of our ecological system, so it has long been present in the food we eat. However, intensive industrial processes in some areas have disturbed the natural ecological balance and thus introduced into the environment natural radionuclides in quantities that can affect the quality of human life and the state of the environment. On the other hand, artificial radionuclides reach the environment only in the case of accident situations in nuclear facilities. In the nuclear era so far, two accidents, Chernobyl in 1986 and Fukushima in 2011, affected the environment globally with the significant impact. Consequently, a system of monitoring radioactivity in the environment was introduced, which includes foodstuffs as well as animal feed. The paper describes the sources of radioactive contamination and the most critical raw materials and products in the human and animal food chain, as well as a critical review of current legislation.

1. Introduction

Natural radioactivity is an integral part of our ecological system, so it has long been present in the food we eat. The human body is thus in a balance between natural and cosmic radiation because there are natural processes of repairing cells damaged by that radioactive radiation. By consuming food during their life, a person also increases the total radioactivity of their body, because part of the ingested radionuclides is retained in the body and is included in metabolic processes. This is the reason we need to monitor the annual consumption and, consequently, intake of natural radionuclides in the population, to keep it at the safe level.

However, intensive industrial processes in some areas have disturbed the natural ecological balance and thus introduced into the environment natural radionuclides in quantities that can affect the quality of human life and the state of the environment. Such materials are called *NORM* (naturally occurring radioactive material), while a special category consists of *TENORM* products (technologically-enhanced NORM), in which the original content of natural radionuclides is increased by the technological process itself, making them even more dangerous to the environment [1].

The rapid alert system for food and feed (RASFF) monitors the results of control of foodstuffs and products, as well as animal feed, from the point of view of all quality parameters, including radionuclides.

2. The source of radioactivity

The concentration of natural radionuclides depends on the location and is highest in places where mineral raw materials are exploited, which includes radionuclides from the uranium and thorium series, together with all their radioactive descendants, the most important among them being radon gas (^{222}Rn). This radionuclide is not characteristic of industrial processes, so we will not consider it further.

Besides the ore and coal mining industries, the technology of production of mineral phosphate and potassium fertilizers for plant nutrition and mineral additives for animal feed also significantly contribute to the introduction of natural radionuclides into the environment. The reason is that most phosphate ores are characterized by the presence of radioactive elements from the uranium and thorium series, especially ^{226}Ra and ^{238}U, so the total specific activity of these ores can reach several thousand Bq/kg. Therefore, crude phosphate as a semi-finished product and mineral fertilizers with potassium and phosphorus also contain significant amounts of natural radionuclides. These radionuclides are regularly introduced into the ecosystem through plant nutrition processes, but also through technology of wastewater and waste treatment, because unwanted contamination and exposures can originate from radionuclides that enter the environment due to inadequate waste treatment, especially *via* phosphogypsum, which is a by-product of phosphate production. Mineral additives for animal feed, based on phosphates, can significantly increase the amount of natural radionuclides in diet and consequently in the meat and other products.

In that way, a part of these radionuclides through the animal nutrition chain, but also deposition, can end up in foodstuffs. It is estimated that the total contribution of this industry, together with the oil and gas exploration industry, is almost 95% of the total input of natural radionuclides into the environment.

In addition to the mentioned natural radionuclides, one of the most important sources of natural radiation is potassium, i.e., its radioactive isotope ^{40}K. Unlike natural radionuclides from the uranium and thorium series, which, due to their chemical structure (heavy metals), are not included in metabolic processes, ^{40}K acts like natural potassium, fully participates in human and animal metabolism and finally is deposited in bones. It is found in soils in quantities that correspond to specific activities in the range of 600-900 Bq/kg, and consequently, it is also found in various products for human and animal nutrition. During life, a person constantly ingests new amounts of ^{40}K through food, with a contribution of approximately 65 Bq/kg body weight, so this radionuclide together with ^{14}C makes up almost the total radioactivity of the human body, which for a person of 80 kg is about 5200 Bq ^{40}K and 3400 Bq ^{14}C.

On the other hand, artificial radionuclides reach the environment only in the case of accident situations in nuclear facilities. The highest global radioactive contamination that has affected the environment was registered after the 1986 Chernobyl and 2011 Fukushima disasters. The accident at the Chernobyl nuclear power plant reactor was recorded as the accident that led to the greatest radioactive pollution of the environment, especially in Eastern and Northern Europe. The consequences of that accident are still measurable, and some of the artificial radionuclides that were emitted at that time are still detected in samples from the environment, including foodstuffs. Of course, due to the natural process of radioactive element decay, their concentration is now much lower than in the years immediately after the accident. Due to its longer half-life of about 30 years, ^{137}Cs is the primary radionuclide that can still be detected in the environment. Significant amounts of radioactive ^{137}Cs originating from this accident are today mostly detected in forest fruits, mushrooms and wild game meat since this forest ecosystem is mostly not mechanically processed and treated, which are procedures that reduce the specific activity of radionuclides in the surface layer of the soil. Given the much smaller share of these foods compared to other agro-industrial products, the contribution of this radioactivity to the total dose received by the population is relatively small.

Forest wild mushrooms can have a ^{137}Cs specific radioactivity of several hundred Bq/kg, and exceptionally, up to 1000 Bq/kg depending on mushroom species, the altitude at which they were collected and the characteristics of the environment itself. In addition to mushrooms, berries can also have increased radioactivity due to the presence of ^{137}Cs.

Wild game, especially boar and deer, also can produce meats that contain increased amounts of radioactive Cs as a result of their diet and the accumulation of radionuclides in the animal's body.

Therefore, some European countries have defined a limit of 3000 Bq/kg for ^{137}Cs in wild meat from certain areas (Norway) [2]. Based on the data published in Germany for 2017-2019, it was concluded that the maximum values of specific activity of radioactive ^{137}Cs in wild boar meat were up to 1600 Bq/kg, which excluded further sale of that meat and its restriction to personal use only [3].

3. The current legislation

European legislation defines the maximum values of permitted contamination by appropriate regulations, especially foodstuffs and feed originating from third countries, are particularly important items to be controlled for radionuclide content and their specific activity (*Commission Implementing Regulation (EU) 2020/1158 of 5 August 2020 on the conditions governing imports of food and feed originating in third countries following the accident at the Chernobyl nuclear power station*).
In relation to the previous edition of this rulebook from 2008, the radionuclide ^{134}Cs was excluded from the control system, considering that 10 periods of its half-life (approximately 2 years) had already passed since the accident, and it no longer needed to be controlled.

The prescribed limits of permitted maximum specific activity of ^{137}Cs are 370 Bq/kg in milk, dairy products and food for infants and young children and 600 Bq/kg for all other products. The stated maximum values do not apply to private consumption, only to products for further sale. It is estimated that the contribution of food and water radioactivity to the total dose received by the population is in the order of 10%, but would be greater in any population that has specific eating habits. In numbers, it would look like this: ingestion of food containing 80 kBq of ^{137}Cs corresponds to exposure with a dose of 1mSv, which is approximately one third of the total dose received by the population during one year from all sources. Most countries in the region that are not EU members have accepted the EU legislation in the accession process, so the same maximum allowed values of ^{137}Cs contamination are used on their markets.

The Russian authorities have defined the issue of radioactivity in food much more precisely, as stated in *Annex No. 4* to the *Technical regulation of the Customs Union On Food Safety (TR TS 021/2011)*, where the maximum values for radionuclides ^{137}Cs and ^{90} Sr are defined (Table 1).

Table 1. Maximum allowable levels of radionuclides cesium-137 and strontium-90

No.	Groups of Food Products	Specific Activity of ^{137}Cs, Bq/kg(l)	Specific Activity of ^{90}Sr, Bq/kg(l)
1	Meat, meat products and by-products	200	-
2	Venison, game	300	-
3	Fish and fish products	130	100
4	Dried fish and stockfish	260	-
5	Milk and products of milk processing (except for condensed, concentrated, canned, dry, cheese, and cheese products, butter, and butter paste from cow milk; cream-and-vegetable spread and cream-and-vegetable melted mixture, concentrates of dairy proteins, lactulose, lactose, casein, caseinates, hydrolysates of dairy proteins)	100	25
6	Concentrates of dairy proteins, lactulose, lactose, casein, caseinates, hydrolysates of dairy proteins	300	80
7	Products of milk processing, dry, freeze-dried	500	200
8	Cheese and cheese products	50	100
9	Products of milk processing, concentrated, condensed; dairy, composite dairy, milk-containing canned food products	300	100

10	Butter, butter paste from cow milk, milk fat	200 (for milk fat 100)	60 (for milk fat 80)
11	Cream-and-vegetable spread, cream-and-vegetable melted mixture	100	80
12	Dry nutritional media on a milk basis	160	80
13	Vegetables, root crops including potatoes	80 (600(2))	40 (200(2))
14	Bread and bake goods	40	20
15	Flour, grits, meals, cereals, alimentary products,	60	-
16	Wild berries and preserved wild berry products	160(800(2))	-
17	Fresh mushrooms	500	-
18	Dried mushrooms	2500	-
19	Specialized baby foods ready to serve(1)	40	25
20	Vegetable oils	40	80
21	Oils (fats) interesterified refined deodorized; oils (fats) hydrogenated refined deodorized; margarines; special purpose fats, including cooking, confectionary, and baking fats; milk fat replacers; cocoa butter equivalents, cocoa butter improvers of SOS-type, cocoa butter substitutes of POP-type, non-tempering cocoa butter substitutes, vegetable-and-fat spread, vegetable-and-fat melted mixtures, sauces on the basis of vegetable oils, mayonnaises,	60	80
22	Vegetable-and-cream spreads, vegetable-and-cream melted mixtures	100	80

(1) – in the case of freeze-dried products, the specific activity is determined for a reconstituted product.
(2) – permissible level for a dry product.

Legislation in the Republic of Serbia regarding radioactivity is within the competence of the Directorate for Radiation and Nuclear Safety and Security of Serbia and is formulated in two documents: *Rulebook on control of radioactivity of goods during import, export and transit, 86/19 and 90/19* and the *Ordinance on the limits of radionuclide content in drinking water, foodstuffs, animal feed, medicines, items of general use, construction materials and other goods placed on the market (Official Gazette 36/18)*.

Goods for which gamma spectrometric analysis is mandatory during import, export and transit are listed in *Annex 1* of the *Ordinance on the control of radioactivity of goods during import, export and transit (Official Gazette 44/11, 86/19 and 90/19)*.

Goods under the jurisdiction of phytosanitary inspection and for which gamma spectrometric examination is performed are:

1. Herbs
2. Mushrooms, in any form and products of which they are a part
3. Blueberries, in any form and products of which they are a part
4. Cranberry, in any form and products of which they are a part
5. Forest fruits, in any form and products of which they are a part
6. Mineral phosphate fertilizers (finished product)

Goods under the responsibility of veterinary inspection and for which gamma spectrometric analysis is performed in accordance with the risk analysis are:

1. Meat and products
2. Milk and milk products
3. Edible products of animal origin

4. Fish caught in the sea and their products
5. Other goods under the jurisdiction of the veterinary inspection

The limits of maximum permitted values are prescribed by the *Ordinance on the limits of radionuclide content in drinking water, foodstuffs, animal feed, medicines, general use items, construction materials and other goods placed on the market* and are based on the values of derived radionuclide concentration in water and food which induces an annual dose of 0.1mSv. These sizes depend on the annual quantities of these products that are consumed, so based on WHO recommendations, an average value of 730 l of annual drinking water consumption per capita was taken.

$$\mathrm{IKv} = \frac{GD}{e_g\, V_v}$$

GD - annual dose, maximum value 0.1 mSv.
Ikv - derived concentration of radionuclides in water.
e_g - received effective dose at unit intake.
V_v - annual amount of consumption (for water 730l).

Table 2. Derived specific activities of some radionuclides in drinking water

	Radionuclide	**IK_v, Bq/l**
Natural radionuclide	^{238}U	3
	^{226}Ra	0.5
	^{210}Pb	0.2
Artificial radionuclide	^{14}C	240
	^{90}Sr	4.9
	^{60}Co	40
	^{134}Cs	7.2
	^{137}Cs	11

The same Rulebook defines the specific activity of ^{137}Cs in individual food categories, which are shown in Table 3.

Table 3. Derived ^{137}Cs radionuclide specific activities in particular product categories

Product categories	**IK_h, Bq/kg, Bq/l**
Milk and milk products, infant formulas, vegetables, fruits, cereals, meat and meat products, eggs, other foods such as lard, oil, sugar, sweets, alcoholic and non-alcoholic beverages	15
Powdered milk, wild berries (blueberries, cranberries, blackberries, strawberries, raspberries, currants, gooseberries), game, fish, seafood, mushrooms (fresh and mushroom products), herbs, teas and coffee	150
Dried mushrooms, flavourings, spices and other foods used less than 2 kg per year	600

These two tables shows that the defined value of IK_h for ^{137}Cs is determined arbitrarily, because it would imply that all these categories of food are consumed in amounts per year of 535 kg or l for the first category and 53.5 kg or l for the second category, which is certainly overestimated value.

4. The critical points

Previously, the system of radioactivity control of goods in Serbia included a large number of products, and control, and sampling was performed at the border, so it had a preventive effect in some way. The

import control system with the new editions of the Rulebook excludes certain categories of foodstuffs that have been regularly controlled so far, so that imported cereals are no longer controlled, even though a large part of these products comes from Eastern Europe. Also, the established control system is more focused on imported products, while domestic products are generally more often controlled during exports or monitoring.

As critical points in the current national legislation, which defines radioactivity as a parameter of food and feed safety as well as of other goods placed on the market, we can list the following:

- inconsistency of ordinances describing the control of goods at import and placing of goods on the market.
- certain product categories are excluded from the control process, although they can significantly increase the radiation load of the population (cereals and plant products).
- the categorisation of animal feed is not defined, but a historical approach is used and boundaries for humans are applied, although the diet and quantities are not the same.
- non-compliance of product and raw material categories, e.g., for forest fruits and products (blueberries).
- lack of information for end users about the content of radionuclides in certain types of products and procedures for their safe use (mineral fertilizers, some mineral premixes for animal nutrition).

Compared with the allowed values of specific activity for ^{137}Cs in national and European regulations and in regulations valid in Russia and some former Soviet republics, we notice deviations and differences that can cause misinterpretations and problems in the trade of such goods in import and export. A significant difference is the introduction of control of the content of the radionuclide ^{90}Sr, which is analytically much more complicated than analysis of ^{137}Cs.

5. Conclusion

In conclusion, we point out the fact that Serbia's national legislation is stricter than the European one in terms of radioactive contamination, but that it will be harmonized with it in time. The current situation can lead to certain problems when importing goods that are safe on other markets but cannot be marketed in Serbia because they do not meet the national legislation. Even in the EU system with higher permitted contamination limits than are allowed in Serbia, during 2019, the RASFF recorded 13 cases of food and animal feed with high radionuclide content [4]. Mechanisms for controlling radioactivity in food and animal feed need to be developed and improved, since there are several older generation nuclear power plants in the vicinity of Serbia that are still operational. The security systems of nuclear facilities are certainly one of the most advanced creations of the human mind, but cases like Chernobyl or Fukushima must suggest the need to constantly improve the process of protecting the population from the harmful effects of radioactivity.

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Obtaining plant growth biostimulants by hydrolysis of animal raw materials

Obtaining plant growth biostimulants by hydrolysis of animal raw materials

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Abstract. This paper describes the process of obtaining and using complex biostimulants for plant growth, based on enzyme-mediated and acid-mediated hydrolysates of animal raw materials. Animal blood at slaughter was enzymatically hydrolyzed with a substance that contains up to 35% of free amino acids. The effect of biostimulants on development of agricultural crops and forest plants grown from seed after long dormancy was studied. The changes in enzymatic activity of the system were assessed. The prominent positive effect of the biostimulants on the rate of seed germination and formation of green biomass was established.

1. Introduction

The successful development of modern agriculture is largely based on use of chemicals that stimulate the growth and development of plants [1, 2]. Plant growth rate is influenced by various factors, including the influence of microorganisms that can suppress or stimulate the accelerated growth of plant cells [3]. The microbiota of the growth medium and accelerated growth of cell biomass often contributes to intensive development of a plant [4, 5, 6].

Eco-friendly agricultural production now means the issue of fertilizers and other chemicals is a most important factor. This is especially important when using plant growth regulators [7, 8]. Advances in chemical synthesis and the improvement of analytical control methods nowadays allow the use of very low concentrations of growth regulators, which increases the danger of their use from the ecological point of view [9, 10, 11].

Today, research is focused on production of small chemical molecules by hydrolysis of natural biopolymers, the structures of which contain minor

components valuable for biological plant cells [12, 13]. The aim of this work was to establish whether effectors based on animal raw material hydrolysates would have a stimulating effect on development of plant biomass.

2. Materials and Methods

Seeds of beans *Phaseolus vulgaris* (L.) Savi, curled mustard *Brassica juncea* L. and the slowly germinating seeds of Jack pine *Pinus banksiana* were used. Before germination the seeds were kept at 5-10°C for 1 month.

The beans were soaked in water media for 4-7 hours. The swollen seeds were placed on a moistened filter paper in bacteriological Petrie dishes and were germinated for 4 days in a glass box. Seeds of *Brassica juncea L.* were germinated on a filter paper for 3 hours with constant wetting and at the room temperature. The substrate under the seeds was periodically wetted. Sprouted seeds were removed from filter paper and transplanted into soil. After 5 days, the growth was assessed by measuring plant root parameters.

In the work, we utilized acid-mediated hydrolysates (AH) and enzyme-mediated hydrolysates (EH) obtained by us from animal protein [14]. The preparations were diluted with water at a ratio of 1:100.

EH and AH hydrolysates are minor free amino acids with admixed impurities of non-hydrolyzed proteins residues. EH is produced with the help of enzymes, and compared with AH, it usually features lower amounts of amino acids and a significant proportion of peptides and oligopeptides of medium and high molecular weight. AH is produced with the help of mineral acids, which result in a greater proportion of pure amino acids than in EH.

The content of amino acids in AH was as follows, %: Ile – 0.4; Leu – 3.6; Lys – 1.6; Met - 0.5; Cys – 0.6; Phe – 0.8; Tyr – 1.1; Tre – 0.5; Trp – 1.3; Val – 1.4; Ala – 0.7; Arg – 2.8; Asp – 1.4; His – 1.3; Gly – 0.5; Glu – 7.3; Pro – 3.3; Ser – 0.7. Total = 30. In AH, up to 70% of short peptides with a molecular weight of 200-300 kDa were found.

In EH, the following free amino acids were found, %: Ile - 4.6; Leu 5.3; Lys 4.7; Met - 1.7; Cys 0.05; Phe 2.3; Tyr 5.1; Tre - 0.9; Trp - 0.2; Val 5.3; Ala - 7.4; Arg 1.3; Asp - 21.6; His 12.5; Gly 9.9; Glu 2.3; Pro - 3.7; Ser - 1.5, total amount of amino acids – 95%.

The following substances were admixed into the stimulating biological products, g/100 g of concentrate: EH (or AH) – 5; amber acid – 0.3; urea – 3; sodium nitrates – 3; ammonium nitrates – 6; monosubstituted potassium phosphate – 3; magnesium sulfate – 4; ammonium sulfate – 2; manganese nitrate – 0.006; zinc nitrate – 0.6; copper sulfate – 0.5.

The moisture content of the seeds was determined by gravimetric measurement. Amylase was determined by a standard test method.

3. Results and Discussion

Table 1 shows quantitative determination of the standard amylase activity in the germinated seeds in media with added biostimulants.

Table 1. Amylolytic activity, unit/mg·hour·g

Time, hour	Control		Biostimulant AH		Biostimulant EH	
	B. juncea L.	*Pinus banksiana*	*B. juncea L.*	*Pinus banksiana*	*B. juncea L.*	*Pinus banksiana*
0	40	30	90	75	205	130
24	70	45	110	80	210	150
48	60	50	105	80	215	155
72	75	60	125	87	225	168

The values in Table 1 prove that amylolytic activity in the different systems was expressed in different ways. Maximum amylolytic activity was reached, as a rule, on the third day, and in seeds with biostimulants, this activity exceeded the activity in the control by around 10-15%. The introduction of biostimulants promoted rapid assimilation of nutrients by cells and provided a positive effect on plants' development and growth. The AH-based biostimulant, with a noticeably high content of free amino acids, quickly activated amylolytic enzymes, which was registered by amylase activity. The use of the biostimulants correlated with the rate of seed germination. It was of scientific interest to assess the efficiency of amylase enzymes on development of the seeds under consideration.

Table 2. Proportion of germinated *Phaseolus vulgaris* (L.) Savi bean seeds in biostimulant medium, %

Time, days	Control	Stimulant EH	Stimulant AH
1	30	36	69
2	32	49	74
3	61	64	103

Table 2 shows the ratio of germinated seeds to the total number of seeds. From Table 2, it is obvious that a greater percentage of the seeds sprouted in the media with added stimulants, which contained high amounts of amino acids that were released during hydrolysate preparation. AH, based on acid

hydrolysate from animal raw materials, contained more than 90% free amino acids. Their ratio corresponds to those ratios that are specific in natural origin tissues and, therefore, AH is extremely favorable for development of plant cells.

EH had a less significant stimulating effect. EH contains many peptides that are not actively involved in cellular bio-exchange processes. The mass fraction of amino acids in EH amounted only to 30%, and therefore, the stimulating effect was less profound.

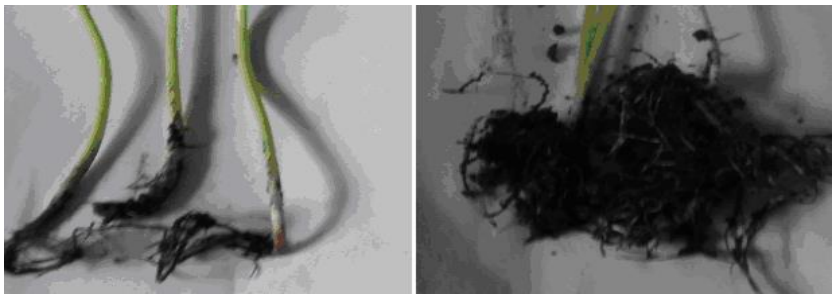

Figure 1. The development of beans in 4 days at 25 º C, humidity 85%, illumination rate 500 lux, in pure water (left) and in media with the biostimulant EH (right), after subsequent plant growth in soil for 5 days

Figure 1 shows the impact of the stimulant on bean shoot and root formation. The biostimulant EH induced powerful development of the plant root system, which is the key to further successful development of green biomass. The efficiency of AH biostimulant was also tested on hard-to-germinate forest plant seeds, Jack pine. These seeds are known for their poor rate of germination and survival rate. Usually, their germination rate does not exceed 45-60%. Pretreatment of seeds with AH and EH biostimulants resulted in a germination rate of more than 65%.

4. Conclusion

Complex biostimulants were developed for activation of plant cells. These biostimulants are based on enzyme-mediated and acid-mediated hydrolysates obtained from raw materials of animal origin. To increase the efficiency of the biostimulants, inorganic nutritional components were additionally included into the composition of the biostimulants. The impact of AH and EH biostimulants on the growth and development of beans *Phaseolus vulgaris* (L.) Savi, curled mustard *Brassica juncea* L. and Jack pine *Pinus banksiana* was studied. Positive effects of biostimulants on the

rate (percentage) of seed germination and the formation of green biomass were established, and these effects are apparently associated with the amino acids used.

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Sorbates and benzoates in meat and meat products: Importance, application and determination

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Sorbates and benzoates in meat and meat products: Importance, application and determination

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Abstract. Recent views on the use of preservatives sorbic and benzoic acids and their salts in meat products are presented from the point of accordance with current legislation in the Republic of Serbia and the EU, food safety and public health risks, and mainstreams in the methodology for their determination. These preservatives are permitted to be added individually or in combination, the maximum level is applicable to the sum and the levels are expressed as the free acid. Currently set values of the recommended daily intake of sorbate and benzoate are 25 mg/kg and 5 mg/kg, respectively. These values vary and depend on regulations in different countries. Considering control of the use of these additives, the most common methods for their determination are chromatographic methods based on high performance, or high pressure, liquid chromatography with diode array detectors.

1. Introduction

In order to prolong the shelf life of food in general, as well as meat products, several methods of their preservation have been developed in the food industry. The use of preservatives is a chemical method of preserving food [1]. Their application is versatile: to prevent reproduction of microorganisms, to reduce the chemical oxidation processes to a minimum and to reduce the darkening of products [2]. The harmfulness of the presence of microorganisms in meat products is multiple [3]. By reducing their number, a reduction is achieved of their metabolic products, which are directly related to faster food spoilage, and are also toxic to humans. Sorbic and benzoic acids, as well as their salts, have been widely used as antimicrobial preservatives in foods with a lower pH value.

2. Sorbic acid and sorbates

The food additive sorbic acid has a role of general food preservative and is known under the number E 200. Beside sorbic acid, its salts (potassium sorbate, E 202 and calcium sorbate, E 203) are also allowed to be used in meat products [4,8,9]. Sorbic acid is an unsaturated fatty acid, chemical name 2,4 hexadienoic acid, primarily extracted from plants [5,6]. Today, sorbic acid and its salts are produced synthetically and marketed in the form of powder or granules. They have a neutral taste and smell, which is a very important characteristic, because they do not affect the sensory properties of the products in which they are used. Due to their physical and chemical properties, sorbates can be used in various food categories such as aqueous solutions, fat emulsions, suspensions, gels and other products with a high water content [7]. Usually, sorbates in amounts of 0.1 - 0.2% are added to food.

The average recommended daily intake (RDI) for humans is up to 25 mg/kg, but these values vary and depend on regulations in different countries [10,11]. The mechanism of their antimicrobial effect is not fully explained. As a weak lipid-soluble acid, sorbic acid dissolves the membrane of microorganisms, penetrates and accumulates in the cell, affects the pH of the cell contents, which results in disruption of transport and metabolic functions, and ultimately causes cell death [12,13]. Sorbates are mostly used as inhibitors of mould and yeast growth, but they are effective in reducing the production of bacterial biofilm [14,15]. If the pH of the product is lower, the effects of sorbates are more efficient. The best effects are in the range of pH 3-6.5 [16]. For use in human nutrition, they show very low harmful effects, which have been shown by numerous studies conducted on laboratory animals [17,18].

3. Benzoic acid and benzoates

Benzoic acid was first described in the 16th century. It is an aromatic carboxylic acid found naturally in prunes, cinnamon and cloves [16]. It is known as a preservative under the number E 210, and its salts that are also widely used as preservatives are sodium benzoate E 211, potassium benzoate E 212 and calcium benzoate E 213 [4,8,9].

Benzoic acid has low toxicity, and its use within the permitted limits does not harm human health. However, the danger is its conversion to benzene, which is a carcinogenic and toxic compound, but studies have shown that there is little chance that this reaction will occur in food [16].

The use of benzoate in food has an antimicrobial effect. Benzoates primarily have an inhibitory effect on yeasts and moulds, but also, like sorbates, they have a bactericidal effect. As benzoates are weak acids, they are used in products with a pH value below 7. The pH range in which they show the best effects is pH 2.5-4 [19]. In the food industry, they are used as additives and are added to products in amounts of 0.05-0.1% [20]. They are widely used in carbonated drinks, where they provide long-term product safety despite the constant opening and closing of products. They are also used in jams, fruit salads, minced meat, ice cream, pickles and non-alcoholic beverages [21]. The RDI of benzoate is up to 5 mg/kg body weight [22]. Excessive use can lead to abdominal pain, enlargement of the liver and kidneys, as well as diarrhoea [21,23].

4. Domestic and EU regulations on preservatives in meat products

Legislation [4,8,9] relating to the use of sorbic acid and benzoic acid and their salts in meat products in various ways regulate their addition in particular categories of products.

4.1. Fresh meat, meat preparations and non-heat-treated processed meat

In accordance with the regulations, sorbic acid and sorbates and benzoic acid and benzoates are not allowed at all in fresh meat and meat preparations. Also, in the non-heat-treated processed meat category, the use of these compounds is allowed only for surface treatment of dried products in the *quantum satis* amount [4,9].

4.2. Heat-treated processed meat

The regulations for heat-treated processed meat are rather different, so the use of sorbic acid and sorbates is allowed up to 1000 mg per kg only in pâtés and in the same quantity only in aspic, and benzoic acid and benzoate only in aspic up to 500 mg per kg. These preservatives are permitted to be added individually or in combination, the maximum level is applicable to the sum and the levels are expressed as the free acid [4,9]. In local Serbian regulation [4], in the same category of processed meat, sorbic acid and sorbates and benzoic acid and benzoates are allowed in the *quantum satis* amount for surface treatment of heat-treated, dried products.

4.3. Casings, coatings and decorations for meat

In this category, the regulation allows the use of sorbic acid and sorbates in the *quantum satis* amount only in collagen-based casings with water activity greater than 0.6, and up to 1000 mg per kg only in jelly coatings for meat products (cooked, cured or dried) [4,9]. As in the previous category, sorbic acid

and sorbates are permitted to be added individually or in combination, and the maximum level is applicable to the sum and the levels are expressed as the free acid. Benzoic acid and benzoates are not allowed in these products.

5. Determination of sorbates and benzoates in meat products

Several analytical techniques have been used for determination of sorbic and benzoic acids and their salts in meat products. The two main classes of methods for determination of these additives are ones based on spectrophotometry and chromatography [24].

Spectrophotometric analytical methods have been seldom used to determine sorbates and benzoates in meat products in recent times [24]. The reason for this is the complex techniques of sample preparation for analysis and the possibility that other compounds present in the sample interfere with the determination.

Today, the most common chromatographic methods are based on the technique of high pressure liquid chromatography (HPLC) or ultra-high pressure liquid chromatography (UHPLC, UPLC). After relatively simple extraction procedure from meat product sample and purification of extract, sorbates and benzoates have been determined by HPLC with various detection techniques. The most common detection techniques for HPLC determination of sorbates/benzoates use photodiode array (PDA) or diode array (DAD) detectors. Also, methods involving mass spectrometer detection were used [25].

In most cases, the same method can be used to determine sorbates and benzoates along with some other additives [26]. The variety of analytes that can be determined by these methods along with sorbates and benzoates, especially when it comes to additives added to meat products, gives them special importance for controlling the use of additives in accordance with legislation, food safety and public health control.

6. Conclusion

Sorbates and benzoates are of great importance for preventing spoilage and prolonging the shelf life of food, including meat products. Despite the fact that there is no clear evidence of their potential harmful effects on the health of consumers, studies are periodically conducted to re-evaluate their use in food and meat products. In order to increase food safety and reduce consumer health risks, RDI values of these additives have been set.

In order to maintain a satisfactory level of food safety and to ensure the achievement of the conditions of health recommendations as required by RDI, legislation has been adopted regarding the use of these additives in food. The use of sorbates and benzoates is very strictly regulated in meat products and is allowed mainly for external use, on surfaces of non-heat-treated processed meat and casings, coatings and decorations for meat. In fresh meat and meat preparations their use is not permitted. The exception is their use in heat-treated processed meat, which is limited to the addition of sorbic acid and sorbates and benzoic acid and benzoates only in aspic, as well as sorbic acid and sorbates only in pâtés. The local Serbian regulation allows the external use of sorbates and benzoates in heat-treated processed meat for use on the surface of smoked products.

Today, HPLC chromatographic methods with diode-array detectors are mostly used to determine the content of sorbates and benzoates because such systems are low-cost and available, analyte extraction is simple, and sample preparation does not require complicated purifications and time-consuming sample manipulation. These methods can usually be used at the same time to determine other additives, such as added colours, which increases the methods' usability for controlling the use of additives in meat products.

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Relationships between broiler final weights and histomorphometric parameters of certain segments of the intestine

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Relationships between broiler final weights and histomorphometric parameters of certain segments of the intestine

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Abstract: The aim of this study was to determine relationships between final mass of broilers and the histomorphological properties of individual segments of the gastrointestinal tract. This is confirmed by the obtained results, which indicate a strong and significant correlation between compared parameters. A strong significant ($p<0.05$) correlation ($r=0.866$) was found between the broiler final weight and the duodenal villus length, and between the broiler final weight and the caecal villus length ($r=0.918$). Correlation between the broiler final weight and the duodenal villus width ($r=0.841$), as well the caecal villi width ($r=0.918$) was strongly significant ($p<0.05$). Between the crypt depths in caecum correlation was medium and significant ($r=0.701$, $p<0.05$). It was determined that between the broiler final weights and the ratio of villus length and crypt depth there is a significant medium correlation ($r = 0.736$, $p<0.05$). A strong ($r = 0.924$) significant ($p <0.05$) correlation was found between the broiler final weight and the ratio of villus length and caecal crypt depth. Between final mass of broilers and other histomorphological properties of individual segments of intestine there was no significant correlation.

1. Introduction

In order to improve genetic potential of highly selected broiler hybrids (Cobb, Ross), appropriate conditions of health care, accommodation, and especially nutrition are required. The gastrointestinal tract (GIT) of poultry has not only the function of digestion and absorption of nutrients, but it is also a metabolic and immunological organ, which serves to limit the presence of harmful agents, primarily bacteria and protozoa (coccidia and intestinal parasites), in the GIT. Maintaining a balanced ratio of harmful and useful microorganisms (eubiosis) in the poultry GIT has special importance for its health, and thus the health of the animals, and hence the production results and economy of meat production. Antibiotics (growth promoters) were used for the protection and health of animals' GIT. Their use has caused two negative phenomena, one of which is the finding of antibiotic residues in meat (eggs), and the other, certainly was a much more serious phenomenon, bacterial resistance, which is why their use is now forbidden. In order to preserve animal health in modern intensive poultry production, alternatives to antibiotics are required. As an alternative to antibiotics, medium-chain fatty acids (MCFAs) are used,

singly or as two or more MCFAs in poultry diets. One of such commercial products, Aromabiotic®, is recommended in poultry nutrition. The use of MCFA in broiler nutrition contributes to presesrving the health of the GIT, and thus to better production results (higher final body weight, higher growth, and better feed conversion). The aim of this study was to examine the correlation between the final mass of broilers and the histomorphological properties of individual segments of the gastrointestinal tract.

2. Materials and methods

The conditions of keeping and feeding broilers are described in previous works [1, 2]. Birds were randomly assigned to one of three dietary treatments (control and two experimental groups), each having six replicates (ten birds in each replicate). Immediately after the animal slaughter, parts of the small intestine (duodenum and ileum) and caecum were collected for histological analyses from each group. The tissue samples were fixed in 10% buffered formalin saline, then dehydrated by immersing through a series of alcohols and embedded in paraffin by standard technique. Sections 5 to 8 µm in thickness were stained with Mayer's haematoxylin and eosin (HE) method [3]. Morphometric examinations of the villi length and width, as well as the crypt depth, were carried out using an ocular micrometer 1:100 [4]. Pearson's correlation was used to determine the correlation between the final mass of broilers and the histomorphometric properties of individual segments of the broiler gastrointestinal tract. Statistical analysis of the data and presentation of the results obtained was conducted using the Microsoft Excel 2013 and GraphPad Prism software, version 8.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

3. Results and Discussion

Final body weight, average daily gain and feed conversion are most often used as indicators of production results in poultry nutrition [5,6]. These parameters can be correlated with the histomophrometric properties of the GIT. The correlation between the broiler final weight (g) and the histomorphometric properties of individual segments of the broilers' digestive tract are present in Figures 1 to 5.

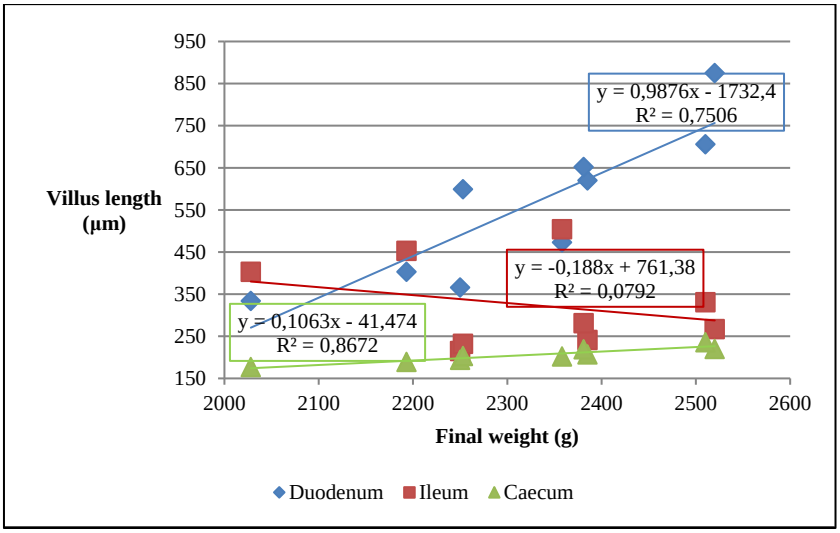

Figure 1. Correlation between the broiler final weight (g) and villus length (µm) in broilers

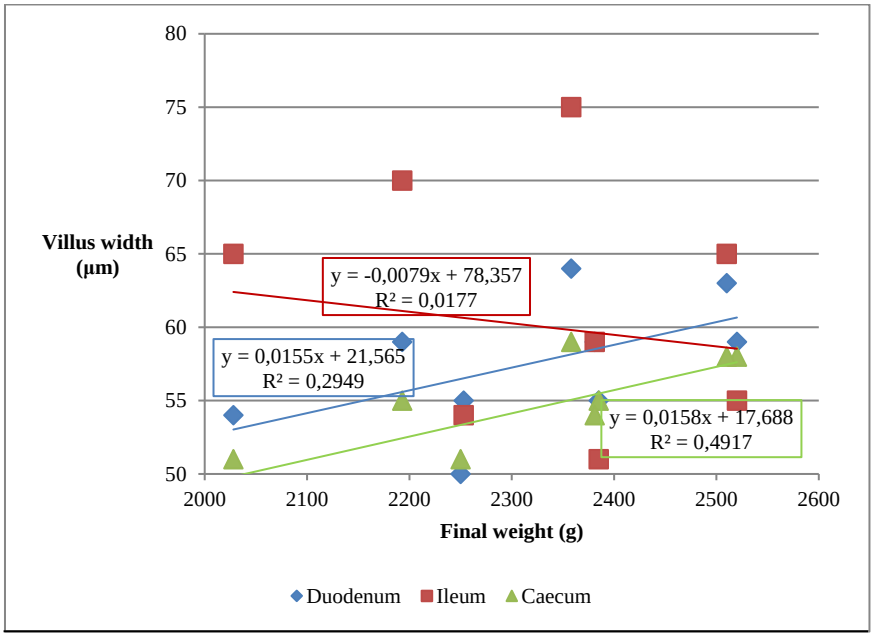

Figure 2. Correlation between the broiler final weight (g) and villus width (µm) in broilers

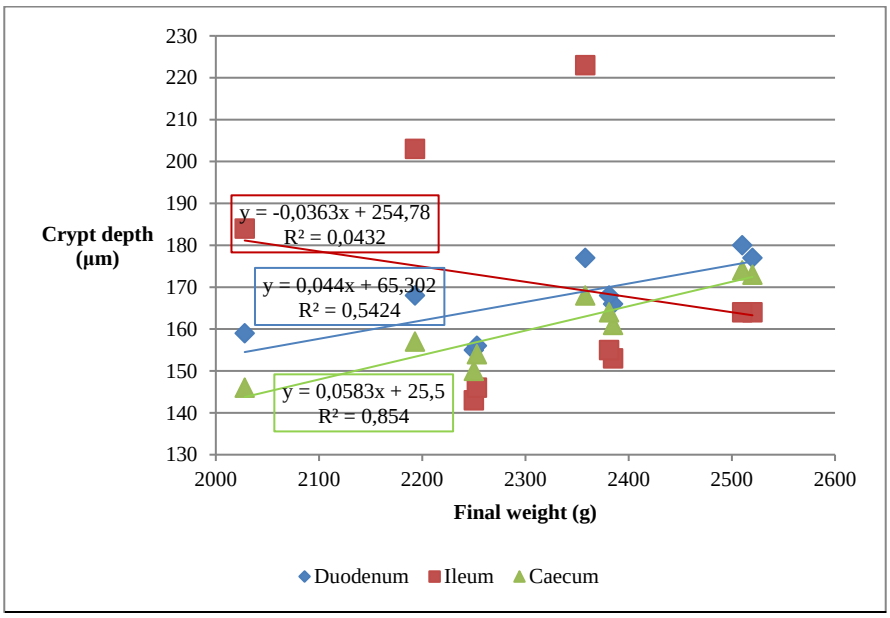

Figure 3. Correlation between the broiler final weight (g) and the crypt depth of the intestinal villi (µm) in broilers

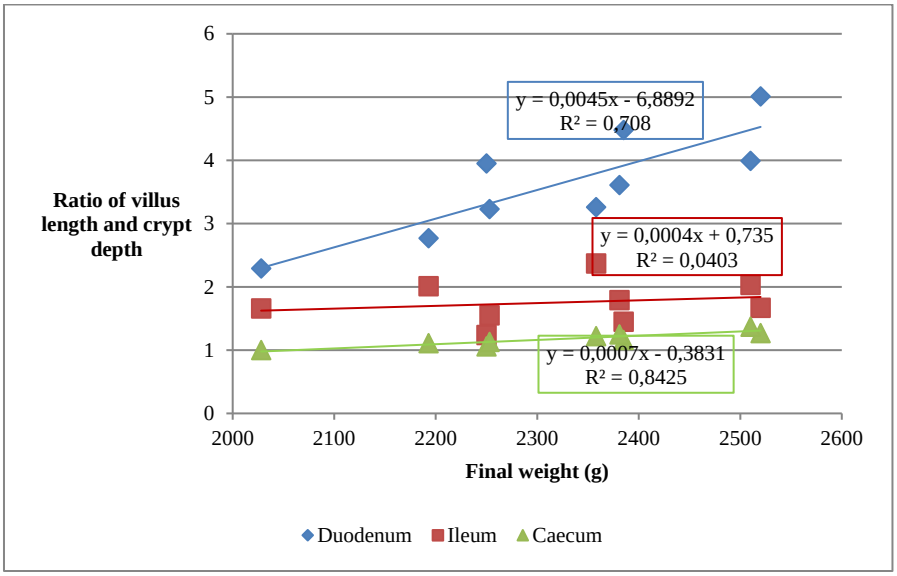

Figure 4. Correlation between the broiler final weight (g) and the ratio of villus length to crypt depth of intestinal villus in broilers

The correlations and significance of differences between the broiler final weight and histomorphometric parameters of individual segments of the intestine are present in Table 1. A strong significant ($p<0.05$) correlation dependence ($r=0.866$) was found between the broiler final weight and the duodenal villus length. Also, a significant ($p<0.05$) strong correlation ($r=0.918$) was found between the broiler final weight and the caecal villus length. There was a weak negative correlation dependence ($r =-0.281$), which was not significant ($p>0.05$), between the broiler final weight and the ileal villus length. A strong ($r=0.841$) statistically significant ($p<0.05$) correlation dependence was found between the broiler final weight and the duodenal villus width, as well as a strong significant ($p<0.05$) correlation ($r=0.918$) between the final body weight of the broiler and the caecal villi width. There was no correlation dependence between the ileal villus width and the broiler final weight ($r=0.201$). A medium correlation dependence was found between the crypt depths in duodenum, i.e. the crypt depth in caecum ($r=0.543$, duodenum, or $r=0.701$, caecum). The correlation dependence ($r=0.543$) between the broiler final weight and the duodenal crypt depth was not significant ($p>0.05$), while the correlation ($r=0.701$) between the broiler final weights and the caecal crypt depths was significant ($p<0.05$). Broiler final weight and the ratio of villus length to crypt depth, exhibited a significant ($p <0.05$) medium correlation ($r=0.736$). A strong ($r=0.924$) significant ($p<0.05$) correlation was found between the broiler final weight and the ratio of villus length to caecal crypt depth. There was no correlation dependence ($r=-0.208$) between the broiler final weight and the ratio of villus length to ileum crypt depth.

Table 1. Correlation dependence and significance of the difference between the broiler final weight and histomorphometric parameters of individual intestinal segments

Histomorphometric parameter	Intestine segment	Correlation coefficient (r)	Interpretation of correlation dependence*	Significance of difference
Intestinal villus length	Duodenum	0.866	Strong	p<0.05
	Ileum	- 0.281	Weak	ns

	Caecum	0.930	Strong	p<0.05
	Duodenum	0.841	Strong	p<0.05
Intestinal villus width	Ileum	0.201	No correlation	ns
	Caecum	0.918	Strong	p<0.05
	Duodenum	0.543	Medium	ns
Intestinal crypt depth	Ileum	- 0.133	No correlation	ns
	Caecum	0.701	Medium	p<0.05
	Duodenum	0.736	Medium	p<0.05
The ratio of villus length /crypt depth	Ileum	- 0.208	No correlation	ns
	Caecum	0.924	Strong	p<0.05

Legend: ns – not significant; * Source: Colton, 1974 [7]

The efficacy of MCFA use is based on their influence on the morphological properties and on the microbiota of the GIT. At beginning of fattening, there is a rapid physical and functional development of the GIT and there are morphological changes in the duodenum, jejunum, and ileum. Thus, in the first few days, changes in the villus length are especially pronounced, and in two to three days, crypts are formed. With the change of morphological properties, the ability to absorb nutrients continuously increases. Also, the activity of pancreatic enzymes, which was observed even before hatching, increases in the first days of broiler life [8]. Intestinal villi and crypts are units of epithelium that allow the absorption of nutrients and are renewed every four to five days [9]. Greater cell regeneration and a lower degree of enterocyte apoptosis, or both, contribute to longer villus length and even greater surface area of the mucosa, which result in greater nutrient resorption. The degree of absorption is particularly important for protein absorption since 20 to 40% of the protein is synthesized in the GIT [10,11]. Morphometric examinations of the GIT (duodenum and jejunum) are also important because fats and other nutrients are absorbed in these parts of the GIT. The villi are covered with enterocytes that form in the crypts of Lieberkühn, from where they migrate to the top of the villi. The migration of enterocytes to the top of the villi and their loss due to apoptosis are in balance in a healthy animal, and the loss of enterocytes occurs as a consequence of numerous pathogenic bacteria, which ultimately causes an increase in the crypt depth. More favorable morphological properties, i.e., an increase in the villus length and width of the intestinal segments, especially the duodenum and caecum, lead to an increase in the resorptive surface of these intestinal segments. The reduced depth of the crypts and the altered ratio of the villus length and the crypt depth indicate a reduced replacement of enterocytes, i.e. a reduced need for the formation of new cells. Growth of new enterocyte cells and maintenance of the normal structure of the GIT requires significantly more energy and an increased need for proteins. In the phase of intensive growth in energy demand, they make up 25% of the needs, i.e. 12% of the protein needs out of the total needs [12, 13, 14]. With increasing broiler age, the crypt depth and the number of enterocytes per cross section of the villi increase. However, the number of villi per unit area, especially in the duodenum, decreases [15]. The density of enterocytes in different segments of the intestine does not change with broiler age. Maximum digestibility and resorption of nutrients and, thus, the impact on production results, are conditioned by the size of the intestinal surface and with the optimal functional maturity of enterocytes [16]. Adding MCFA to the broiler's feed leads to a significant increase in the villus length and width and, thus, to an increase in the absorption surface area of the duodenal, ileum, and caecum epithelia in forty-day-old broilers [17, 18, 19, 20, 21]. Also, the addition of MCFA increases the villus length and the crypt depth, but only in the ileum. Lesson et al. [22] and Panda et al. [23] report that MCFA in broiler nutrition increases the villus length and the crypt depth in the duodenum, from

which it can be concluded that this can greatly help young individuals in the development of the digestive tract. This was confirmed in another study as well [24]. It has also been confirmed that the use of MCFA in broiler feed leads to increased villus length and increased enzyme production, better digestion, increased resorption area, and, thus, better absorption of food nutrients [25]. MCFAs reduce intestinal colonization of pathogens and the occurrence of infections, reduce the frequency of inflammatory processes in the mucosa, increase villus length and secretion, and increase digestion and resorption of nutrients [26].

4. Conclusion

Broiler feed with added MCFA contributes not only to the health preservation of the GIT, but also significantly affects strong correlations between broiler final weight and duodenal and caecal villus length, duodenal and caecal villus width, and caecal ratio of villus length/crypt depth. Significant medium correlation was determined between broiler final weight and caecal crypt depth, and also duodenal ratio of villus length/crypt depth. The results obtained showing the correlation between broiler final weight and histomorphometric parameters of individual segments of the intestine indicate the justifiable use of MCFA in broiler nutrition.

Acknowledgments

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Impact of COVID-19 pandemic on food supply chain: An overview

Impact of COVID-19 pandemic on food supply chain: An overview

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Abstract. Since 2020, the rapid spread of the SARS-CoV-2 virus has caused the global pandemic COVID-19, generating health, economic and social impacts. The rapid spread of the infection in the human population required an accelerated adaptation to the new circumstances to protect human health and mitigate financial losses. As the ongoing pandemic has caused reported cases in the multi-millions, all stakeholders need to prevent further outbreaks and mitigate associated risks. Hence, besides government, health care systems, business stakeholders, public authorities, non-governmental organizations, and other socially responsible associations, the food sector has a crucial role in combating COVID-19. The food sector in this context is referred to as every actor in the food supply chain. This paper explores the difficulties in the entire food supply chain's reactions to the pandemic crisis and underlines the meat sector's response.

1. Introduction

Last year, 2020, was challenging due to the pandemic of COVID-19, a highly contagious disease caused by a new virus, officially named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The first cases of infection arose in December 2019 and were linked to the Huanan Seafood Market (Wuhan, China) [1]. The virus was isolated on January 7, 2020, and complete genome sequencing was performed. On January 12, 2020, the World Health Organisation (WHO) named the coronavirus the 2019-novel coronavirus (COVID-19). After studying the genome sequence results and evolutionary analysis, bats were suggested as the natural host of SARS-CoV-2 [2]. On March 11, 2020, WHO declared COVID-19 a pandemic due to its rapid spread and severity. According to WHO data, so far, SARS-CoV-2 has affected more than 170 million people worldwide, with a mortality rate of about 2%. More than 700,000 COVID-19 cases were registered in Serbia from the beginning of the pandemic, with a mortality rate of approximately 0.97% [3].

Although there is a lack of information and evidence of how COVID-19 is transmitted, it is known that this virus can infect humans. The most common signs of illness shown by patients affected with COVID-19 were fever, cough, and sometimes gastrointestinal disorders [4]. The prevention of virus spread and protection of human health quickly became priorities for multi-stakeholders, including the food sector as one of the responsible and key actors. In that context, it is essential for the whole food

supply chain to identify, control, and decrease food safety risks during the current pandemic crisis. Preventive measures at governmental level included travel restrictions, border controls, and country lockdowns.

In contrast, within the food sector, preventive actions cover measures from the implementation of good hygiene practices (GHP) to improving already established food safety management systems (FSMS). However, preventive actions differ among countries and food sectors within them, at the same time causing severe global consequences. Thus, the need for food security protocol alignment on a worldwide scale arose [5]. To date, the possibility that SARS-CoV-2 can survive in vegetables, meats or other foods is based only on assumptions with no direct and unmistakable evidence to show that SARS-CoV-2 can be transmitted from food or food packages to humans [6]. Available data about COVID-19 outbreaks showed that slaughterhouses and meat processing plants are suitable environments for spreading SARS-CoV-2. Additionally, in previous literature, several outbreaks in slaughterhouses and meat processing plants worldwide have been analysed [7]. This study aims to explain how COVID-19 disease affected the food supply chain, highlighting the meat sector's concerns.

2. Impact of the COVID-19 pandemic on the food supply chain

2.1. Affected actors of the food supply chain

The COVID-19 pandemic introduced unexpected stresses on food systems, inducing the rapid response of food supply chains [8]. The range and type of responses varied depending on the stage within the food supply chain, but food safety is a priority in each phase. The common bottlenecks for almost every actor within the food supply chain were shortage of workers, maintaining social distancing, transport restriction, etc. Food processing industries are especially vulnerable, as they have an intensive number of production staff in the facilities' limited closed space. On the other hand, the absence of workers due to prevention measures or disease isolation can cause short or long-term shutdowns in production, especially in less automated systems such as meat industries and slaughterhouses. The challenge that emerged from the current crisis is how to balance the need to keep production going and the need to protect the workers.

According to Djekic et al. [9], retailers were identified as the food supply chain link affected mainly by the pandemic, in contrast with food storage facilities, ranked as least affected. From the perspective of consumer food-related demands, the pandemic has led to a drastic shift. In that sense, hotels, restaurants, catering, and cafés were most affected due to COVID-19 lockdowns on a global level [8]. As the last actor in the food supply chain, consumers have a significant role in influencing behaviour and food choices. In other words, besides the legislative measures established, consumer demand for safe food induces the whole food supply chain to implement all necessary steps to prevent zoonoses and reduce other risks for humans [5]. Besides this consumer role in the food supply chain at the point of purchase, consumer actions can directly affect food safety at the moment of consumption. These actions are mainly adequate personal hygiene and ensuring the hygiene of food preparation surfaces. Thus, to avoid cross-contamination in various directions, such as cooked-uncooked food, human factor-food, or contaminated surfaces-food, every actor in the food supply chain exposed to this type of risk should follow WHO suggested measures referring to food handling and preparation practices [10].

Additionally, with the pandemic crisis, hygiene procedures need to be more stringent in the retail and food industries. More stringent hygiene practice was followed by other requirements, such as the need for more additional personal protective equipment. Requirements associated with the implementation of pandemic prevention procedures varied from hygiene improvement, staff awareness, recommended measures from WHO/government, temperature checks of workers, the physical distance between workers, limited visits to the facilities, etc. It was revealed that increasing staff awareness and improving hygiene are the two most critical prevention actions. On the other hand, temperature checks of workers were declared as less important [9].

The integrative overview of the current COVID-19 pandemic considering food supply chain issues is concerned with food security, food safety and food availability. Some consider that authorities should

encourage food enterprises to gather information about their suppliers of raw materials and fresh products (e.g., vegetables) and connect them with sellers to improve food availability. Furthermore, specialized mobile device shopping applications can assist farmers in finding alternative buyers in small city centres. Many countries react to pandemic crises by ensuring food reserves. While China assured the sufficient nourishment of the local population by releasing at least 300,000 tons of pork reserves, Italy implemented relevant laws to force food makers to keep resources for emergency purposes [10]. When it comes to emergency preparedness within food companies, it was revealed that less than a half of food companies had documented any emergency plan associated with pandemics [9].

2.2. Changes within meat supply chain during COVID-19

Although meat is not necessary for the diet, the inclusion of meat and meat products makes it easier to ensure a good and healthy diet. In Serbia, meat in general, especially barbecued meat and different types of roast meat, has an important role in daily meals [11]. From the nutritional quality aspect, meat is a good source of high-quality proteins as it contains essential amino acids that must be ingested. Furthermore, meat and meat products are characterized as important sources of all the B-complex vitamins and excellent sources of some minerals, such as iron. On the other hand, fresh meat is a perishable food [12]. In that context, providing safety assurance of this food is a concern, especially in emergencies. Safety assurance within the meat sector is not a simple task for every meat supply chain, especially in terms of greater potential risks. As a risk-based meat safety assurance system needs to be periodically reviewed and its development constantly in focus [13], system revisions, including of COVID-19 associated risks, should be done. Moreover, it was recognized that livestock farmers, slaughterhouse workers, meat processors, traders, and policymakers need mutual support to ensure a stable meat supply chain [14].

The COVID-19 pandemic has direct and indirect impacts on meat production. Direct impacts were based on decreasing numbers of production workers due to infection and adverse market conditions for farmers. Furthermore, for meat producers, the market was not desirable due to the reduced income status of consumers. Although consumers started to stock items with longer shelf life during COVID-19, the demand for particular meat products such as hamburgers and minced meat also increased [14]. Additionally, in recent research conducted in Serbia, authors confirmed that risks and associated preventive actions linked with the COVID-19 pandemic significantly influenced consumers' eating habits and food shopping behaviour [15].

3. Response of meat sector in Serbia to the pandemic crisis

According to available public statistics data from the Statistical Office of the Republic of Serbia [16], some trends within the meat sector emerged. Observations are based on the statistical indicator of meat production (Figure 1).

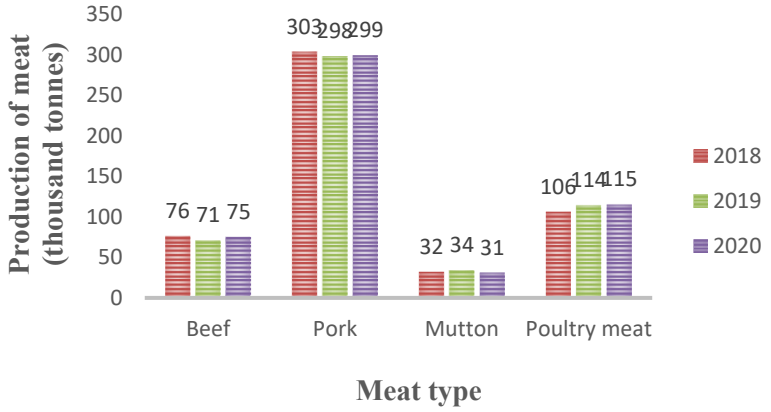

Figure 1. Meat production in Serbia during 2018 to 2020

Data on production of the most common types of meat produced in Serbia were obtained for potential determination of differences. The analysed period covered one year before the critical year of the pandemic.

4. Conclusion

According to relevant representative studies of how COVID-19 affected the food sector, it was revealed that all actors of the food supply chain were affected in particular ways. The most common triggers originating from pandemics, such as lockdowns, social distancing, and worker illness, caused changes in every sector in the food supply chain. Implemented preventive measures varied from increasing staff awareness and improving hygiene to making food reserves and revising safety assurance systems. For the meat sector, adverse conditions on the global market were detected. In contrast, public statistics data from Serbia for the critical pandemic period showed increased beef, pork, and poultry production. However, studies are leading meat scientists, producers, and other actors in the meat supply chain to develop preventive actions and safety assurance systems to overcome food insecurity under pandemic situations.

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Toxoplasma gondii in pork and pigs in Serbia – a real food safety hazard

Toxoplasma gondii in pork and pigs in Serbia – a real food safety hazard

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Abstract. Infection with the apicomplexan protozoon *Toxoplasma gondii* is one of the most prevalent parasitic zoonotic infections globally, with existing seroprevalences varying between continents, countries, and even within countries and between individual communities. It is estimated that one third of the world's human population is infected with *T. gondii*, with many studies showing that the dominant mode of infection is consumption of undercooked meat harbouring *T. gondii* tissue cysts. Prevalences of infection in food animals in different countries range from 0 to 93%. Because of the absence of clinical symptoms in infected animals, and the unfeasibility of rapid and unequivocal detection of microscopic tissue cysts in pork, infected pigs remain unrecognized, and their meat becomes an essential source of infection for humans. The data on *T. gondii* infection in pigs in Serbia from several studies, as well as on the detection of the parasite in different food categories, from fresh pork to heat-treated products, are discussed.

Introduction

Among the pathogens transmitted through food, parasites occupy a significant place. Many parasitic infections pose a major public health challenge, among which the European Food Safety Authority has identified as critical risks *Cryptosporidium* spp., *Echinococcus* spp. and *Toxoplasma gondii* [1]. It is estimated that one third of the world's human population is infected with the protozoan *T. gondii*, making it the most widespread parasitic zoonosis globally [2]. Although the domestic cat and other members of the cat family are the only permanent hosts of *T. gondii*, many studies have shown that the dominant mode of infection for humans is consumption of undercooked meat (from pigs, lambs, cattle, and horses), and that differences in prevalence correlate with food preparation culture [3]. The World Health Organization (WHO) estimates that over 1 million cases of toxoplasmosis in the European region are caused by contaminated food every year [4]. The infection is peroral in over 90% of cases, i.e., caused by ingestion of contaminated food or water. Research indicates that 40-60% of these are infections are caused by consumption of contaminated meat, especially pork. Due to the absence of clinical symptoms in infected animals and the lack of

appropriate, specifically commercially feasible methods for rapid and unequivocal detection of microscopic cysts of *T. gondii* in meat [1], infected pigs remain unrecognized, and their meat becomes an essential source of infection for humans [5]. That is precisely why *T. gondii* remains one of the most studied parasites due to its importance for veterinary and human medicine.

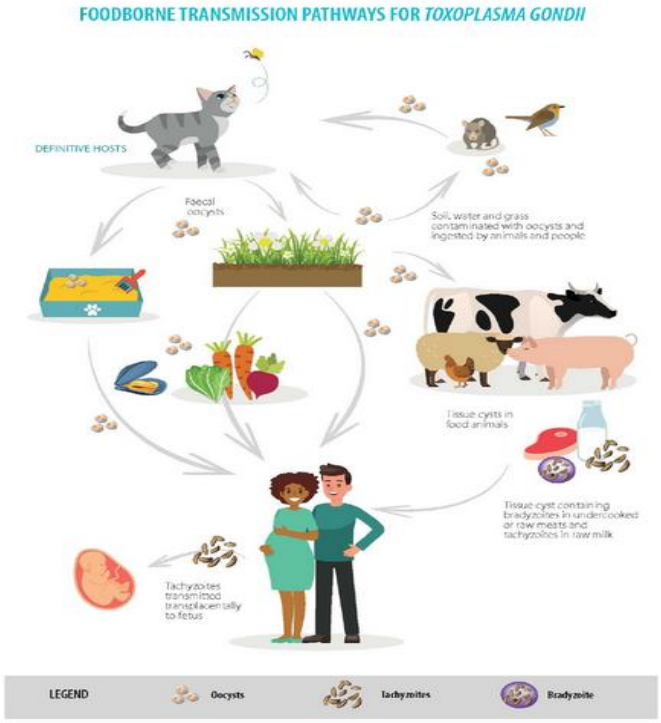

Figure 1. Foodborne transmission pathways for *T. gondii* infection [1]

Presence of *T. gondii* in pigs. What are the data for Serbia?

Traditionally, pork is the most preferred type of meat among domestic consumers in Serbia, corresponding to the volume of pig production in the primary sector. According to data from the Statistical Office of the Republic of Serbia, pigs bred in Serbia number about 3 million units, while about 2.2 million pigs are slaughtered annually in slaughterhouses [6]. Given the importance of *T. gondii* in the pig meat production chain, it is necessary to know the presence of this parasite in pigs at the national level. By considering risk factors and biosecurity systems on farms, whether and to what extent certain parameters affect parasite transmission among animals can be determined [7]. The prevalence of *T. gondii* can vary depending on the type of management practices used in the farms, the number of animals tested, and the age and type of the pigs tested (fattening vs. sows; indoor pigs vs. organic pigs) [8]; globally, including data from 47 countries from all continents, *T. gondii* prevalence in pigs averages about 19%, while the European average is 13% [9]. The first

seroepidemiological study in Serbia was conducted in 2002/2003 [10], when a seroprevalence of 28.9% (40.9% in weaned sows, 15.2% in fatteners) was determined, while a subsequent study in Belgrade showed a similar distribution of infection among age categories (30% in weaned sows, 8.3% in fatteners) with a total prevalence of 9.2% [11]. In the latest research in Vojvodina, an overall prevalence of 17% was determined in a total of eight farms, and viable *T. gondii* parasites were isolated from pig tissues [12].

Control of *T. gondii* in pork and pork products

Although predilection organs have been shown to be more parasite-laden than muscle tissue [13], an increasing number of studies have shown the presence of parasites in retail fresh pork [14,15]. In addition to fresh pork, *T. gondii* has been demonstrated in vacuum packaged meat; this packaging is used commercially for pork to enhance palatability and prolong shelf life. In control (non-vacuum packaged) pork stored at 4 °C, *T. gondii* survived storage for 28 days. In vacuum-packaged pork, *T. gondii* survived for 14 days but not 21 or 28 days [16]. Minced meat has also been investigated for the presence of *T. gondii*, as various commercial cuts are often used in mince production. In a study from Poland, *T. gondii* DNA was detected in 4.5% of 756 samples of retail minced pork and 5.8% of 1355 samples of raw sausage [17]. It has even been confirmed that the parasite survived in cured hams in Spain after 12 months of maturation [18]. On the other hand, three studies in America showed that *T. gondii* cannot survive salting procedures for ready-to-eat pork products [19, 20, 21]. However, all heat-treated products, cooked sausages, or products preserved by sterilization [22, 23] are free of *T. gondii* parasites; for example, Hill et al. [24] showed that *T. gondii* is killed in 5.6 minutes at 49 °C, in 44 seconds at 55 °C and in 6 seconds at 61 °C if the temperature is evenly distributed and maintained throughout the thickness of the meat. So far, there is no data on the detection of *T. gondii* in retail pork or pork products in Serbia.

Conclusion

All new knowledge on the transmission and risk factors for *T. gondii* infection is vital for improving educational programmes for groups of people considered to be at high risk concerning infection with this parasite, primarily pregnant women (because of the risk to the foetus) and immunocompromised persons. For food to be safe for consumption, it is necessary to carry out actions and activities to prevent or eliminate food safety hazards following the *Codex Alimentarius* [25]. The purpose of control measures is to produce food that is safe and suitable for human consumption. To prevent foodborne toxoplasmosis, one should follow the WHO five keys [4] to safer food.

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The controversies of genetically modified food

The controversies of genetically modified food

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Abstract. The increasing use of genetically modified (GM) foods and feeds attracts the interest of media and public, causing great concern among consumers about the consequences of their consumption. The issues of concern are mainly focused on the impact on consumer health and the repercussions on the environment. The biggest fears are the possible negative consequences on human and animal health, which encompass allergic reactions, side effects such as toxicity, damage to individual organs, gene transfer and differences in nutritional value. Consumers are unsure and confused as to whether consuming GM foods is harmful to their health or not. According to a Pew Research Center survey conducted between October 2019 and March 2020, 48% of respondents said GM foods are harmful, 13% responded GM foods are safe, while 37% of respondents could not express their opinion due to lack of knowledge about it. Numerous studies have been undertaken to examine the effects that GM foods and feeds exert on humans and animals. The results differ in many ways that issue numerous questions. In this paper, we will try addressing questions that concern the public, as well as the activities and measures that science and competent institutions are taking to confront them.

1. Introduction

1.1 What are genetically modified organisms (GMOs)?

GMOs are plants, animals or microorganisms with genetic material altered by gene insertion from another organism, carrying information about a desired trait not naturally present in the organism [1]. This gene manipulation results in a new organism that does not occur spontaneously by natural methods of crossbreeding. The gene with the desirable trait is introduced to the host organism by a biolistic or *Agrobacterium tumefaciens* mediated approach [2]. Gene transfer either can occur between two organisms of the same species or closely related species (the genes are called cysgenes), or between organisms not closely related (transgenes) [3]. An example of the latter is the insertion of a gene from marine fish into spinach, potatoes or tomatoes, which makes them resistant to frost [4].

The history of cultivating plants with higher yields, faster growth, disease resistance or containing a specific nutritional characteristic goes back to the early beginning of humankind. Ever since, humans have been producing crops by selecting those with desirable phenotypes. Because of that selection, we began to produce new varieties of plants with desirable traits much faster than they would naturally occur. Now though, our GM crops can carry genetic material, for example, for a particular trait that allows them to survive in adverse and/or extreme climatic conditions, or improves their quality. These include herbicide and pesticide tolerant crops, crops resistant to pests and viruses, drought or frost, or crops with improved yields and quality. In addition, the benefit of foods that contain these genetic modifications is the continued use and preservation of their nutritional value.

More than a hundred research studies comparing the effects of consuming traditional foods and GM foods have tried to give answers to the many questions raised around the use of GM foods. The outcomes of the studies have been published in numerous papers in professional and scientific journals. The accordance or not of GM foods with the regulations is visible in the database of GM cultures in different countries on the website of International Service for the Acquisition of Agri-biotech Applications (ISAAA) [5]. Moreover, the U.S. Department of Agriculture provides a list of bioengineered foods available to consumers throughout the world, where they can get information about the history of GM cultures, type of modification and regulation in the world [6].

1.2 Why did humans start producing GMOs?

According to the United Nations forecast, the world's population will reach 9 billion by 2050 [7]. This will impose an increased demand for livestock products, followed by an urgent need for increased crop production. The ISAAA brief form 2018 states that per capita consumption of meat, milk and will rise by 2% per year in most parts of the developed world [8]. Therefore, the demand for feed crops will significantly increase, taking into consideration the fact that livestock unit's average needs per kg of meat production are approximately 3 kg of feed grain and less than 1 kg of feed grain per kg of milk production [9].

Every year, we are witness to a drastically increased climate change that results in floods, droughts and spread of plant diseases, which poses a growing threat to the cultivation of many of these crops. So, in the decades to come it will be increasingly difficult to feed the world's population, which is a big challenge for genetically modified crops – will they be able to do that?

In order to produce crops that will perform better than traditional crops and give higher yields with higher nutritional value, and are resistant to pesticides, especially insecticides, to limit the action of toxic herbicides, genetic modifications have been used. GM crops resistant to the organophosphorous herbicide glyphosate enable increased yields despite the much greater use of glyphosate during plant growth [10]. Unlike this type of herbicide-resistant GM crop, with the production of GM insect-resistant crops, the use of insecticides in crop cultivation is significantly reduced, indirectly affording better protection of the environment [10]. Furthermore, GM crops are resistant to various diseases, are more tolerant to stress factors of diverse nature, have a long storage time, and can be used in medicine and industry.

1.3 The first genetically modified food produced

The first genetically modified food was the red tomato, so-called Flavr Savr™ tomato, which contained a gene that delays ripening [11]. This prevented the softening of the tomato, making it resistant to spoilage, while maintaining its natural colour and taste. This product was approved for use in 1994 by the US Food and Drug Administration [11]. The most important commercially produced bioengineered crops are soybean, corn, cotton and oilseed rape, which are resistant to herbicides and pesticides. Such is the case with GM crops that are resistant to glyphosate, which is then widely used during crop growth to destroy weeds and grasses. A gene (CP4 epsp) is inserted into these crop plants from the bacterium *Agrobacterium tumefaciens*, and which reduces the affinity for glyphosate binding, thus increasing the plant's tolerance to the herbicide. Currently, soybean, corn, alfalfa, oilseed rape, sugar beet and cotton carrying a genetic modification that gives them resistance to glyphosate are present on the market.

Furthermore, a gene from the bacterium *Bacillus thuringiensis (Bt)* and responsible for protein synthesis i.e. crystal toxin, referred to as Cry toxin, has been inserted into corn, cotton, potatoes and tobacco. This protein is toxic for larvae of some insects in the Lepidoptera, Coleoptera, Hymenoptera, Diptera and Nematoda classes that attack these cultures [12]. After ingestion of the GM food, in the digestive system of the insects, a protein coded by this gene is synthesized and binds to the intestinal wall. Within a few hours, the insect's intestinal wall disintegrates, allowing the normal gut microbiota to enter the abdominal cavity, causing septicaemia followed by death. The commercial production and distribution of Bt crops were approved by the Environmental Protection Agency in USA in 1995 [12].

Another example of GM culture is the production of "Golden Rice", with a genetic modification carrying genetic information for the synthesis of 20-fold more β-carotene than other varieties. Golden Rice is produced by inserting two genes into the rice genome: one from daffodil, responsible for synthesis of the enzyme phytoene synthase, and the second from the bacterium *Erwinia uredovora*, which produces the enzyme phytoene desaturase [13]. These two enzymes participate in the biosynthesis of β-carotene, which accumulates in the edible rice grains, and which in the human liver is converted into vitamin A.

Another variety of GM rice, used to fight the iron deficit in humans comprising almost 30% of the world's population, has also been produced. The genome of this GM variety of rice contains ferritin gene from *Phaseolus vulgaris*, responsible for the synthesis of protein capable of binding iron, thus increasing the iron content up to twofold. To increase iron bioavailability, a gene is inserted into the rice from *Aspergillus fumigatus*; the gene is responsible for the synthesis of the enzyme phytase that breaks down phytate, an iron inhibitor [14].

1.4. Feeding animals with GM crops – are there implications for animal's health and animal derived products?

As previously pointed out, in the past decade, food demand has rapidly grown, primarily because of climate change and environmental degradation that are reducing the amount of fruitful agricultural land. At the same time, the increase of world's population creates serious challenges in food production, so making food safety a main issue. Taking into account this fact, as well as the fallout of COVID-19, and the UN Report from 2021 that clearly indicates that the number of people suffering from malnutrition is close to 811 million [15], serious measures should be undertaken. In response to supplying the growing world's population with high quality protein, the global livestock population has grown, followed by an increased demand for feed crops. Producing feed crops able to persist in spite of a severe climate, especially in rural and dry areas with limited water resources, is laborious [16]. The use of modern biotechnology, including genetic modification techniques, has been proposed as a way to increase productivity and improve food/feed quality and at the same time, reduce the environmental footprint [17].

More than 70-90% of harvested GM crops are used in food-producing animal nutrition [18]. The question that arises is whether using GM feed crops can wield potential adverse effects on animals and consequently on humans. Therefore, it is crucial to review the safety of products such as milk, meat and eggs derived from animals fed GM crops, and this step should be a mandatory part of the regulatory process.

Independent studies have been undertaken to show if feeding GM crops affects the health and safety of animals. Some of the studies evaluated the health effects of feeding GM crops to ruminants, pigs and poultry, by observing parameters related to immune response, body condition score, organ weight, haematology, serum biochemistry, histopathology or gastrointestinal microbiota [19, 20]. Results have shown that despite the observed significant differences in some of the parameters, most of the effects measured were not of biological significance and were within normal biological ranges [19]. Studies where lactating dairy cows, pigs, poultry, lambs and rats were fed non-GM feed or GM-feed show the presence of fragments of GM DNA only in some parts of the gastrointestinal tract, while GM DNA was not detected in blood or any visceral tissue [21, 22, 23, 24, 25].

Research studies focused on analysing the safety of animal-derived products for the presence of GM DNA/genes have not proven their presence [26, 27]. Agodi et al., 2006, reported presence of GM DNA from maize and soybean in 25% and 11.7% of analysed samples respectively, during screening of milk samples from 12 different brands from the Italian market [28]. Within a normal diet, humans daily consume between 0.1 and 1 g of DNA originating from animal and plants [29]. Most of the digestion takes place in the small intestine, where nuclease and protease enzymes break down DNA nucleotides (nucleoproteins) into smaller parts. About 90% of nucleotides are absorbed by the cells of the intestinal epithelia, out of which 5% are retained for DNA or RNA synthesis, and 20-25% remains in the epithelial cells [30]. Therefore, since the GM DNA remains in the gut, concerns are expressed about the possibility

of horizontal gene transfer to the bacteria of the normal intestinal microbiota [21]. Despite the ability for horizontal gene transfer among certain bacterial species, *in vivo* experimental studies have not detected horizontal transfer of GM DNA/genes so far [21, 22].

2. What is the opinion about GMOs in scientific circles?

In the decades since the first use of GM foods, negative health consequences for consumers have not been registered. This does not mean they do not exist, but that they have not yet been definitively identified.

The European Union has invested more than €300 million in GMO biosafety research. The latest report from 2017 states: "The main conclusion that can be drawn from more than 130 research projects, conducted over a period of 25 years, involving more than 500 independent research groups, is that botany, especially GMOs themselves, do not pose a risk compared to plants obtained with conventional cultivation technologies". A report by the American Medical Association, the National Academy of Sciences and the World Health Organization, based on research by independent groups around the world, also found that 90% of the scientific community believed that GMOs were safe to use. However, only a little over a third of consumers share this opinion. Fears about the side effects of GMO use still persist among consumers, as does the fear that inserting one or more genes could have a negative effect on other genes that are naturally present.

3. What are the fears that consumers face from the use of GM foods?

Most often, consumers fear GM foods that have inserted genes that improve nutritional value, primarily because they could increase the risk of allergic reactions, antibiotic resistance, toxic effects on various organs, mutations, or affect pregnancy, offspring and potential gene transfer to the consumer.

Concerning the fact, that in the past decades food allergy has become significant threat to humans, the relation with consuming GMOs, has raised many scientific questions. The transfer of genetic material from one organism to other results in the creation of a new protein, which could be a potential allergen, in the GM organism. Therefore, with the use of genetic engineering, the percentage of allergens naturally present in food increase. This was the case with a GM soy produced in the mid-1990s. Its genetic material was altered by the insertion of a gene from the Brazilian walnut coding information for synthesis of high quality protein with a favourable ratio of amino acids. The new protein in soy caused allergic reactions in people who were allergic to Brazilian nuts and consumed soy, which was proven by immunoassays. Fortunately, this GM soy was never approved or placed on the market. The review paper of Dunn et al. (2017) summarizes data from 83 studies of GM crops: corn, wheat, rice, soy, milk, peanut and others, addressing the question "Are GM products more allergenic than their conventional counterparts?" In this review, the authors thoroughly analysed studies that involved ingestion of GM food and feed by humans and animals. No animal or human study showed that consuming GM food or feed is more allergenic than its conventional counterpart [31].

In creating GMOs, antibiotic resistance genes are used as markers. This poses a concern of possible horizontal gene transfer between these genes and human and animal gut microbiota, as well as with environmental microorganisms. Scientists from China demonstrated the presence of a plasmid coding for β-lactamases in six Chinese rivers [32]. This very same plasmid is used for GMO production. To avoid even the very rare possibility of horizontal gene transfer, only genes coding for antibiotics that are not used in human and veterinary practice should be used in creating GM seeds and plants [33].

4. GMO regulation

There are differences between countries regarding GMO regulation, which are the result of different socio-economic and political factors. Constraints regarding GMO regulation are assessed through several indices, such as: approval process, risk management, labelling, traceability, coexistence and participation in international agreements. Thus, the European Union and Japan are in favour of more stringent measures in terms of GMO regulation, unlike the United States, where in some of the states currently it is not mandatory to label GMOs present in products, while other states have mandatory

labelling only if GMOs are present in quantities over 5%. However, starting from January 2022, the United States will begin to implement mandatory GMO labelling in consumer products. In the European Union, Japan and New Zealand, it is mandatory to label the presence of GMOs in food, if the authorized GMOs are present at levels above 0.9% for. Non-authorized GMOs must not be present at all.

Even though GMOs cause great controversy worldwide, especially in Europe, still in the European Union, 107 GMOs in food have been approved for use so far. After a call by the European Commission, 19 of 27 states of the European Union voted to either fully or partially ban GMOs. Spain and Portugal still allow GM corn MON 810 to be grown.

5. Detection and testing of GMOs

GM poses a huge challenge for analysts in developing methods that are precise and sensitive enough, can provide accurate data on whether or not the food or feed contains genetic modifications, and which will help consumers in their choice of products. Testing for the presence of GMOs in food is more than just screening for a few genes. The increase in GM crops, global trade routes and complex approval conditions in different countries require optimization of protocols for analysis and development of national strategies for their testing.

5.1 The strategy for classical GMO analysis

5.1.1. GMO screening – faster and more efficient detection of most GMOs

GMO analysis strategies generally begin with qualitative screening, which provides a reliable determination of whether or not there is a genetic modification in the food tested. Especially for food business operators who want to sell their GMO-labelled products, screening methods are essential.

5.1.2. GMO identification – providing product sales

In many countries around the world there is 0% tolerance or a tolerance threshold for unapproved GMOs. If the GMO is not approved in one country but is already approved and planted in another country, this asynchronous approval status can lead to problems in the sale of products that contain even traces of this GMO that is not approved in the importing country.

With the help of specific detection methods, unapproved GMOs can be identified and later excluded from market sale. Therefore, specific qualitative methods are an important aspect of confirming the status of raw materials and processed products derived from unapproved GMOs.

5.1.3. GMO quantification – is labelling required?

In addition to the approval of GMOs, the labelling of GMO content above a certain threshold is differently regulated by country. For example, in the European Union, unexpected or technically unavoidable pollution with approved GMOs of up to 0.9% is exempt from labelling. GMO quantities in food and feed above the threshold of 0.9% must be labelled according to EU Regulation no. 1830/2003 [34]. With the help of quantitative detection of GMOs, it can be determined whether the content of GMOs in the product is above or below the legal threshold. In animal feed, unauthorized GMOs are tolerated up to a threshold of 0.1% as long as they meet the requirements set out in EU Regulation no. 619/2011 [35]. For questions such as these, quantitative analysis is the method of choice for verifying sales and labelling requirements.

6. Conclusions

Although technologies for the production of genetically modified food are very promising in some areas that are challenges for the 21st century, still like all new technologies, they bear certain risks, known and unknown. The controversy and public concern over GM food and feed are primarily focused on human and animal health, as well as on environmental safety, labelling and consumer choice, intellectual property rights, ethics, food safety, poverty reduction and environmental conservation. What effects will GM food/feed have on the environment? What are the dangers to human health? Are we challenging

"Mother Nature" with these innovative technologies? These are the questions that we will leave to evolution itself to answer.

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Possibility of partial replacement of sodium chloride with potassium chloride and ammonium chloride in chicken *ćevapčići* (kebabs)

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Possibility of partial replacement of sodium chloride with potassium chloride and ammonium chloride in chicken *ćevapčići* (kebabs)

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Abstract. The goal of this study was to investigate the influence of reducing sodium chloride content in chicken *ćevapčići* (kebabs) by partial replacement of sodium chloride with potassium chloride and ammonium chloride, with the target of achieving the optimal salty taste. The trial consisted of five groups. In the control group of chicken *ćevapčići*, only sodium chloride was added. In group 1, one third of sodium chloride was replaced with potassium chloride; in group 2 one half of the sodium chloride was replaced with potassium chloride; in group 3, one third of sodium chloride was replaced with ammonium chloride. In group 4, sodium chloride was half reduced and one quarter of ammonium chloride in the relation to control group was added. Sensory evaluation was performed by ten trained assessors using numeric scales. Evaluations of colour acceptability and consistency showed there were no statistical differences ($P \geq 0.05$) between the *ćevapčići*. The most expressed saltiness was evaluated in the control *ćevapčići* group due to it having the largest amount of added sodium chloride, as well in group 3 *ćevapčići*, wherein one third of the sodium chloride was replaced with ammonium chloride.

1. Introduction

Sodium chloride is the first of the known food preservatives ever to be used, particularly where meat is concerned, and has remained in use to this day. The main source of sodium in food products is derived from sodium chloride i.e., table salt, and its consumption and intake significantly exceeds nutritional recommendations. This is particularly the case in modern, highly industrialised human societies. Salt is the prototypical stimulus for salty taste [1] and it improves the sensory properties of food by increasing saltiness, decreasing bitterness and increasing sweetness and other congruent flavour effects [2].

Overconsumption of sodium through food is the main cause of essential human hypertension [3], as well as cardiovascular disorders. In 2012, the World Health Organisation (WHO) adopted new guidelines concerning the intake of salt and potassium, which recommends a daily intake of less than 2,000 mg of sodium or 5 grams of salt and less than 3,510 mg of potassium for adults [4]. The American Heart Association recommends no more than 2,300 mg of sodium per day and moving toward no more than 1,500 mg per day for most adults [5].

One of the most common foods with high amounts of sodium are meat products. Of the total daily amount of table salt ingested into the body through usual amounts of food, approximately 20% originates from meat products [6].

Minced meat products are extremely popular among all cultures, due to their specific aroma and taste, especially when these types of meats are prepared on a grill. In recent times, people are increasingly using minced meat made from poultry, the first choice being chicken meat. In addition to containing proteins with high biological value, fats and essential amino acids, vitamins and minerals, poultry meat is also rich in vitamin B complex, specifically thiamine, riboflavin, niacin and pantothenic acid [7]. One of the methods used to reduce the salt content of meat products is through the partial substitution of sodium chloride with other chloride salts (KCl, CaCl and $MgCl_2$) [8, 9]. Potassium chloride is the most common substitute for sodium chloride; however, complete substitution of sodium chloride is not an option, as even with 50% substitution, many studies indicate that this results in an increase in bitter taste and a decrease in salinity.

The goal of this paper was to investigate the possibility of reducing the sodium content in meat preparations i.e., chicken *ćevapčići* (kebabs), by partially substituting sodium chloride with potassium chloride and ammonium chloride.

2. Materials and Methods

The experiment consisted of five sample groups of chicken *ćevapčići* (kebabs) produced with different salt mixtures. The composition of chicken *ćevapčići* is presented in Table 1. Chicken *ćevapčići* were prepared from chilled chicken minced meat category I (breast 70%, drumstick and thigh without skin 30%).

In the control group (C) only sodium chloride was added in an amount of 8.5 g which is common for this type of product. In group 1, one third of sodium chloride was replaced with potassium chloride, while in group 2 one half of the sodium chloride was replaced with potassium chloride. In group 3, one third of sodium chloride was replaced with ammonium chloride. In group 4, sodium chloride was half reduced and one quarter of ammonium chloride in the relation to control group was added.

Table 1. Composition of chicken *ćevapčići* (g)

Sample groups	Chicken minced meat	Sodium Chloride	Potassium chloride	Ammonium chloride
C	491.5	8.5		-
1	491.5	5.66	2.84	-
2	491.5	4.25	4.25	-
3	491.5	5.66		2.84
4	493.6	4.25		2.13

2.1. Sensory evaluation

Sensory evaluation was performed by ten trained assessors [10] using numeric scales. Colour acceptability, consistency, saltiness acceptability and taste acceptability were evaluated with a 1-5-point scale, where 1 was at least acceptable and 5 was the most acceptable attribute. Saltiness intensity was evaluated with a 1-5-point scale, whereby 5 was the most expressed attribute and 1 was the least expressed attribute. Preparation and presentation of the samples to the assessors (number, coding and randomization) as well as the fitting out of the serving area (isolation of panellists, lighting conditions) were performed according to [11]. The final ranking was done according to the sum of all sensory evaluation results, where the best scored chicken *ćevapčići* was ranked first and the worst ranked in fifth place.

2.2. Statistical evaluation

The obtained results were statistically evaluated using Microsoft Excel 2010 and are presented as mean ± SD. Statistical differences between means of the examined parameters were determined at the levels 0.05 and 0.01 by Student's t-test.

3. Results and discussion

The results of the sensory evaluation of chicken *ćevapčići* are presented in Table 2. Samples of chicken *ćevapčići* from all experimental groups were evaluated as having high colour acceptability and consistency and between means, there were no statistically significant differences ($p > 0.05$).

The mean saltiness acceptability of control *ćevapčići* (4.40 ± 0.54) was significantly higher ($p < 0.05$) than that of group 3 *ćevapčići* (3.25 ± 1.03), which had the lowest saltiness acceptability. Group 3 *ćevapčići* had the most expressed saltiness (4.35 ± 0.78). Group 4 *ćevapčići* were significantly less salty ($p < 0.05$), as were group 2 *ćevapčići* ($p < 0.01$), than those from group 3. The results obtained were in accordance with results published by Lilić et al. [12] for meat balls. They found significant differences in saltiness intensity between control and groups 2 and 4 for burgers ($p < 0.05$), but the saltiness of *ćevapčići* did not accord to saltiness differences of meatballs from groups 2 and 3 ($p < 0.01$).

Due to the *ćevapčići* from the control group being produced only with added sodium chloride, the bitter taste was the least expressed in this product. However, in *ćevapčići* where sodium chloride was replaced with larger amounts of potassium chloride i.e. ammonium chloride (group 2: 3.20 ± 1.14 and group 4: 3.15 ± 1.36), bitter taste was intensified compared to the control *ćevapčići*, evaluated as 1.75 ± 1.12 ($p < 0.05$). In group 2 *ćevapčići*, half of the sodium chloride was replaced with potassium chloride, resulting in increasing bitter taste intensity, leading to lower overall acceptability of the product. The presented results are in accordance with the results obtained by De Almeida et al. [13] and Inguglia et al. [14], who concluded that the partial substitution of sodium chloride with potassium chloride had a negative impact on product taste, because of bitter taste. Despite that, Lilić et al. [12] found no statistically significant differences between samples of meatballs from the control and experimental groups produced with the same salt mixtures as samples in this trial.

The highest taste acceptability was achieved by *ćevapčići* from the control group (4.45 ± 0.57). The taste acceptability of group 2 *ćevapčići* (3.45 ± 0.52), in which sodium chloride was partially substituted with potassium chloride, and taste acceptability of group 4 *ćevapčići* (3.25 ± 1.31), in which half the amount of sodium chloride was added, and to which ammonium chloride in the amount of a quarter of the amount of salt added to the control group was added, was statistically significantly lower than the taste acceptability of the control *ćevapčići* ($p < 0.05$). Group 3 *ćevapčići*, in which a third of the sodium chloride was substituted with ammonium chloride, were awarded the lowest taste acceptability (3.15 ± 0.87), which was a statistically significant difference compared to the control group ($P < 0.01$). The results obtained were in accordance with results of Rašeta et al. [15], who reported very similar numeric evaluation for taste acceptability and statistical significances.

Overall acceptability of *ćevapčići* from the control group (4.45 ± 0.52) was statistically higher ($p < 0.01$) compared to the overall acceptability of *ćevapčići* from groups 2 and 4 (3.10 ± 1.07 and 3.05 ± 1.29, respectively), and statistically significantly higher ($p < 0.05$) compared to the overall acceptability of group 3 *ćevapčići* (3.25 ± 0.78). There were no statistically significant differences ($p > 0.05$) between the overall acceptability of control and group 1 *ćevapčići* (3.85 ± 0.78).

Despite the significance of the differences in terms of certain sensory attributes, the samples of chicken *ćevapčići* taken from all experimental groups were sensorially acceptable in terms of saltiness, taste, colour, consistency and salt and bitter taste intensity.

Based on the results of this study, it can be concluded that chicken *ćevapčići* with reduced sodium chloride and/or potassium chloride content could be prepared by the partial substitution of sodium chloride with potassium chloride and ammonium chloride.

Table 2. Sensory evaluation of chicken *ćevapčići*, Mean ± SD, n = 10

	Colour acceptability	Consistency	Saltiness acceptability	Saltiness intensity	Taste acceptability	Bitter taste intensity	Overall acceptability
C	4.65±0.55	4.45±0.65	4.40±0.54 ^{a}	4.05±0.82 ^{a}	4.45±0.57^{ax}	1.75±1.12 ^{a}	4.45±0.52 $^{a, x}$
1	4.40±0.80	4.20±0.75	4.20±0.90	3.65±0.74	4.00±1.00	2.65±1.10	3.85±0.78
2	4.50±0.63	3.80±0.68	3.65±1.18	3.00±0.81 ^{b,x}	3.45±0.52^{b}	3.20±1.14^{b}	3.10±1.07^{y}
3	4.55±0.57	3.80±0.71	3.25±1.03 ^{b}	4.35±0.78 $^{a, y}$	3.15±0.87^{y}	2.80±1.10	3.25±0.78^{b}
4	4.65±0.63	4.00±0.89	3.50±1.10	3.15±1.16 ^{b}	3.25±1.31 ^{b}	3.15±1.36 ^{b}	3.05±1.29^{y}

$^{(a,b)}$ Values (mean±SD) in columns with different superscript letters are significantly different (P≤0.05)
$^{(x,y)}$ Values (mean±SD) in columns with different superscript letters are significantly different (P≤0.01)

4. Conclusion

Despite the significance of the differences in terms of certain sensory attributes, the experimental chicken *ćevapčići* taken from all groups were sensorially acceptable in terms of saltiness, taste, colour, consistency and salt and bitter taste intensity. Partial replacement of sodium chloride with other chloride salts, potassium chloride and ammonium chloride, in the different ratios did not influence colour acceptability or consistency. However, use of these salts had direct impacts on the salt intensity, bitterness and overall impression. The *ćevapčići* with salt replacements had less intensity of saltiness, except *ćevapčići* with a large amount of added ammonium chloride, in which the saltiness was the most expressed and that were more bitter than control *ćevapčići*, which resulted in low sensory evaluation scores for taste and overall acceptability.

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The effect of Spirulina inclusion in broiler feed on meat quality: recent trends in sustainable production

The effect of Spirulina inclusion in broiler feed on meat quality: recent trends in sustainable production

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Abstract. This review covers the current situation of the use of Spirulina in poultry diets and discusses its benefits and challenges with particular emphasis on the effect of Spirulina supplementation on production performances and meat quality. Feed enriched with Spirulina influences broilers' health by improving their immune response and gut function and increasing PUFA and pigment content in the meat. However, despite numerous studies, the effect of Spirulina on broiler performance remains unclear.

1. Introduction

Poultry meat is among the most common animal-origin foods consumed worldwide due to its nutritional composition. Poultry production is one of the fastest-growing agricultural subsectors, especially in developing areas, effectively contributing to food security [1]. Increased competition for land with other agricultural sectors, climate change, and the constant need to increase productivity and improve meat quality has resulted in demand for novel feed resources. Moreover, in recent years, there has been an increased interest in the production of innovative functional foods using eco-friendly sources [2]. One of the promising sustainable ways to meet the aforementioned criteria is the use of Spirulina (*Arthrospira* spp.), which is a filamentous, spiral-shaped, blue-green microalga [3]. Spirulina is suitable for use as a feed supplement and feed ingredient for poultry due to its high protein content (up to 65%), rich in all essential amino acids [4]. In addition, Spirulina contains high amounts of physiologically active substances, including carotenoids, phycocyanin, polyunsaturated fatty acid (PUFA), vitamins, macro, and micro-minerals. These substances exhibit antimicrobial, antioxidant, and anti-inflammatory properties [5,6,7]. Considering recent trends, toward sustainable production, this review aims to discuss the benefits and challenges of using Spirulina as a feed ingredient in poultry feeds and its consequent effect on meat quality.

2. Materials and Methods

A review was performed by analysing scientific research and review papers from the scholarly databases and technical reports published in the domain of microalgae use in poultry diets.

3. Discussion

3.1. The effect of Spirulina supplementation on production traits

Results regarding production parameters in broilers fed Spirulina differ between studies. Some authors [8,6,9] reported that even low (10g/kg diet) levels of inclusion of *Spirulina platensis* significantly increases body weight gain (BWG), while decreasing feed conversion ratio (FCR). Moreover, Mariey *et al.* [10] found that broilers supplemented with 0.2 and 0.3g Spirulina/kg diet improved FCR by 10 and 12%, respectively, compared with the control group. They also reported increased dressing percentage and decreased relative abdominal fat weight. An increase in feed intake (FI), BWG, and better FCR were observed with 2 g of Spirulina/kg feed supplementation by Khan *et al.* [9]. Similar results were reported by Alwaleed *et al.* [11], with the inclusion of 10 g Spirulina/kg feed. These results are attributed to previously reported healthy effects of Spirulina, in terms of increased immune response and beneficial influence on gut histology and microbiota. Various studies showed that Spirulina enhances the immune response [7]. The proposed hypothesis is that Spirulina increases macrophage functionality and, overall, the mononuclear phagocyte system [12]. Seyidoglu *et al.* [13] suggested that β-glucan and phycocyanin from Spirulina influence the development and maturation of leukocytes. Lokapirnasari *et al.* [14] and Jamil *et al.* [15] found a higher number of leucocytes in broilers supplemented with Spirulina, compared to control broilers, while Fathi *et al.* [16] reported that the addition of *S. platensis* resulted in a higher weight of immune-system related organs. Park *et al.* [6] reported the antioxidative activity of Spirulina. They found that low levels (2.5-10g/kg diet) of Spirulina supplementation led to an increase in the serum superoxide dismutase and glutathione peroxidase activity. Furthermore, many authors reported that the inclusion of Spirulina in diets influences gut health by reducing the number of *Escherichia coli* and increasing the counts of beneficial lactic acid bacteria (LAB) in ileum and caecum of broilers [6,16]. The fibrous cell wall materials and chlorophylls in the microalgae could provide substrates for the growth of LAB, and so act as prebiotics. Shanmugapriya *et al.* [8] reported that dietary Spirulina inclusion of 10g/kg diet (1%) resulted in a significant increase in the villi height in broilers intestines. Thus, a lower number of pathogenic bacteria and increased villi area is attributed to better nutrient absorption and, consequently higher performances. Contrarily, other studies reported that the addition of this microalgae to broiler diets [2,17] had no effect or had an adverse effect on growth performances. El-Bahr *et al.* [18] found that the inclusion of 1g *S. platensis*/kg diet did not influence FI, FCR or body weight. Altmann *et al.* [17] reported no significant effect on live weight or carcass weight when 50% of the soy protein was replaced by *Arthrospira platensis* in broiler diets. In addition, Pestana *et al.* [19] found lower improvement to BWG and higher FCR in birds fed 150g *S. platensis*/kg feed (15%), individually or in combination with exogenous enzymes, during the finishing period (from 21 to 35 day), while no effect was observed on FI. A possible explanation can be that incorporation of higher amounts of Spirulina into the diet results in protein gelation, increasing digesta viscosity, and lowering amino acid digestibility [20].

These contradictory data between studies could result from several factors: levels of *Arthrospira* used for different broiler hybrids, broiler age, housing conditions, feed preparation, or the way the supplement/feed is administration.

3.2. The effect of Spirulina supplementation on meat quality and meat sensory attributes

The supplementation of different microalgae, including Spirulina, in broiler diets, alters meat quality with regard to consumer expectations of a healthy diet. In different studies, the colour of meat was affected by the addition of Spirulina in broiler feeds. Toyomizu *et al.* [21] reported that Spirulina incorporation in the broiler diet resulted in redder and more yellow meat due to higher pigment content, in particular, due to the accumulation of zeaxanthin. Furthermore, the replacement of 50% of the soy protein in broiler diets with Spirulina influenced both the yellowness and redness of broiler meat [17]. In agreement with such findings, Pestana *et al.* [19] reported that addition of 15% Spirulina with or without enzymes increased the b* value of breast muscle up to three times, and both a* and b* values in thigh muscle. Also, these authors found a significant increase in the total amount of carotenoids. Thus,

Spirulina supplementation is a good strategy, not only to manipulate broiler meat colour but also to enrich meat in antioxidants.

From the available literature, it seems that the addition of Spirulina does not significantly influence the pH of the meat [6,19]. In addition, previous studies did not show that Spirulina dietary treatments affect shear force. El-Bahr et al. [18] reported that even low Spirulina levels (1g/kg) in broiler diets significantly decreased drip loss. Similar results were obtained by Athman *et al.* [17], who found a significant increase in cooking loss and storage loss after seven days when Spirulina replaced 50% of the soy protein in broiler diets. Park *et al.* [6] found that the addition of Spirulina (0.25-1%) in broilers diets resulted in a significant reduction of drip loss after seven days of storage. These results are related to the delayed oxidation of the cell membrane caused by antioxidative compounds from Spirulina. Other studies did not report relevant differences in drip loss and cooking loss between the supplemented and control diets [22]. Moreover, *S. platensis* supplementation increased essential amino acid levels (lysine, methionine, tryptophan, histidine, and aspartic acid) in breast muscle. Also, Spirulina inclusion led to significantly lower microbial growth on breast meat, resulting in a longer shelf life of this meat [18].

As far as sensory attributes, Altmann *et al.* [17] reported that meat from Spirulina-fed broilers, after seven days of MAP storage, was less metallic in flavour and more tender than the control. Another study showed that breasts from Spirulina-fed birds scored higher in terms of umami and chicken flavour [22].

Regarding fatty acids, numerous studies confirmed that the addition of Spirulina in feeds increases omega-3 PUFA in meat, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content, and beneficially affects the n6/n3 ratio. El-Bahr *et al.* [18] found that even low Spirulina supplementation led to a significant increase in the levels of EPA, DHA, n-3 FA, total PUFA, and arachidonic acid in breast meat. Moreover, Bonos *et al.* [2] reported a significant enrichment of polyunsaturated fatty acids in thigh muscle, especially in EPA and DHA, when adding Spirulina into broiler diets. The ultimate nutritional goal is to reach an n6/n3 ratio of 4:1 and a PUFA/SFA ratio above 0.4. Thus, the addition of Spirulina in broiler diets could turn meat into a healthier product for consumers from the fatty acid perspective. Furthermore, and although some studies reported an increase in omega-3 fatty acids, the malondialdehyde levels remained the same or lower [18,2] than in the controls. How the method for including Spirulina in feed affects muscle lipid oxidation is not completely clear and there is lack of information on antioxidant properties related to Spirulina in poultry. Some authors suggest that the phycocyanin is the main component responsible for Spirulina's antioxidant activity, but also highlight the role of carotenoids in cells protection from oxidative stress [23,6]. Contrarily, Altmann *et al.* [22] found increased lipid oxidation levels of meat from broilers fed Spirulina when stored under high oxygen MAP (80% O_2). This higher susceptibility to oxygen should be considered when packing meat from broilers fed Spirulina.

3.3. Spirulina as a sustainable feed ingredient

Spirulina is crop that does not use arable land, involves simple growth requirements and, it is produced through economical and environmentally friendly processes. Its production uses less energy and water per kilogram than proteins from plant sources [24]. Therefore, Spirulina is a sustainable feed alternative with reduced environmental impact. Taking into consideration the rapid and progressive increase of the global population and growing demand for food, Spirulina use, along with other microalgae, in animal feeds could relieve the pressure on agricultural yield for domestic animal feeding [4] and help to preserve the agricultural land for food production for humans. Moreover, Park *et al.* [6] suggested that dietary inclusion of 1% Spirulina powder in broiler diet might reduce ammonia emissions in the excreta.

3.4. Challenges of introducing Spirulina to poultry diets

From a commercial point of view, the application of Spirulina is limited by the high costs of the large-scale production compared to other feeds [3]. To overcome this barrier, developments in low-cost production and drying units and an improvement in operational management are needed [25]. Furthermore, the cost of broiler feed increases with the addition of vitamin-mineral premixes. Venkataraman *et al.* [26] reported that vitamin-mineral premixes can be excluded from broiler diet when

Spirulina is included due to its nutritional composition. Pandav and Puranik [27] confirmed this in a study with *S. platensis* enriched with iron and zinc. Thus, despite the higher price of Spirulina production, by reducing premix costs, this microalgae can be used in broiler nutrition in a cost-effective manner. Other deterrents to the practical use of Spirulina are poor palatability, production costs for the dried powder form and odours [5].

4. Conclusion

Based on the current scientific data, blue-green microalgae, Spirulina, are promising candidates to replace traditional, high protein feed crops, such as soybean meal, the major protein source for broiler feed. Further research should be focused on a deeper understanding of the mechanisms of Spirulina actions, delivery methods, and optimization of the added concentrations in order to achieve maximum beneficial effects for poultry health, productivity, meat product nutritional and organoleptic quality and the environment.

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Examination of the influence of conjugated linoleic acid in broiler nutrition on the economic efficiency of fattening

Examination of the influence of conjugated linoleic acid in broiler nutrition on the economic efficiency of fattening

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Abstract. The aim of this study was to examine the influence of the use of CLA (2%), from days 1, 11 or 22 of fattening, on selected production results (broiler weight after each fattening phase including at the end of fattening, viability, average daily gain and feed conversion) on the efficiency of fattening during the whole fattening period (42 days). The obtained production results were used to calculate the European production efficiency factor (EPEF) and the European broiler index (EBI) values for each fattening phase as well as for the whole fattening. The results obtained indicate that, in the later stages of fattening, the use of CLA during the whole fattening period is economically more justified than the non-CLA diets used for control broilers. With the use of CLA throughout the whole fattening, the EPEF and EBI values are consistent with these values calculated for the Cobb 500 standard. In addition to economic justification, the use of CLA also has human nutritional significance, since the broiler meat is enriched with CLA and has a more favourable n-6/n-3 fatty acid ratio.

1. Introduction

World poultry meat production, after decades of the primacy of pork production, equalized three years ago with pork production, and in 2020, it will be higher than the pork production. In total world meat production for the five-year average (2016-2020) of 323.25 million tons (mt), the amount of poultry meat was 122.82 (37.99%) mt. In Serbia, the total meat production for the five years (2015-2019) was 501.6 thousand tons, of which 97.8 thousand tons (19.59%) was poultry meat production [1, 2, 3]. In the last sixty years, poultry meat production has intensified in both developed and developing countries. In underdeveloped countries, poultry breeding has an extensive character, but it has great importance because it provides the poorest part of the population with meat and eggs as high-value foods, employs mostly female labour and enables the human population viability in unfavourable climatic conditions with underdeveloped agricultural production, e.g. with a small amount of water. In such circumstances, it is not possible to raise other types of domestic animals, except perhaps sheep and goats.

There has been progress in intensive poultry breeding, thanks primarily to genetic selection, reduction in fattening duration, and good feed conversion (below 1.77 kg of feed per kg of meat). Among

meat species, poultry meat production is the most economically viable. In addition, poultry meat is a highly valuable food, especially breast meat (low in fat, high in protein). It has no religious restrictions, is easy to prepare, all parts of the carcass are usable, and it is the most common type of meat on the market.

One of the advantages of poultry meat is the ability to increase its nutritional value by choosing nutrients and supplements for live birds. This means that poultry meat can also be a functional food (it was first mentioned a little over 40 years ago in Japan and means food that, in addition to its usual ingredients, also contains ingredients that support certain human body functions). To date, there are several definitions of functional food, and from all definitions, we can conclude that functional food has additional nutritional value and serves to preserve human health via human nutrition. When we talk about poultry meat as a functional food, we primarily mean the possibility of increasing the content of n-3 fatty acids in meat (eggs), a more favourable n-6/n-3 fatty acid ratio, and enriching poultry meat and eggs with conjugated linoleic acid (CLA) [4, 5]. The fatty acid composition of poultry meat (eggs) is directly dependent on the fatty acid composition of broiler/laying hen diets. Thus, increasing n-3 fatty acids in poultry feed (with preparations of flax, green algae) improves the n-6/n-3 fatty acid ratio. Non-ruminant animals do not have the ability to synthesize CLA, so its occurrence in meat (eggs) is conditioned by its addition to animal diets. There are data that CLA can be found in meat (eggs) and poultry in whose diet CLA has not been added. However, these are insignificant amounts that are sometimes even below the detection threshold [6].

Of the numerous CLA isomers in poultry nutrition, two isomers are used: *cis*-9,*trans*-11 and *trans*-10,*cis*-12, which are in approximately the same ratio in commercial preparations. The isomer *cis*-9,*trans*-11 is most often stated to have an anti-carcinogenic effect in the human diet (cancer of the skin, breast, intestines, and liver), to reduce the occurrence or alleviate symptoms of diabetes, mitigate bone density loss and atherosclerosis, and to have a positive effect on reduction of chronic cardiovascular diseases [7, 8]. Isomer *trans*-10,*cis*-12 is considered to contribute to the reduction of obesity and to be of primary importance [7, 8]. In addition to meeting market needs, meat production (primary production) is especially interested in the economic viability of broiler fattening. In recent years, the economic viability of broiler fattening is most often expressed through the European production efficiency factor (EPEF) and the European broiler index (EBI).

The aim of this study was to examine the effect of using CLA in broiler feed on the economic efficiency of fattening.

2. Materials and Methods

The study was conducted on 240 one-day-old chickens of both sexes and the same origin (Cobb 500) during a 42-day period. At the beginning of the study, broilers were randomly allocated to one of four dietary treatments. Each experimental group contained 60 animals housed in groups of 10 birds per pen in six repetitions (stocking density = 0.15 m^2/head). Conditions in the facility (ventilation, heating, lighting, and relative humidity) were according to the technological standards and recommendations for this hybrid [9]. Pens were bedded with straw and provided with fresh water and feed *ad libitum*. At the beginning of the study, the temperature of the room was 32°C, and then was gradually lowered to 22°C that was maintained until the end of the study. During the trial, relative humidity was 60-70%. A continuous period of six hours of darkness was provided during the night. From the start of the trial, each group of broilers was fed with one of four experimental diets which comprised the same basal diet, but differed only in the content of CLA. Basal diet was formulated according to the recommendations for the Cobb 500 strain [10] (Table 1).

Table 1. Ingredients of diets

Component	***Starter K***	***Starter O***	***Grower K***	***Grower O***	***Finisher K***	***Finisher O***

Maize	50.85	48.85	44.15	42.15	44.95	42.95
Wheat	-	-	10.00	10.00	15.00	15.00
Soybean semolina	15.00	15.00	17.00	17.00	20.00	20.00
Soybean meal	12.40	12.40	1.00	1.00	1.00	1.00
Soybean cake	17.00	17.00	23.30	23.30	14.70	14.70
Monocalcium phosphate	1.20	1.20	1.00	1.00	0.90	0.90
Limestone	1.60	1.60	1.60	1.60	1.60	1.60
Salt	0.35	0.35	0.35	0.35	0.35	0.35
Premix	1.00	1.00	1.00	1.00	1.00	1.00
Lysine	0.20	0.20	0.20	0.20	0.10	0.10
Methionine	0.20	0.20	0.20	0.20	0.20	0.20
Adsorbent	0.20	0.20	0.20	0.20	0.20	0.20
CLA	-	2	-	2	-	2

Diets fed from days 1 to 42 were starter (days 1–10), grower (days 11–21) and finisher (days 22–42). The proximate composition of all feed mixtures was analysed according to [11] (Table 2).

Table 2. Chemical composition of diets.

Mixture	Diet	Proteins	Moisture	Fat	Ash	Cellulose
				$\overline{X}$ ± SD		
Starter (0-10)	K	24.98±0.57	8.04±0.24	6.09±0.37	5.45±0.14	2.04±0.05
	O	24.97±0.47	8.06±0.27	6.96±0.35	5.50±0.15	2.04±0.04
Grower (11-21)	K	22.17±0.21	9.38±0.09	7.03±0.26	4.88±0.13	2.16±0.04
	O	22.11±0.47	9.38±0.10	7.09±0.29	4.92±0.12	2.16±0.05
Finisher (22-42)	K	20.91±0.87	9.98±0.07	5.44±0.11	4.76±0.21	2.38±0.26
	O	20.78±0.80	10.00±0.06	5.46±0.06	4.72±0.22	2.57±0.24

The control (K) group was without addition of CLA in the feed mixture, the O-I group received starter feed with 2% CLA from the beginning, the O-II group received feed with 2% CLA from day 11 of growing, and the O-III group received feed with 2% CLA from day 22 of finishing. The mixtures were balanced and fully met the needs of the animals at all stages of fattening.

We examined the production results (average final weight gain BWG at each fattening period, average daily gain ADG, feed conversion ratio FCR) and mortality (%), and then calculated the economic efficiency of broiler production using the European production efficiency factor (EPEF) [12] and the European broiler index (EBI) [13]. The following formulas were used to calculate these indicators:

BWG (grams on period) = BW (g) at the end period- BW (g) in first d;
ADG (g/chick/d) = BWG/ days number of growth period;
FCR (kg feed/kg gain) = cumulative feed intake (kg)/total weight gain (kg);
Viability (%) = chicks remaining at the end of period (%);
European Production Efficiency Factor (EPEF):

$$EPEF = \frac{viability\ (\%) x\ BW\ (kg)}{age\ (d)\ x\ FCR\ (kg\ feed/\ kg\ gain)}\ x\ 100$$

European Broiler Index:

$$EBI = \frac{viability\ (\%)x\ ADG\ (g/broiler/d)}{FCR\ (kg\ feed/\ kg\ gain)x\ 10}$$

Results were compared by statistical analysis using Microsoft Excel 2010 and GraphPad Prism software, version 8.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). To determine the significance of the differences between the examined groups of compared parameters, we used analysis of variance (ANOVA), followed by Tukey's *post hoc* test. Testing the significance of the difference between the arithmetic means of the compared parameters and the standard value (prefixed according to the recommendations for this hybrid [9]) were conducted according to Petz et al. [14]. Differences were considered significant if the observed value was $p < 0.05$.

3. Results

Table 3 presents the production results and economic efficiency indicators of the Cobb 500 broiler fattening. There were no statistically significant differences between the average broiler weights ($p>0.05$) on days 10, 21 and 42. There were no statistically significant differences between the average daily gain (from 31 to 32 g) on day 10. On day 21, the average daily gains were from 92.8 to 95.5 g and also did not differ statistically significantly. As there were no differences in the average broiler weights at the end of fattening, there were no statistically significant differences in the average daily gain on day 42. Compared to the standard values from the Cobb 500 Guide, the average broiler weights (Figure 1) on days 10, 21, and 42 were not statistically significantly different for control and experimental groups. Group O-I had a numerically higher average weight (2862 g) compared to the average standard Cobb 500 weight (2857 g). The statistical significance of the differences in feed conversion between our control and experimental groups and the Cobb 500 standard is present in Figure 2. On day 10, feed conversion values were from 1.46±0.12 (O-I) to 1.79±0.15 (O-III). Feed conversion values on day 21 were from 1.56±0.13 (O-I) to 0.169±0.14 (O-III). Feed conversion values of broilers that received CLA in feed were statistically significantly higher than the standard Cobb 500 feed conversion value on day 10 (1.05 kg) and day 21 (1.26 kg). At the end of fattening, the average feed conversion values were from 1.69±0.14 (O-I) to 1.87±0.14 (O-III). There was no statistically significant difference between the average feed conversion value of group O-I and the standard Cobb 500 feed conversion value or of the group O-II feed conversion value and the standard Cobb 500 feed conversion value, and these values were statistically significantly less than the feed conversions of the control (no CLA) and group O-III.

Table 3. Production results and parameters of economic efficiency of broiler fattening.

Fattening days	Parameters	K	O-I	O-II	O-III
1 to 10	BW (kg)	0.320	0.310	0.320	0.310
	ADG (g)	27.69	26.30	27.69	27.69
	FCR (kg feed/kg gain)	1.67	1.46	1.66	1.79
	Viability (%)	100	100	100	100
	EPEF	191.62	212.33	192.77	173.18
	EBI	165.81	180.14	166.89	154.69
1 to 21	BW (kg)	0.947	0.928	0.955	0.952
	ADG (g)	47.35	44.10	46.58	45.18
	FCR (kg feed/kg gain)	1.66	1.56	1.66	1.69
	Viability (%)	100	100	100	100
	EPEF	285.24	297.44	291.16	281.66

	EBI	272.11	282.69	278.54	267.34
1 to 42	BW (kg)	2.762	2.862	2.672	2.717
	ADG (g)	65.18	67.19	62.66	62.68
	FCR (kg feed/kg gain)	1.88	1.69	1.72	1.87
	Viability (%)	100	100	100	100
	EPEF	350.56	403.21	370.08	346.11
	EBI	346.17	397.53	364.30	336.26

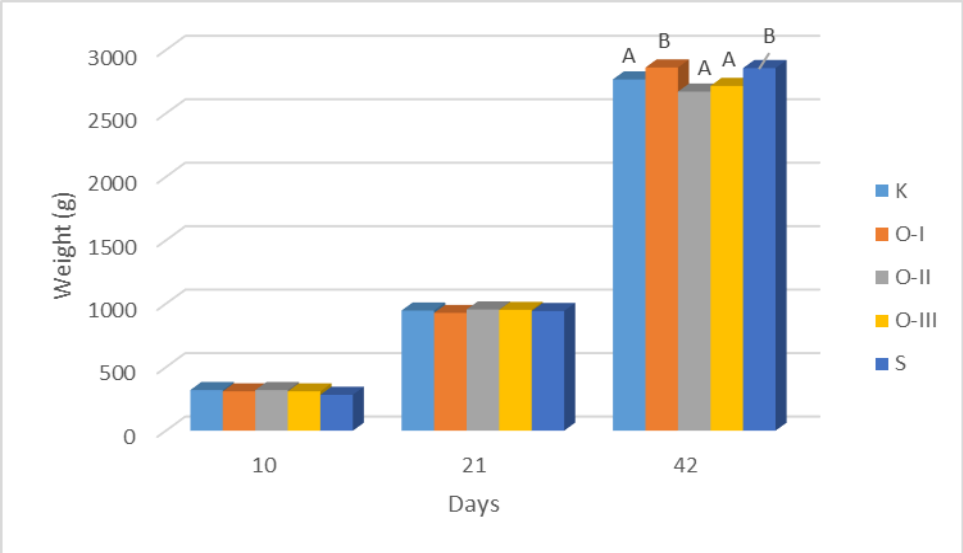

Figure 1. Average broiler weights and standard Cobb 500 weights during fattening.
Different letters A and B indicate different average broiler weight, $p<0.05$

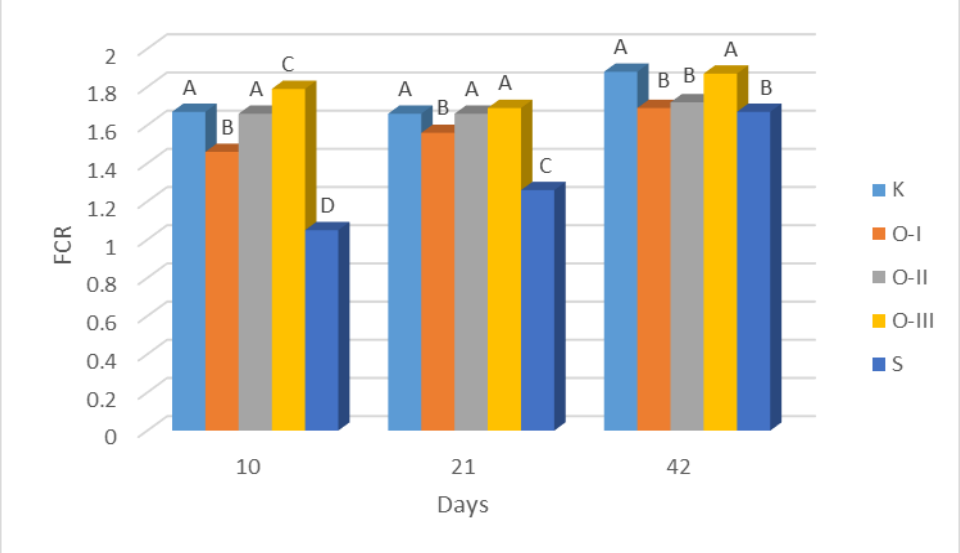

Figure 2. Average feed conversion values (kg) and standard Cobb 500 feed conversion values.
Different letters A, B, C and D indicate different feed conversion values, $p<0.05$

Figure 3 presents the EPEF values of the studied broilers and the EPEF value calculated for the standard Cobb 500. The EPEF value of group O-I on day 10 was statistically significantly higher (p

<0.05) than the EPEF values of K, O-II, and O-III groups. For the same period, the standard Cobb 500 EPEF value was statistically significantly higher compared to the control and experimental groups. On day 21, a statistically significantly higher EPEF value was calculated for the Cobb 500 standard in relation to the EPEF value of the control and experimental groups of broilers. On the same day, there were no statistically significant differences between the average EPEF values of the control and experimental groups. At the end of the fattening, EPEF values of O-I and the Cobb 500 standard did not differ statistically, but they were both statistically significantly higher compared to the control, O-II, and O-III groups.

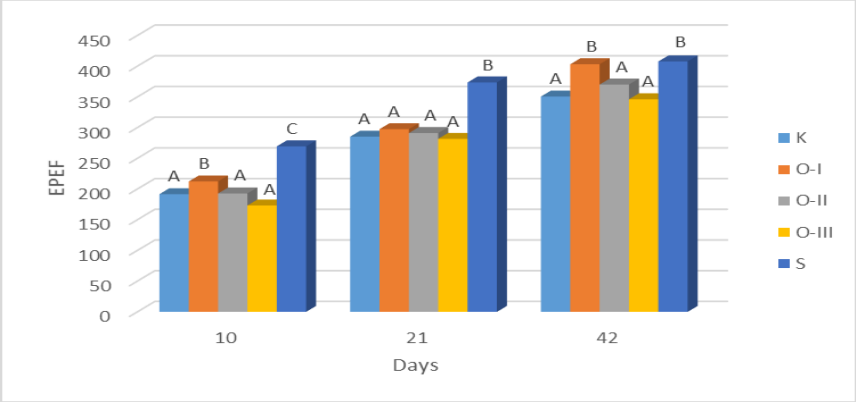

Figure 3. EPEF values of broilers and standard Cobb 500 EPEF values.
Different letters A, B and C indicate different EPEF values, p<0.05

On days 10 and 21, the EBI of the Cobb 500 standard was statistically significantly higher than the EBI values of the control and experimental groups (Figure 4). By the end of fattening, the EBI value of group O-I and the EBI value of the Cobb 500 standard did not differ significantly, and they were statistically significantly higher than the EBI values of groups K, O-II, and O-III.

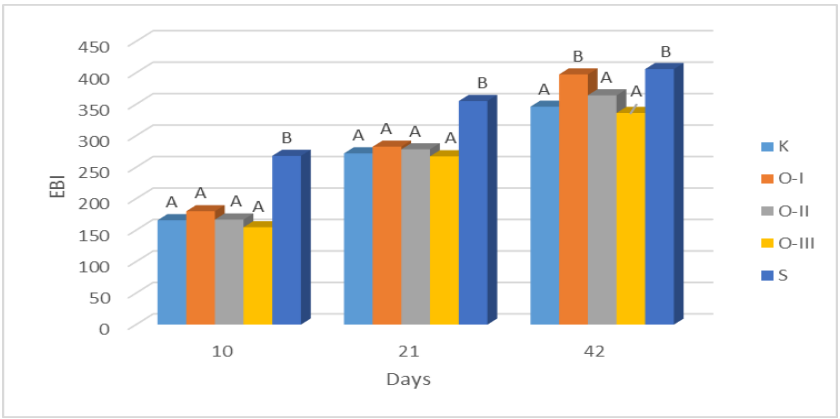

Figure 4. EBI values of broilers and standard Cobb 500 EBI values.
Different letters A and B indicate different EBI values, p<0.05

4. Discussion

The cost-effectiveness of broiler fattening is one of the particularly important factors that has contributed to the increase in broiler meat production in the past few decades. It depends on the total value of fattening and the cost of fattening. As the value of fattening increases and the cost of fattening decreases,

the economy of fattening increases. In recent years, two indices have been used to calculate the cost-effectiveness of fattening: EPEF and EBI. To calculate the EPEF, we used a formula that includes the viability of broilers, their weight, and also the feed conversion for each fattening stage and for the whole fattening. The EBI value is calculated from a formula that includes viability, average daily gain, and feed conversion for each stage of fattening [12, 13, 16, 17]. Higher EPEF or EBI values indicate a better fattening economy [12, 18]. The key factor in both these economic efficiency indices is the feed conversion ratio, because feed accounts for about 70% of the costs in today's large broiler fattening systems. The other 30% of costs are fixed costs (depreciation, one-day-old chicks, energy costs, labour, broiler health care) [15]. The feed conversion value in broilers has been reduced thanks primarily to genetic selection, while diet was far less important for feed conversion reductions [18, 19].
Our results indicate that the feed conversion values (Figure 2) had the greatest impact on the EPEF and EBI values. On days 10 and 21, broiler weight did not affect the EPEF values, and on day 42, it had an impact but far less so than the feed conversion values. The standard Cobb 500 feed conversion values were significantly lower on days 10 and 21 than feed conversion values for the control and experimental groups of broilers, which resulted in higher values of EPEF and EBI. ADG (g) did not have a significant effect on the EBI values, since the values of this parameter on day 10 were between 26.30 g and 27.069 g, on day 21 were between 44.10 g and 47.35 g, and on day 42 were between 62.66 and 67.19 g (Table 3). However, on day 42, the feed conversion values for groups O-I and O-II were at the level of that of the Cobb 500 standard, so an impact on the EPEF and EBI values was seen at the end of fattening. The high viability of the broilers can be explained by optimal breeding conditions, primarily by the small number of broilers in the groups and good initial performance of the one-day-old chicks (vitality, uniform weights).

There are numerous data in the literature on the effect of adding CLA to the broiler diets on production results in fattening, and they are often contradictory [20, 21, 22, 23]. However, the use of CLA in non-ruminant diets should not be seen only in terms of production results (final weight, weight gain, conversion) but also from the standpoint of the nutritional value of meat. In non-ruminants, the use of CLA in the diet is important because it provides meat with added value, since the meat contains CLA in an amount that depends on the amount of CLA added to the diet. The animal fattening duration also plays a role. This added value is not only the CLA content but is also the improved n-6/n-3 fatty acid ratio in the meat, as has been seen in pork with CLA addition. The importance of CLA and the n-6/n-3 fatty acid ratio is well known in human nutrition. Meat with added value can be labelled as a functional food, so it would understandably fetch a higher price, which should be reflected in the economic profitability of fattening [24, 25, 26, 27]. Poultry meat enriched with CLA is of special importance given the fact that the total production and consumption of poultry meat in the world have increased to 39% of total meat production and to 15 kg per capita per year, respectively [1]. The use of meat with high CLA content in human nutrition could gradually result in lower mortality from chronic non-communicable diseases and contribute to a lower number of people suffering from these diseases.

5. Conclusion

The results in this paper support the use of CLA in broiler nutrition, since this is economically justified given the good production results (broiler weight at the end of fattening, feed conversion, growth) and the resultant broiler meat has added value and can be defined as a functional food. To calculate the economic justification of fattening, the use of EPEF or EBI values are justified since these indices are based on broilers' production results. These indices can be applied in different conditions, such as comparison of the cost-effectiveness of fattening different broiler hybrids, different feed compositions, different lengths of fattening period, the influence of different climatic conditions on production results, etc.

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Arugula and pre-converted arugula extract as natural Nitrate/Nitrite sources for heat-treated sausages

Arugula and pre-converted arugula extract as natural Nitrate/Nitrite sources for heat-treated sausages

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Abstract. The aim of this study was to investigate using arugula or pre-converted extracts as nitrite alternatives in heat-treated fermented sausages. Sausages with nitrite, 150 mg/kg of $NaNO_2$, 1.2% arugula extract, and 1.5% pre-converted arugula extract were formulated. Natural nitrate sources added resulted in significantly lower oxidation content compared to negative control groups at initial storage. The addition of natural nitrate sources influenced colour, pigments, and conversion rate of sausages. Pre-converted arugula extract showed little effect on the residual nitrite content. The result of colour, oxidation, and nitrite analysis suggest pre-converted arugula is a potential nitrite replacer, but arugula as a nitrate source is limited to provide the functions of nitrite.

1. Introduction

Curing is one of the meat preservation methods that uses as ingredients salt, nitrate, and nitrite [2]. Nitrate/nitrite are utilized to form cured meat pigment, enhance the flavour, and provide antimicrobial and antioxidant effects in meat products. It is well known that nitrite is also responsible for producing carcinogenic N-nitrosamines in certain meat products under some processing conditions [3]. This concern has led researchers to seek natural nitrite alternatives. In recent years, much attention has been paid to products formulated with natural additives [4,5]. An alternative curing method to avoid the direct addition of synthetic nitrite is by utilizing those vegetables that have high nitrate content and starter cultures that possess nitrate reductase activity to convert the nitrate to nitrite (pre-conversion).

Spinach, beets, radishes, celery, lettuce, and cabbage are vegetables with high nitrate content [6]. It is stated that serious health concerns about nitrate and nitrite are mostly associated with high intake doses. However, the use of nitrate and nitrite obtained from natural sources does not have harmful toxicological effects on human health [6,7]. Arugula species (*Eruca* spp. and *Diplotaksis* spp.) are commercially important salad crops grown around the world [8]. The European Food Safety Authority (EFSA) found that the nitrate content in arugula ranged from 4800 to 6400 ppm in various regions and seasons in Europe [8]. Despite the high nitrate content of arugula species, there is no study regarding the effects of using arugula as a nitrite alternative in meat products. Therefore, the objective of this study was to determine the effects of arugula extract or pre-converted arugula extract on some quality parameters, lipid and protein oxidation of heat-treated fermented sausages.

2. Materials and methods

Preparation of pre-converted nitrite sources from arugula extract (PAE)

Ten grams of dried arugula was ground into powder form, mixed with 100 mL of distilled water, then 0.025% of an active nitrate reductase culture containing *Staphylococcus carnosus* (S-B-61 BactofermTM, Chr. Hansen Inc., USA) was added, and the mixture was incubated with shaking at 30°C for 24 h. The mixture was filtered using Whatman No. 1 filter paper and evaporated in a rotary evaporator (Heidolph Hei Vap ValG1, Germany) at < 50°C. The extract was stored in amber flasks in the dark (pre-converted arugula extract: nitrate content = 514.21 ppm, nitrite content = 56.38 ppm, pH = 5.33, L*=14.24±0.47, a*=-0.46±0.03, b*=13.96±0.32).

Preparation of nitrate sources from arugula extract (AE)

Ten grams of dried arugula powder was mixed with 100 mL of distilled water, then the mixture was left 20 minutes at 80°C in a water bath. The solution was filtered from a 0.45 µm filter, and the mixture was evaporated in a rotary evaporator (Heidolph Hei Vap ValG1, Germany) at < 50°C. The extract was stored in amber flasks in the dark. (Arugula extract: nitrate content = 4,689.27 ppm, nitrite content = 23.82 ppm, pH = 5.34, L*=14.24±0.54, a*=-0.47±0.84, b*=13.96±0.24).

Preparation of heat-treated fermented sausages

Fresh boneless beef cuts and beef fat were obtained from a local manufacturer. Production of heat-treated fermented sausages was carried out according to Zungur et al. [9]. Beef was trimmed of visible fat and connective tissue. Four different formulations of heat-treated fermented sausage, 4 kg each, were prepared: C (negative control, nitrite free), CN (positive control, 150 ppm sodium nitrite), PA (1.5% pre-concerted arugula extract), AR (1.2% arugula extract). Fat and meat were separately minced through a plate with 3 mm holes and mixed with spices, curing agents (salt, sodium ascorbate, saccharose, and nitrite or natural nitrite source). Arugula extracts (150 ppm nitrate equivalent and 150 ppm nitrite equivalent) were added to the formulation to replace sodium nitrite. Sausage formulations are given in Table 1.

Table 1. Formulation of heat-treated fermented sausages

Groups	Beef (g)	Fat (g)	Salt (g)	Spice (g)	Sugar (g)	Ascorbate (g)	$NaNO_2$ (g)	AE (g)	PAE (g)
C	4000	800	96	180	19.2	1.92	0	0	0
CN	4000	800	96	180	19.2	1.92	0.76	0	0
PA	4000	800	96	180	19.2	1.92	0	0	75.51
AR	4000	800	96	180	19.2	1.92	0	60.64	0

C (negative control, nitrite free), CN (positive control, 150 ppm sodium nitrite), PA (1.5% pre-concerted arugula extract), AR (1.2% arugula extract).

The nitrate/nitrite amounts in arugula and pre-converted arugula extracts were calculated to be equivalent to 150 ppm nitrate and 150 ppm nitrite, respectively.

Physicochemical parameters

Total moisture and ash analysis were performed according to AOAC [10], fat content determined according to Flynn and Bramblet [11], protein content was determined by using an automatic nitrogen analyser (FP 528 LE-CO, USA) based on the Dumas method. pH was measured three times by using a pH-meter (WTW pH 330i/SET, Germany) equipped with a penetration probe. The colour of the samples was measured in triplicate using a portable colorimeter (CR-200, Konica Minolta, Japan) with D65 illuminant setting and 10^{o} standard observer and expressed as CIE L* (lightness), a* (redness) and b* (yellowness).

Lipid and protein oxidation analyses
Oxidative stability of sausages was analysed by determining peroxide value (PV) [12] and 2-Thiobarbituric acid reactive substances (TBARS) [12]. Protein carbonyls were measured by estimation of total carbonyl groups according to Levine et al. [13] with some modifications as described by Srinivasan et al. [14]. Sulfhydryl groups (thiol content) were determined according to Srinivasan et al. [14].

Residual Nitrite content
Residual nitrite content of heat-treated sausages determined by using AOAC method [10].

Nitrosomyoglobin and total pigments contents
The content of nitrosomyoglobin and total pigments were identified with the method described by Horsey [15]. Conversion ratio was calculated from the nitrosomyoglobin concentration divided by the total pigment concentration.

Statistical analyses
The data grouped by treatment were analysed by one-way ANOVA using the SPSS software version 11 (SPSS, 2001). A significance level of $p < 0.05$ was used for all evaluations.

3. Results and discussion

Chemical composition and pH values of sausages grouped by treatment are given in Table 2. The moisture content of all treatments was lower than 50% which is a limit set by the Turkish Food Codex [16]. The highest moisture content was found in PA and AR treatments due to the addition of extracts in liquid form. Similarly, Ozaki et al. [17] reported an increase in moisture content when radish powder and chitosan were added to the formulation as nitrite alternatives. Fat contents of the sausages were between 18.52%-24.45%. The highest fat content was found in PA treatment ($P<0.05$). The highest protein content was recorded in CN and AR treatments ($P<0.05$). Addition of chard powder [18] and beetroot flours [6] to fermented sausage formulation as nitrite alternatives did not change the chemical composition of the sausages. In our study, the pH was between 5.56-5.58 and significant differences were recorded among the pH of the sausages. pH 5.6 is the maximum pH value allowed for heat-treated fermented sausages in Turkey [16]. Similarly, cooked pork loins treated with nitrite, spinach juice, and fermented spinach juice had similar pH values [4]. Even though some researchers reported addition of acidic or basic additives to product formulation for nitrite replacement [18,19], in our study, arugula extract did not affect pH.

Table 2. Physicochemical parameters of heat-treated fermented sausages

	Samples	C	CN	PA	AR
Physicochemical parameters	**Moisture (%)**	47.71^{b}±0.92	47.43^{b}±0.54	49.53^{a}±0.36	49.44^{a}±0.26
	Fat (%)	21.09^{bc}±1.75	21.95^{ab}±1.77	24.45^{a}±1.25	18.52^{c}±1.01
	Protein (%)	23.76^{b}±1.22	26.30^{a}±0.69	22.44^{b}±0.09	27.42^{a}±0.45
	Ash (%)	4.11^{a}±0.15	3.71^{ab}±0.33	3.66^{b}±0.14	3.76^{ab}±0.21
	pH	5.58±0.01	5.56±0.01	5.56±0.01	5.56±0.01

All values are mean ± standard deviation of three replicates (n = 3).
[a-b] Means within a row with different letters are significantly different ($P<0.05$).
C (negative control, nitrite free), CN (positive control, 150 ppm sodium nitrite), PA (1.5% pre-concerted arugula extract), AR (1.2% arugula extract).

The L* and a* values of internal surfaces were not affected by the treatments (Table 3), and regarding b*, it was found that C sausage, with no added nitrite or extract, was yellower than other sausages ($P<0.05$). The use of fermented celery and beet extracts did not affect L* values; however, the additives resulted in increasing effect on b* values [20]. In our study, the lowest external L* value was found in

CN treatment (P<0.05). External L*, a*, and b* values recorded were between 33.10-37.79, 10.27-13.74 and 10.81-14.95 respectively. The results showed that significant differences were obtained in colour features depending on formulations (P<0.05). The positive control formulated with nitrite had a more intense red colour as expected (P<0.05). The most visible change was seen in L* values, where PA and AR treatments were lighter compared to C and CN treatments. L* values did not change with the addition of 2% celery juice powder whilst a* values were higher in Bologna sausages formulated with celery juice [19]. The reason for lower a* values in our PA and AR treatments is probably due to the natural behaviour of extracts in giving a darker colour when mixed with water.

Nitrosomyoglobin, total pigment concentrations, and pigment conversion rates were lower in treatments without nitrite (CN; negative control) than other treatments, as expected. The pre-conversion process had a significant effect on nitrosomyoglobin formation, and similar results have been reported by other researchers [18]. Total pigment concentrations were between 109.63-155.93 ppm. The bio-conversion process was found to be effective with regard to total pigment content, (P <0.05), as PA treatment had a greater amount of total pigment compared to other treatments (P <0.05). The conversion rate of fermented sausages was between 22.64-67.14%. CN had the highest conversion rate, followed by PA (P<0.05). The highest total pigment amount was found in the PA sausages. This result supports the idea that natural nitrate sources as nitrite alternatives would be more effective in curing if the nitrate is converted to nitrite. Sausages without sodium nitrite showed the lowest conversion rate (P<0.05). The curing efficiency of pork patties ranged between 9.16-82.66% and the highest efficiency was found in patties formulated with 60 ppm nitrite + 1 % pre-converted nitrite from Swiss chard powder, and addition of pre-converted nitrite from Swiss chard powder increased the total pigment concentration in pork patties [21].

Residual nitrite content of our heat-treated fermented sausages was between 8.45-22.59 ppm, and sausages formulated with 150 ppm sodium nitrite had the highest residual nitrite, as expected (p<0.05). Residual nitrite contents of the sausages treated with extracts were the same as the negative control. This result indicates that residual nitrite content was likely influenced by the presence of pre-converted nitrite. Kim et al. [22] reported that residual nitrite levels of pork sausage was affected by the presence of pre-converted nitrite in spinach extract. Similar to our results incorporation of celery, parsley, red beet, and spinach powders into cooked sausages lowered the residual nitrite levels [18], and Sindelar et al. [23] showed that the residual nitrite content of meat products treated with vegetable juice powder was higher when a greater amount of pre-converted nitrite was added to meat products.

Table 3. Colour parameters (L*, a*, b*), nitrosomyoglobin, total pigment, residual nitrite concentrations and conversion rate

		Samples	C	CN	PA	AR
Color parameters	Internal surface	L*	49.23±0.63	49.65±1.19	48.37±1.15	49.21±0.95
		a*	10.40±0.35	10.98±0.66	11.34±0.83	11.38±0.66
		b*	10.88±0.36^{b}	12.23±0.75^{a}	12.36±0.39^{a}	12.81±0.75^{a}
	External surface	L*	34.52±0.90^{b}	33.10±0.63^{c}	37.79±1.15^{a}	37.30±0.10^{a}
		a*	11.76±0.77^{b}	13.74±0.54^{a}	10.27±0.48^{c}	10.48±0.06^{c}
		b*	14.95±1.11^{a}	10.81±0.47^{c}	13.25±0.61^{b}	13.37±0.72^{b}
	Nitrosomyoglobin (ppm)		24.77±1.30^{d}	102.64±2.48^{a}	96.77±1.34^{b}	42.76±1.86^{c}
	Total pigment (ppm)		109.36±0.98^{d}	152.89±0.15^{b}	155.93±0.77^{a}	124.14±1.14^{c}
	Conversion rate (%)		22.65±1.35^{d}	67.14±1.68^{a}	62.06±1.15^{b}	34.44±1.51^{c}
	Residual nitrite (ppm)		8.45±0.13^{b}	22.59±1.09^{a}	8.78±0.54^{b}	9.50±1.15^{b}

All values are mean ± standard deviation of three replicates (n = 3).
[a-d] Means within a row with different letters are significantly different (P<0.05).
C (negative control, nitrite free), CN (positive control, 150 ppm sodium nitrite), PA (1.5% pre-concerted arugula extract), AR (1.2% arugula extract).

PVs and TBARS values are shown in Table 4. The PV of treatments ranged between 5.63 and 20.64 meq0_2/kg. Initially, C and AR sausages had the highest PVs (P<0.05), whilst CN and PA had similar

PVs. PVs decreased in the second month then remained stable. CN and PA had similar PVs at the end of the storage. It could be said that primary oxidation was more pronounced in C treatment, which was formulated without nitrite.

The incorporation of nitrite effectively retarded TBARS values. Lipid oxidation in AR and C sausages was more intense compared to other treatments. Nevertheless, CN had the lowest TBARS value followed by PA ($P<0.05$). This result indicates the potential of PAE to replace nitrite up to some point. A study carried out by Ozaki et al. [17] reported that the use of beet and radish powders as natural nitrite sources did not affect the TBARS values on days 45 and 60 of storage. On the other hand, Kim et al. [4] stated that replacing nitrite with pre-converted spinach juices lowered lipid oxidation.

The total amount of carbonyl and sulfhydryl is accepted as one of the indicators of protein oxidation [24]. Carbonyl and sulfhydryl contents are shown in Table 4. Protein oxidation of PA and AR, which contained added with natural nitrite sources, did not differ from each other ($P>0.05$). The treatment with pre-converted nitrite source had higher sulfhydryl content compared to C ($P<0.05$). During the storage, pre-converted arugula extract was more effective in inhibiting protein oxidation than arugula extract. Sulfhydryl content of all treatments decreased throughout the storage; however, the addition of arugula extract or pre-converted arugula extract was effective against sulfhydryl loss. Similar to our result, Martínez-Zamora et al. [24] indicated that natural nitrate sources such as chard+beet with rosemary or citrus extract protected the chorizo against thiol loss. Initial carbonyl contents of our sausages were similar. At the end of the storage, the carbonyl contents of the sausages ranged between 5.07-5.67 nmol/mg. The addition of natural nitrite sources was found to be effective in maintaining carbonyl contents. It has been observed that nitrite and nitrite alternatives added to the formulation have a significant effect on lipid oxidation, resulting in a lower carbonyl content amount, thus providing a slower progression of protein oxidation in these examples.

Table 4. Oxidation parameters of heat-treated fermented sausages

		Samples	C	CN	PA	AR
Oxidation parameters	**Peroxide values (meq02/kg)**	**0th day**	$18.62^{a.y} \pm 0.41$	$13.71^{b.y} \pm 1.17$	$14.78^{b.x} \pm 1.03$	$17.53^{a.y} \pm 0.98$
		1st months	$24.53^{a.x} \pm 1.15$	$19.86^{b.x} \pm 1.03$	$16.72^{c.x} \pm 0.47$	$20.64^{b.x} \pm 1.65$
		2st months	$8.05^{a.z} \pm 0.49$	$3.72^{b.z} \pm 1.06$	$6.63^{a.y} \pm 1.29$	$7.24^{a.z} \pm 0.09$
		3st months	$8.83^{a.z} \pm 0.61$	$3.40^{c.z} \pm 1.85$	$5.63^{bc.y} \pm 1.15$	$7.77^{ab.z} \pm 0.37$
	TBARS values (mg MA/kg)	**0th day**	$1.01^{b.t} \pm 0.01$	$0.54^{d.y} \pm 0.10$	$0.75^{c.y} \pm 0.04$	$1.70^{a.x} \pm 0.02$
		1st months	$1.35^{b.y} \pm 0.02$	$1.07^{c.x} \pm 0.06$	$1.13^{c.x} \pm 0.01$	$1.67^{a.x} \pm 0.02$
		2st months	$1.38^{a.x} \pm 0.01$	$1.20^{b.x} \pm 0.10$	$0.81^{c.y} \pm 0.01$	$1.36^{a.y} \pm 0.04$
		3st months	$1.23^{b.z} \pm 0.03$	$0.67^{d.y} \pm 0.01$	$1.10^{c.x} \pm 0.10$	$1.37^{a.y} \pm 0.06$
	Sulfhydryl (nmol SH/mg)	**0th day**	$36.70^{c.x} \pm 0.70$	$43.86^{a.x} \pm 1.08$	$38.79^{b.x} \pm 0.91$	$38.67^{b.x} \pm 0.68$
		1st months	$35.13^{c.y} \pm 0.23$	$41.60^{a.y} \pm 1.38$	$39.05^{b.x} \pm 0.59$	$35.60^{c.y} \pm 0.52$
		2st months	$32.18^{c.z} \pm 0.95$	$40.02^{a.y} \pm 1.10$	$38.32^{a.xy} \pm 0.93$	$35.24^{b.y} \pm 0.86$
		3st months	$30.53^{c.t} \pm 0.28$	$34.80^{b.z} \pm 0.61$	$37.16^{a.y} \pm 0.70$	$35.72^{b.y} \pm 0.28$
	Carbonyl (nmol/mg protein)	**0th day**	$5.18^{a.y} \pm 0.22$	$4.31^{b.y} \pm 0.29$	$4.74^{ab.z} \pm 0.25$	$4.87^{ab.y} \pm 0.64$
		1st months	$5.44^{a.x} \pm 0.02$	$4.51^{d.y} \pm 0.08$	$5.04^{c.y} \pm 0.06$	$5.27^{b.xy} \pm 0.02$
		2st months	$5.52^{a.x} \pm 0.02$	$4.57^{d.y} \pm 0.02$	$5.24^{c.xy} \pm 0.04$	$5.37^{b.xy} \pm 0.03$
		3st months	$5.62^{a.x} \pm 0.02$	$5.07^{d.x} \pm 0.05$	$5.42^{c.x} \pm 0.02$	$5.54^{b.x} \pm 0.02$

All values are mean ± standard deviation of three replicates (n = 3).
[a-d] Means within a row with different letters are significantly different ($P<0.05$).
[x-y] Means within a column with different letters are significantly different ($P<0.05$).
C (negative control, nitrite free), CN (positive control, 150 ppm sodium nitrite), PA (1.5% pre-concerted arugula extract), AR (1.2% arugula extract).

4. Conclusion

In this study, it was shown that replacing nitrite with arugula extract or pre-converted arugula extract resulted in lower redness and nitrosomyoglobin and residual nitrite levels. At the same time, adding pre-converted arugula extract resulted in sausages that had similar quality parameters and oxidation levels

to sausages treated with nitrite. Therefore, arugula extract has promising potential for use in heat-treated sausages as a nitrite source.

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Protective effects of honeybee products against COVID-19: a review

Protective effects of honeybee products against COVID-19: a review

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Abstract. According to the World Health Organization, coronavirus disease (COVID-19-global pandemic) is defined as highly contagious viral infection that causes severe acute respiratory syndrome-coronavirus-2 (SARS-CoV2). This disease is very rapidly transmitted among humans. To date, 30 May 2021, at least 170,689,920 confirmed cases of COVID-19 have been reported while global deaths reached 3,550,093 (https://www.worldometers.info/coronavirus/). In Serbia (to date 30 May 2021) at least 712,046 confirmed cases of COVID-19 have been reported, while deaths reached 6,844 and recovered 694,492. Also, Serbia is at the top of the list of country vaccination rates against COVID-19. New concerns regarding vaccine safety and efficacy are coming with the mutated strains of SARS-CoV-2 virus. Natural products of known antiviral activity can be used for various therapeutic purposes. Honey, propolis, pollen, bee bread, bee venom and royal jelly might potentiate the immune system of patients and reduce their susceptibility to COVID-19 infection.

1. Introduction

In December 2019, COVID-19 infection firstly manifested in Wuhan, China, where a number of patients shared similar symptoms to respiratory syndrome [1-4]. According to Kandeel and Al-Nazawi [5] and Harrison [6], scientists started to massively investigate drugs and vaccines for COVID-19 treatment.

Recent years have seen wide application of apitherapy and bee products in medicine. Many studies have investigated health benefits (Figure 1) of different bee products, especially bee bread and pollen, as a means to increase the chemical and nutritional value of food or create functional food [7]. Also, many studies investigated honeys as food, in terms of contaminants, chemical composition and risk for consumer health [8-11]. In addition, the benefits of honey bee products to the immune system are remarkable (induction of antibody production, maturation of immune cells, and stimulation of the innate and adaptive immune responses [12]. Honey might have beneficial effects as an antioxidant, antiviral, antimicrobial, anti-inflammatory, antidiabetic, and cardiovascular and nervous system protective agent (Table 1) [13]. Similar to honey, propolis (*bee glue*) has high antiseptic, antibacterial, anticancer, antioxidant and immunomodulatory effects [13]. The major compounds for these effects are caffeic acid phenethyl ester and artepillin C.

Figure 1. Schematic presentation of the main effects of bee products

Pollen and bee bread are produced by flowering plants and collected by bees. This food is composed of protein (5%-60%), essential amino acids, reducing sugars, lipids, vitamins (provitamin A [β-carotene], vitamin, flavonoids and organic carotenoid pigments [7,14]. Bee venom is composed of several active components, including phospholipase A2 and hyaluronidase, peptides, amino acids, phospholipids, biogenic amines, volatile compounds and pheromones [15,16]. Nowadays, bee venom is use in apitherapy worldwide [13,17,18]. Royal jelly is an acid colloid composed of water sugar, proteins, lipids and low amounts of vitamins [16,19]. Šver et al. [20] showed the high therapeutic and prophylactic potential of royal jelly and its components against human respiratory syndromes, similar to SARS-CoV-2.

Table 1. Antimicrobial activity of honeybee products

Honeybee products	Effect	References
Honey	Antioxidant, chemopreventive, antiproliferative	[13,21,22]
Propolis	Antifungal, antitumor, antimicrobial	[21,22,23]
Bee venom	Antiviral, anti-inflammatory, nociceptive, antimicrobial, antitumoral	[13,16]
Royal jelly	Antiviral, immunomodulatory	[21]
Bee bread	Antibacterial, antiviral	[16,24,25,26,27]

In this review we present current understanding of honey bee products (honey, propolis, pollen, bee bread, bee venom and royal jelly) as antiviral defence mechanisms against COVID-19 infection.

2. Potential protective role of honey

Honey is composed of several bioactive chemicals, phenols, polyphenols, vitamins, bioflavonoids and sugars [28, 29, 30]. This honeybee product is used in traditional medicine to treat viral respiratory diseases [30]. Many studies showed different types of honey (manuka, acacia, multi-floral and mono-floral honey) inhibited viral replication [31]. According to [32], honey antiviral activity is explained by

hydrogen peroxide and the peptide, defensin-1. Similarly, flavonoids from honey have some antiviral activity [33, 34]. Hashem [35] showed the potential action of honey against SARS-CoV-2. According to Lima et al. [16], clinical trials and preclinical studies will indicate the benefit of honey in the therapy of COVID-19.

According to Al Naggar et al. [13], the physical properties of honey might help to disinfect COVID-19 before passing to the lungs. Beside protective effects on lungs, honey promotes health by supporting the growth of positive intestinal microflora [36, 37]. These effects are explained to its low pH [38], high content of prebiotics [39, 40] and major species of beneficial lactic acid bacteria (*Bifidobacterium*, *Fructobacillus* and *Lactobacillaceae*) [41,42].

3. Potential protective role of propolis

According to Berretta [43] propolis has been widely studied and is already extensively consumed (as sprays or extracts) in many countries. Also, propolis, with high antibacterial, antifungal, antiviral, and immunomodulatory activities, is used in veterinary medicine [44]. Propolis is composed of several bioactive chemicals: limonin, quercetin, kaempferol, myricetin, caffeic acid phenethyl ester, hesperetin and pinocembrin [43].

Effect of propolis against SARS-CoV-2 infection [43]:

- Inhibitory potential with high binding energy to viral components from -9 to -7.1 kcal/mol (*in silico*)
- Blocks the 3a channel that is encoded by ORF 3a of SARS-CoV (*in vitro*)
- Inhibitory potential with high binding energy to ACE2 (*in silico*)
- Inhibitory potential with high binding energy to ACE2 (*in silico*)
- Inhibits PAK-1 directly or up-stream, blocking coronavirus infection
- Regulates IFN-γ, IL-6, and IL-10 cytokines in an experimental asthma model

4. Potential protective role of pollen and bee bread

Many of the bioactive compounds (polyphenols) present in pollen and bee bread have promising activity against SARS-CoV [26,27]. Beside polyphenols, flavonoids and isoflavones have high antiviral activity. According to Yi et al. [27] quercetin inhibited the entry of SARS-CoV into cells (*in vitro*).

5. Potential protective role of bee venom

Bee venom is well known to have antimicrobial activity [45]. The potential protective role of bee venom has been studied [46]. Yang et al., [46] showed that bee venom might potentiate the immune system and reduce the susceptibility to SARS-CoV infection. Also, bee venom induced immunity through substantial upregulation of Th1 cytokines (IFN-γ and IL-12) and several forms of immune cells, which could be very significant for SARS-CoV-2 related pneumonia [16].

6. Conclusion

Honeybee products (honey, propolis, pollen, bee bread, bee venom and royal jelly) are well known for their several medicinal and nutritional values, which have been long explored for different therapeutic effects. Honeybee products have antiviral effects and the ability to stimulate the immune system, so could be used in the therapy of severe viral respiratory infections, such as COVID-19. Also, many studies suggest potential antiviral effects of honeybee products by direct effects of different bioactive components (peroxide, flavonoids and phenolics). The immunomodulatory and antiviral effects of different honeybee products can be useful in preventive treatment, and even symptomatic treatment, of COVID-19.

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Biofilms in the food industry – impact on human health

Biofilms in the food industry – impact on human health

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Abstract. Biofilms are complex microbial communities formed by one and more species embedded in an extracellular polymeric matrix of different compositions depending on the attached microbial species and the type of food manufacturing. Attachment of bacteria to food contact surfaces and the subsequent formation of biofilms can cause equipment damage, food spoilage and even human diseases. Foodborne diseases associated with biofilms in the food industry can be intoxications or infections and can have great impact on human health. Foodborne pathogens that express capacity for biofilm formation under different conditions in the food industry, and that are in the scope of our investigations, are *Salmonella* (which, on contaminating a food pipeline biofilm, could induce massive outbreaks and even death in children and elderly) and *Listeria monocytogenes* (a ubiquitous bacterium that can cause abortion in pregnant women and other serious complications in children and the elderly).

The associations of bacteria we nowadays refer to as biofilm were discovered several centuries before we recognised the significance of biofilm in the pathogenesis of infectious diseases and biofilms' importance in the food industry and other fields. Results from numerous studies on the importance of biofilm led to the realisation that this form of bacterial existence predominates in nature and that practically all bacteria possess the ability to form biofilm [1]. The fact that life in biofilm is the preferred form of bacterial existence in nature clearly indicates that this form of bacterial community is widespread in our surroundings and thereby has an impact of all aspects of human life [2].

Biofilm was observed for the first time by Antoine Von Leeuwenhoek in the 17th century as he studied the types of creatures on his own teeth. Later, at the beginning of 20th century, Claude Zobell and his co-workers stated that more than 99% of microbes in aquatic systems live inside biofilms. However, the most important figure for the discovery of biofilm was William Costerton, who defined biofilm for the first time and introduced new terms related to biofilm into microbiology language [1-4].

Biofilm is defined as a complex community of microorganisms [single or several species] that are irreversibly attached to the surface and to each other, and embedded into extracellular polymeric substances [EPS], i.e., matrix, that they produce [1]. Biofilm formation is a complex process and, according to different researchers, it occurs in several common steps: initial contact and attachment to the surface, followed by microcolony formation, biofilm maturation, and finally detachment/dispersion of the biofilm [2,3].

Biofilm formation commences with bacteria adhering to the surface of animate or inanimate materials and is a complex process influenced by numerous factors – characteristics of the material and the surroundings of the material surfaces on which bacteria adhere, hydrodynamics, i.e., the speed of fluid movement and characteristics of the bacterium cell surface [3]. In the first stage of biofilm

formation, initial contact and microbial attachment to the surface occur, individual bacterial cells physically adhere to the surface of animate and inanimate materials (via van der Waals forces, spatial interactions or electrostatic forces) or by means of specialised appendages, cilia and flagella. When forces of attraction between reversibly adhered bacteria and the surface overcome repulsive forces, the irreversible adherence stage, when bacteria adhere firmly to the surface, begins. The connection between bacteria and the surface they adhere to strengthens [4].

After attachment of microorganisms to the surface occurs and becomes stable, a process of bacterial multiplication starts. This process then leads to the formation of microcolonies. Colonies in a biofilm usually consist of various types of micro-communities that coordinate with one another in multiple aspects. This coordination plays a crucial role in substrate exchange, distribution of important metabolic products and excretion of metabolic end-products. Additionally, at this stage, bacteria actively produce EPS, which comprises around 90% of the biofilm. The content and the structure of polysaccharides, the chief components of EPS, determine biofilm conformation, and the large quantity of water in EPS prevents it from drying. Furthermore, EPS contributes to antibiotic and disinfectant resistance of biofilm, because it mechanically prevents active compounds from entering and being transported through biofilm [3-4].

In the biofilm maturation phase, the number of microcolonies increases, and the thickness of the entire structure reaches approximately 100 μm. Microcolonies often consist of bacteria with differing metabolic needs, and they function in a complex coordination. Moreover, due to the higher concentration of O_2, biofilm surface primarily hosts aerobic bacteria, whereas anaerobes usually populate the interior. Spatial proximity enables bacteria to exchange metabolites and eliminate toxic metabolites from the population, and each bacterium performs a precisely defined task that contributes to the wellbeing of the entire community. However, because of their spatial orientation, bacteria in the deepest structures of biofilm receive less water, food and O_2 than bacteria at the surface, and the elimination of metabolites and CO_2 from these areas becomes mostly unsatisfactory. Then, bacteria inside the biofilm shift to the dormant state, performing a minimum of metabolic activities. To summarize, the maturation phase includes biofilm adaptation to surrounding conditions in terms of structure modification, physiology and metabolism of the entire bacterial population that form the biofilm [3]. As in previous biofilm formation stages, the biofilm maturation phase is strictly controlled at the entire bacterial population level by the quorum sensing system. Quorum sensing is the ability to detect and respond to bacteria population density by gene regulation. Many microorganisms use quorum sensing to coordinate gene expression according to the density of their local population [4].

The final stage is characterised by dispersion, i.e., detachment of bacteria from biofilm and colonisation of new surfaces. Biofilm dispersion involves detachment of daughter cells during bacterial division on the biofilm surface, detachment of smaller bacterial clusters instigated by water movements in the surroundings and detachment of bacteria from biofilm due to the lack of nutritive materials. Microcolony structure changes over time, from entirely compact to a very rudimentary cohesion of individual bacterial cells, which obtain unanimous approval from the entire population to move, leave the biofilm and colonise a new surface. In contrast, dormant bacteria remain inside biofilm, maintain its structure and regenerate the biofilm over time [5].

Bacteria in biofilm exhibit different phenotypic characteristics than the non-biofilm bacteria outside. Biofilm bacteria, compared with free-living bacteria, are more resistant to external factors, drying, UV radiation, and changes in pH and temperature, but they are also less sensitive to disinfectants, antibiotics and mechanisms of innate and acquired immunity from individual microbial cells. Bacterial resistance to antibiotics in biofilm can be explained in several ways. One of the explanations states that slow and incomplete penetration of antibiotics through EPS that functions as a mechanical barrier also leads to neutralisation and dilution of some antibiotics. According to numerous authors, biofilm antibiotic resistance is also determined by a lower rate of bacterial growth in biofilm, because antibiotic efficacy largely depends on a certain degree of bacterial cell activity. Finally, horizontal transfer of the resistant gene occurs much more frequently in biofilm than in individual bacterial cells, because of the spatial proximity of biofilm bacteria [3,4]. Additionally, it has generally been accepted that the biofilm structure

and mode of growth induce its resistance to disinfection that can lead to significant health and economic concerns. Mechanisms of biofilm resistance to disinfectants are similar to those responsible for antibiotic resistance [3-5].

Modern food processing lines are applicable environments for biofilm production on food contact surfaces [6]. Many foodborne bacteria can adhere to the contact surfaces present in these areas, which could contribute to an increase in the risk of foodborne infectious diseases [7]. Thus, it does not come as a surprise that 80% of bacterial infections in the USA are believed to be related specifically to foodborne pathogens in biofilms [6].

In the food industry, after bacteria adhere to the surfaces of food matrixes or factory equipment, biofilms start to develop. During the washing process, biofilms resist disinfection and sanitisation, and this allows bacteria to spread across the product. Some bacteria can spoil the products readily, and contaminated products could pose a health risk to consumers. Foodborne diseases associated with biofilms in the food industry can be intoxications or infections. Bacterial exotoxins can be secreted by some bacteria in biofilms found within food processing plants. Subsequently contaminated food can cause human intoxication, so biofilms in food factories pose a risk to human health [6,7].

Salmonella are one of the most important foodborne pathogens. More than 95% of cases of infections caused by these bacteria are foodborne, and these infections account for about 30% of deaths resulting from foodborne illnesses [8]. *Salmonella* are capable of easily attaching to meat (poultry meat is a common reservoir in processed food) and other food matrixes, and they can subsequently form biofilm [8,9]. This will eventually lead to cross-contamination between food batches in a manufacturing plant, a fact that further underscores the serious health concern this bacterium poses with respect to risk of an outbreak. These bacteria are capable of adhering and forming biofilms on different surfaces [8]. In our previous investigation, we studied the influence of different environmental conditions on biofilm formation by *Salmonella in vitro* [9]. The highest quantity of biofilm was formed at 30°C after 24 h of incubation and at 22°C after 48 h of incubation. The multicellular behaviour in *Salmonella*, associated with biofilm formation, is regulated by environmental conditions that target the *agfD* promoter. Expression of this promoter leads to the production of polymers, thin aggregative fimbriae and cellulose, which form EPS and induce biofilm formation. It was shown that production of the thin aggregative fimbriae in *S.* Typhimurium took place in a temperature-dependent manner, at 28°C but not at 37°C, and this could explain the highest amount of biofilm formed at 30°C after 24 h of incubation [9]. On the other hand, in the stationary phase of growth, nitrogen and phosphate depletion were found to induce the *agfD* promoter and favour the multi-cellular state. This could partially explain more biofilm being produced at room temperature after 48 than after 24 h of incubation [9,10].

In the food industry, the type of atmosphere might not be known, and there are circumstances in which an anaerobic atmosphere or variable concentrations of O_2 or CO_2 occur. Moreover, an oxygen gradient is present in biofilms. Our results on *Salmonella* biofilm forming capacity suggest that microaerophilic and CO_2-rich conditions provide the best environment for biofilm formation, while the least biofilm was formed under anaerobic conditions [9]. High biofilm production under microaerophilic and CO_2-rich conditions correlates with the observations of other authors, who found that the expression of *agfD* in a microaerophilic atmosphere was maximal compared with aerobic and anaerobic conditions [9].

Another foodborne pathogen in the scope of our interest was *Listeria monocytogenes*. Food products known to transmit this pathogen are seafood, dairy products, meat, processed food, fruits, soft cheeses, ice cream, unpasteurised milk, candied apples, frozen vegetables, and poultry [11,12]. This pathogen causes gastroenteritis in healthy individuals. However, in pregnant women, infants, the elderly and immunocompromised individuals, this bacterium causes listeriosis, a critical disease which also involves septicaemia, meningitis and meningoencephalitis. In pregnant women, listeriosis can lead to miscarriage or damage to the foetus [12]. *L. monocytogenes* biofilms can form on polypropylene, steel, rubber or glass surfaces throughout the food industry. From there, this pathogen spreads to food batches, where it can replicate at refrigeration temperatures. Together with this low temperature replication ability, this bacterium induces biofilm status as a response to cold temperatures, increasing its attachment to surfaces

and its resistance to cleaning procedures in many food factories [11,12]. Evaluation of biofilm formation by *L. monocytogenes* in our study revealed that this bacterium possesses a high capacity for biofilm formation. In spite of that, biofilm-forming capacity depends on the availability of nutrients in the environment, and *L. monocytogenes* prefers nutrient-rich media for biofilm production [13].

Food industry biofilms constitute a serious economic and health issue. These biofilms can contain microorganisms that are pathogenic in healthy individuals or that target immunocompromised people and cause life-threatening infections.

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Coccidiostats in table eggs, liver and poultry meat on the market in Bosnia and Herzegovina

Coccidiostats in table eggs, liver and poultry meat on the market in Bosnia and Herzegovina

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Abstract. Poultry meat production is one of the most dynamic sectors in agriculture, recording the quickest growth in the food industry, while egg production has shown strong growth in the last twenty years. Combined with meat production, it is achieving the highest growth when it comes to meeting protein needs for the global population. In economic terms, coccidiosis is one of the most significant poultry diseases. Effective application of coccidiostats in poultry feed has been playing a key role in development of commercial poultry production for more than 50 years. The aim of this research was to estimate occurrence and residue concentrations of coccidiostats in table eggs, poultry liver and meat, available on the market in Bosnia and Herzegovina (B&H). Residues of lasalocid were found in table eggs, while residues of nicarbazin, maduramicin and diclazuril were detected in broiler meat and liver.

1. Introduction

Large bird agglomerates in poultry facilities are at risk of fast, massive spread of various infectious diseases. From an economic perspective, coccidiosis is one of the most significant invasive diseases in intensive poultry farming, especially in young animals [1].

Coccidiosis is a parasitic disease of various food production animal species, and is one of the most frequent health problems in intensive poultry farming [2,3]. The prevalence of coccidiosis in commercial poultry flocks is directly proportionate to production intensity, which is characterized by high animal density per unit, i.e., population density, in commercial production facilities [3]. Dense populations, along with other stress factors, help the occurrence and spread of the disease in flocks [4].

The prevention of coccidiosis is based on fundamental factors: zoohygienic measures, genetics, vaccination and administration of coccidiostats [5]. Among them, prudential and effective use of coccidiostats in poultry is essential for modern commercial poultry production. Coccidiostats interfere

in various stages of intestinal development of *Eimeria*, and are the only veterinary drugs allowed as additives in animal feed to prevent and treat poultry coccidiosis [1,6].

This paper reports the results of a study conducted on products at retail in B&H to estimate the occurrence and residue concentrations of coccidiostats in table eggs, poultry liver and meat.

2. Materials and methods

Ninety samples of table eggs of domestic origin were collected to study the presence of coccidiostat residues. One representative sample was made up of twelve table eggs. Sampling of broiler meat and liver on B&H's retail market was conducted in three groups. The first and second groups (Group A and Group B) included samples from two different local poultry producers that had been distributing their products to the whole B&H market. The third group (Group C) included samples from an importer, whose products were also available on the entire market in B&H. Random sampling was conducted between April 2017 and April 2018 in large retailers across B&H.

Screening identification of residues of diclazuril (DICL), lasalocid (LAS), maduramicin (MAD), monensin (MON), narasin (NAR), nicarbazin (DNC), robenidine (ROBN), salinomycin (SAL) and toltrazuril sulfone (TOLT) in samples of table eggs, broiler meat and liver was conducted using liquid chromatography with triple quadrupole mass spectrometry (LC-MS/MS) with a Waters ACQUITY detector connected to TQD mass spectrometer (Waters, Milford, MA, USA), controlled by MassLynks software version 4.1 in the Laboratory for Residues and Food Quality Testing of the Veterinary Faculty, University of Sarajevo, B&H. Confirmation testing was conducted at the Institute of Meat Hygiene and Technology of Serbia, using a Shimadzu LCMS-8040 Triple Quadrupole Liquid Chromatograph Mass Spectrometer (Shimadzu, Japan), operated in positive ion mode, controlled by LabSolution software.

Statistical data analysis of grouped data was conducted using the IBM SPSS Statistics v. 17 software (IBM Inc., USA). Fisher's exact test and chi-square test ($\chi 2$) were used to test the statistical significance of difference in frequency of coccidiostat-positive samples that contained residues of DNC, MAD, LAS and/or DICL among three types of tested matrices (eggs, broiler meat and liver), as well as to test statistical significance of difference in frequency of samples positive for residues of MAD, DNC and/or DICL, given the difference in sample origin (local and imported). P-values less than 0.05 were considered statistically significant.

3. Results and discussion

Table 1 shows the results of screening tests for presence of residues of the nine coccidiostats by using the LC-MS/MS technique.

Table 1. Occurrence and frequency (%) of coccidiostat residues in table eggs, broiler meat and liver that screened positive[a)].

		Group A[b)]		Group B[c)]	
	Table eggs (n=90)	Broiler meat (n=30)	Broiler liver (n=30)	Broiler meat (n=30)	Broiler liver (n=30)
Lasalocid	10 (11.1%)	-	-	-	-
Nicarbazin	-	4 (13.3%)	12 (40.0%)	2 (6.6%)	4 (13.3%)
Diclazuril	-	-	-	-	2 (6.6%)

[a)] concentration of coccidiostats > CCß (5 µg/kg)

[b)] samples from local producer A

[c)] samples from local producer B

Note: Residues of maduramicin, narasin, monensin, salinomycin, toltrazuril sulfone and robenidine were not detected in any of the tested samples. None of the nine tested coccidiostats were detected in the imported broiler meat and liver (Group C).

Confirmation testing included 36 samples, out of which 10 samples of table eggs were positive for LAS residues, 6 samples of muscle tissue and 16 samples of liver for DNC residues, and 2 liver samples for

DICL and MAD residues. LAS residues were confirmed in all 10 samples of table eggs (m/z: 613.2→359.3 and 613.2→377.3), with concentrations ranging from 7 µg/kg to 33 µg/kg (mean value of 20.8 µg/kg), while DNC residues (m/z: 301→107 and 301→137) were confirmed in all 22 samples of muscle tissue and liver of broilers. The concentrations of DNC residues in broiler liver samples ranged from 6 µg/kg to 165 µg/kg, with a mean value of 43.4 µg/kg, and in broiler meat from 6 µg/kg to 41 µg/kg (mean value of 25.0 µg/kg). In two liver samples, DICL residues (m/z: 406.60→336.00 and 405.0→334.0) were confirmed in concentrations of 49 µg/kg and 65 µg/kg, as well as MAD residues (m/z: 934.8 →629.5, 934.8 →647.5) at 1 µg/kg concentrations in both matrices (Figure 1). This finding may be explained by the fact that the used screening method could not identify such low concentrations of MAD, given that its CCß value for broiler meat and broiler liver was estimated at 5 µg/kg. According to the relevant B&H legislation [7,8], the estimated concentrations of the detected coccidiostats were far below maximum residual limits (MRL).

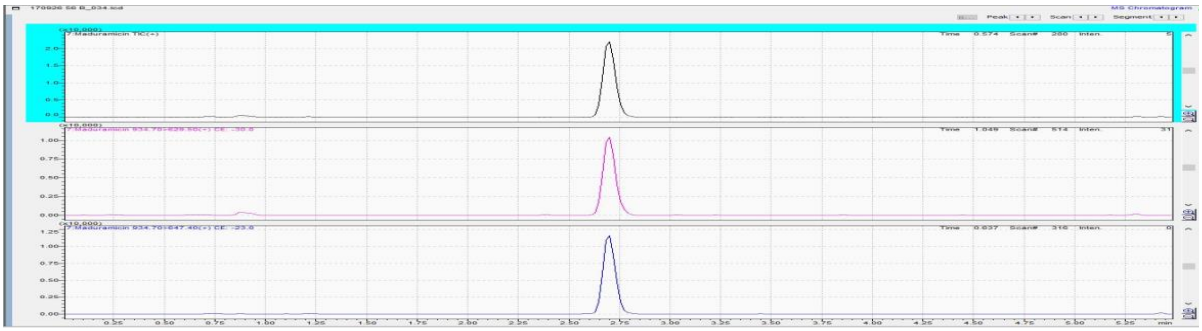

Figure 1. MRM chromatogram of maduramicin (m/z MAD: 934.7→629.5 and 934.7 →647.4) in a sample of broiler liver (1 µg/kg of MAD).

A statistically significant difference (p=0.001) in the presence of LAS residues was found among three tested types of matrices (table eggs, broiler meat and liver). Similarly, a statistically highly significant difference (p<0.001) was observed in the presence of DNC residues in the three types of tested matrices. Also, statistically significantly higher (p<0.001) occurrences of DNC residues in muscle tissue and broiler liver were found in the domestic products as compared to the imported ones.

Our results were also compared with results of coccidiostat residue monitoring in table eggs and broiler livers, provided by the Veterinary Office of Bosnia and Herzegovina (VOB&H) for the period 2010-2016 [9]. In 2011, two samples (1.6%; n=122) of table eggs were positive for MAD residues, while in 2012, five samples (3.1%; n=160) were positive for coccidiostats, with four being positive for MAD and one for SAL. In addition, one positive sample (0.7%) was found among 143 samples tested for SAL residues in 2014. Our study found 11.1% of 90 table egg samples were positive for LAS residues, but in very low concentrations ranging from 7 µg/kg to 33 µg/kg. In 2010, 2013, 2015 and 2016, coccidiostat residues were not identified in table eggs.

According to the VOB&H results [9], coccidiostat residues in samples of broiler liver were not identified in 2010 and 2012, while 4 out of the 11 broiler liver samples tested (36.4%) in 2011 were positive (3 samples positive for MAD and 1 for MON). Out of 20 samples tested in 2013, 6 positive samples were reported (30%), of which 3 were for DNC, 2 for MAD and 1 for DICL. In 2014, out of 19 tested samples of broiler liver, 7 (36.8%) were positive, of which 5 were for DNC and 2 for DICL. These results argue in favour of our findings of DNC, MAD and DICL in broiler liver.

Additionally, an increasing trend in the number of broiler livers positive for DNC residues was detected in 2015 and 2016 [9]. Of 20 samples of broiler liver tested during 2015, 15 samples (75%) were positive, of which 14 (70%) contained DNC residues. The residues were observed in low concentrations, ranging from 17 µg/kg to 229 µg/kg, which includes the DNC residue concentration range observed in our current study (from 6 µg/kg to 165 µg/kg).

High rates of positive broiler livers and animal feeds were also recorded in 2016 [9]. Out of 27 tested samples of broiler liver, 13 (48.1%) were positive, of which 11 (40.7%) contained DNC residues in high concentrations ranging from 8 to 1,200 µg/kg. However, the frequency of broiler feed (finisher) samples positive for LAS and SAL residues (40%) was recorded during the same year, which probably points to the occurrence of cross-contamination from non-target feed for broilers. In that, the highest concentrations of coccidiostat residues in finisher feed were 5,850 mg/kg for LAS, and 1,030 mg/kg and 900 mg/kg for SAL.

The presence of coccidiostat residues in broiler meat and table egg samples, as identified in our research, was most probably the consequence of cross-contamination of animal feed that occurred during the feed production and/or in further processing of the feed on the farm itself, as confirmed by others [10,11,12,13,14,15]. Feed cross-contamination is possible at farm level [16,17]. Inappropriate storage and/or marking of animal feed intended for different animal categories or species, inadequate cleaning of feed reservoirs and equipment, illegal addition of coccidiostats in feed, and disrespecting the coccidiostat withdrawal periods are all known risk factors for cross-contamination of non-target feed and the consequential detection of coccidiostats residues in broiler meat and table eggs [18].

4. Conclusion

This study is the first research into coccidiostat residues in poultry products on the B&H retail market. Study results show that concentrations of identified coccidiostats were below MRL values imposed by the current B&H legislation. This finding probably implies satisfactory handling of poultry feed, as well as adequate preventive and therapeutic applications of coccidiostats. The control and surveillance of coccidiostats in animal feed and poultry products has an essential role to maintain Bosnia and Herzegovina's status as a poultry exporting country on the EU market.

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Effects of handling procedure during unloading on welfare and meat quality of market-weight pigs

Effects of handling procedure during unloading on welfare and meat quality of market-weight pigs

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Abstract. This study aimed to examine the effects of handling procedure during unloading on blood glucose level, carcass lesions and meat quality of market-weight pigs. Rough handling during unloading was related to higher blood glucose level and frequency of slipping and falling. In contrast, gentle handling during unloading was related to the lower blood glucose level and frequency of slipping and falling, but the higher frequency of reluctance to move and turning back. Rough handling during unloading resulted in a higher carcass lesion score, and the higher tendency towards lesions on the middle part of the carcass and handling-type carcass lesions. Pigs subjected to rough handling during unloading had a higher meat temperature 45 minutes after slaughter, lower meat pH value 45 minutes and 24 hours postmortem, higher drip and cooking loss, higher L* and b* values and lower sensory colour score, and consequently, produced a higher prevalence of pale, soft and exudative meat. In contrast, pigs exposed to gentle handling during unloading produced a lower percentage of pale, soft and exudative meat, but a higher percentage of pale, firm, and nonexudative. In conclusion, gentle handling during unloading resulted in improved animal welfare, decreased stress intensity, and increased pork quality.

1. Introduction

Unloading at the abattoir is one of the most stressful moments on the day of slaughter, since pigs are exposed to an unknown environment and contact with unfamiliar people, which can cause stress and negatively affect animal welfare and pork quality [1]. Pig welfare at this pre-slaughter stage can be assessed according to animal-based (slipping/falling, reluctance to move, turning back, vocalization and lameness) and resource-based (unloading ramp angle, corridor appearance, type of handling tools, etc.) measurements [1]. In addition, physiological stress indicators and pork quality traits could be useful indicators of welfare conditions at unloading [2]. Rough handling in the unloading area, which includes the use of electric prods and sticks, compromises pig welfare and results in lower pork quality [2-3]. Several studies reported that alternative handling tools (sorting boards, rattle paddles and flags) to the electric prods and sticks, used to move pigs from the lorry to the lairage pens, were more effective devices because they induced fewer behavioural problems and reduced stress intensity and pork quality defects [1-3]. Therefore, the aim of this study was to examine the effects of handling procedure during unloading on blood glucose level, carcass lesions and meat quality of market-weight pigs. The research hypothesis was that gentle handling during unloading would improve pig welfare and pork quality.

2. Materials and Methods

2.1. Experimental animals and management procedures

The study was conducted in April 2021 on 69 market-weight pigs (29 barrows and 40 gilts) with average live weight of approximately 105 kg and 6 months old. All pigs were of the same genetics ([Yorkshire × Landrace] sows sired with Pietrain boars) and originated from the same farm and, thus, were reared under identical conditions. At the farm, the pigs were loaded in groups of 4-5 pigs by the same lorry driver. Lorry departed from the farm immediately after loading. Transportation of pigs was conducted in two groups (group 1 = 35 pigs; group 2 = 34 pigs) in an interval of two weeks by the same driver and the same lorry. Both groups of pigs were transported to the abattoir for about two hours at the loading density of ~0.43 m^2/100 kg. Both groups of pigs were unloaded as soon as possible (~5 min of waiting time for both groups) after the arrival at the abattoir using a 5-m long metal ramp with a slope of 20°. Pigs from the first group were unloaded by abattoir personnel using electric prods and sticks (in groups of 10–15 pigs) in compliance with standard abattoir practices (unloading time: 5 minutes). Pigs from the second group were unloaded by the first and second author in a slow and calm manner with a PVC sorting board and rattle paddle (in groups of 4-5 pigs), and without the use of electric prods and sticks (unloading time: 15 minutes). After the pigs were unloaded, they were moved to a lairage pens for 30 minutes at a lairage density of 1 m^2 per pig. Pig slaughter and carcass processing were identical for both groups and were performed at the same commercial abattoir in compliance with the standard industry-accepted practices.

2.2. Behavioural and physiological stress indicators

Pig behaviour (slipping, falling, turning back, reluctance to move) was monitored at unloading by direct observation of two trained observers based on the Welfare Quality® protocol [4], as outlined in Table 1. For the measurement of blood glucose levels, blood samples were collected from 15 slaughtered pigs per group (n = 30). Samples were collected in a plastic cup from the bleeding wound at sticking and results were immediately determined (within 15 s) using a handheld device analyser (Gluco Sure Auto Code, ApexBio, Taiwan).

Table 1. Indicators of pig behaviour and health recorded during unloading at the abattoir

Item	Description [4]	Thresholds [5]
Slipping	Loss of balance without the body touching the floor.	Excellent: 3%; Advanced: 6%; Acceptable: 8%; Unacceptable: >8%
Falling	Loss of balance in which a part of the body other than the legs is in contact with the floor.	Excellent: 0%; Advanced: 0.4%; Acceptable: 0.8%; Unacceptable: >0.8%
Turning back	Pig facing towards the unloading zone makes a 180° turn in the direction of the lorry area.	Excellent: 0%; Advanced: 1.5%; Acceptable: 4%; Unacceptable: >4%
Reluctance to move	Pig showing reluctance to move when it stopped walking, without moving its head and body and failed to explore for at least 2 s.	Excellent: 0%; Advanced: 0.5%; Acceptable: 1.5%; Unacceptable: >1.5%

2.3. Carcass lesion evaluation

Carcass lesions were visually assessed on the left carcass side (n = 30; 15 slaughtered pigs/group) by two trained observers in the cold chamber 45 minutes after slaughter using a visual scoring system based on Welfare Quality® protocol [4]. Also, carcass lesions were classified as handling type-carcass lesions, fighting-type carcass lesions and mounting-type carcass lesions by visual assessment of shape and size to recognize their origin [6].

2.4. Pork quality measurements

Pork quality measurements were carried out 45 minutes, 24 hours and 72 hours postmortem on the *Musculus longissimus dorsi* at the level of the 10th and 11th ribs on the left carcass side. Meat samples

were collected from 15 slaughtered pigs per group (n = 30). Both pH (pH_{45min} and pH_{24h}) and temperature (T_{45min} and T_{24h}) were measured 45 minutes and 24 hours postmortem using a pH meter (Testo 205, Testo AG, Lenzkirch, Germany). For determination of pork colour and water holding capacity, two boneless loin samples (2.54 cm thick, 100±4.5 g) were cut 24 hours postmortem by trained abattoir personnel from each selected carcass. Instrumental colour was determined using a portable colorimeter (Konica-Minolta, Chroma Meter CR 410, Osaka, Japan). Subjective colour of meat samples were evaluated 24 hours postmortem by three trained sensorists by using the scaling method according to the National Pork Producer Council colour standard [7]. To evaluate water holding capacity, drip loss, thawing loss and cooking loss were determined, as described in Čobanović et al. [8]. Pork quality classes were determined using pH values measured 24 hours postmortem, drip loss variations, and lightness (L* value) based on Čobanović et al. [8] (Table 2).

Table 2. Determination of pork quality classes based on Čobanović et al. [8]

Pork quality class	pH_{24h}	Drip loss (%)	L* value
PSE pork	<6.0	≥5	≥50
RSE pork	<6.0	≥5	42-50
RFN pork	<6.0	2-5	42-50
PFN pork	<6.0	2-5	≥50
DFD pork	≥6.0	≤2	<42

Abbreviations: pH_{24h} – pH value measured 24 hours after slaughter; Drip loss – meat juice loss at 4°C for a period of 24 to 72 h after slaughter**;** L* value – lightness; PSE – pale, soft, and exudative; PFN – pale, firm, and nonexudative; RSE – red, soft, and exudative; RFN – red, firm, and nonexudative; DFD – dark, firm, and dry.

2.5. Statistical analysis

Statistical analysis of the results was conducted with SPSS software (version 23.00, IBM Corporation, Armonk, NY, USA). According to handling procedure during unloading, the pigs were allocated to two groups: gentle handling = the group of pigs handled in a slow and calm manner with a PVC sorting board and rattle paddle, and without the use of electric prods and sticks (n=35) and rough handling = the group of pigs handled by using the electric prods and sticks (n=34). Student's t-test was used to examine the effects of unloading practice on the blood glucose level, carcass lesion score and meat quality traits of market-weight pigs. Data were described by descriptive statistical parameters as the mean value and standard error of the mean (SE). The effects of unloading practice on behavioural indicators, carcass lesion type, lesion distribution on carcass regions and pork quality classes were determined by Fisher's exact test. Since none of the slaughtered pigs had lesions on the ears and legs nor produced DFD pork, these carcass regions and quality class were not considered for this statistical test. Each pig was considered an experimental unit. In all tests, statistical significance was accepted at $P < 0.05$, while tendencies were accepted at $0.05 < P < 0.10$.

3. Results and Discussion

Effects of handling procedure during unloading on behavioural and physiological stress indicators of market-weight pigs are shown in Table 3. A higher occurrence of slipping (P=0.0356) and falling (P=0.0416) was recorded in pigs subjected to rough handling in the unloading area. The same group of pigs had a percentage of slipping and falling above the threshold level for unacceptable welfare conditions at the abattoir (Table 1) [5]. High frequency of slipping and falling of pigs during rough handling in the unloading area could be attributed to the use of electric prods and sticks, but also to the too steep unloading ramp (slope of 20°) [3]. An unfamiliar environment and a too steep unloading ramp (>15º) lead to agitation and stress in pigs, which make them rush (as shown by the shorter unloading time), and resulting in much more difficult handling during unloading [1]. This results in nervousness and frustration in abattoir personnel, leading to rougher handling of pigs and more frequent use of electric prods and/or sticks during unloading [1]. Considering that unloading ramp was identical for both

groups of pigs, it can be argued that rough handling by unqualified abattoir personnel had the greatest impact on the frequency of slipping and falling of pigs in the unloading area.

Table 3. Effects of handling procedure during unloading on behavioural and physiological stress indicators of market-weight pigs (n=69)

Handling procedure during unloading	Rough	Gentle	*P*-value	Significance
Number of pigs	34	35		
Behavioural stress indicators				
Slipping (%)	23.53[a]	5.71[b]	0.0356	*
Falling (%)	17.65[a]	2.86[b]	0.0416	*
Turning back (%)	2.94[a]	31.43[b]	0.0029	*
Reluctance to move (%)	0.00[a]	14.29[b]	0.0221	*
Physiological stress indicator				
Blood glucose (mmol/L)	6.89±0.57[a]	5.51±0.33[b]	0.0445	*

Rough handling = pigs handled using the electric prods and sticks; Gentle handling = pigs handled in a slow and calm manner with a PVC sorting board and rattle paddle.
* Statistical significance at (P<0.05); T: tendency (0.05 < P < 0.10); NS: not significant (P>0.05)
[a, b] Different letters in the same row indicate a significant difference at P<0.05.

In contrast, pigs exposed to gentle handling during unloading had higher frequencies of turning back (P=0.0029) and reluctance to move (P=0.0221) (Table 3). The same group of pigs had percentages of turning back and reluctance to move above the threshold level for unacceptable welfare conditions at the abattoir (Table 1) [5]. Scientific and professional attitudes about the cause of the aforementioned forms of pig behaviour are not consistent. Some authors [3] suggest that the use of electric prods and/or sticks during unloading leads to the higher frequencies of turning back and reluctance to move in pigs. On the other hand, other authors [1-2] reported more turning backs and reluctance to move in pigs handled during unloading without electric prods and sticks, indicating that gentle handling is connected with these forms of behaviour. Thus, great care is needed when interpreting the causes of turning back and reluctance to move during unloading, since there are indications that these forms of pig behaviour are not reliable indicators of animal welfare at the abattoir [1].

In the present study, pigs that underwent rough handling during unloading had a higher blood glucose level at exsanguination (P=0.0445), indicating the higher degree of stress after inadequate handling procedures. It has been observed that the use of electric prods and/or sticks immediately before slaughter leads to the activation of behavioural and physiological responses to stress, so such pigs have higher blood cortisol, lactate and glucose levels at exsanguination [2-3]. Stress stimuli just prior to slaughter provoke activation of the sympathetic-adrenal-medullary axis, causing the release of catecholamines (noradrenaline and adrenaline), which accelerate metabolism and lead to the breakdown of glycogen reservoirs in skeletal muscle and in the liver, and consequently resulting in increased blood lactate and glucose levels [1-2].

Effects of handling procedure during unloading on carcass lesions of market-weight pigs are displayed in Table 4. Pigs subjected to rough handling during unloading had a higher (P=0.0463) carcass lesion score. Also, there was a higher tendency (P=0.0656) for the occurrence of lesions on the middle region of the carcass and handling type-carcass lesions in pigs subjected to rough handling (Table 4). Rough handling, although it shortens the unloading time, causes fear and panic in pigs, which makes them rush, and leading to a higher occurrence of slipping, falling, mounting and hitting the floor or walls of the unloading ramp and corridors, consequently resulting in a higher prevalence of carcass lesions [2]. As a consequence of the use of electric prods and sticks during unloading of a large number of pigs at the same time, they jump on each other's backs in an attempt to escape from a source of stress, resulting in the higher occurrence of carcass lesions [1]. To reduce the prevalence of carcass lesions and

to facilitate unloading procedure, it is strongly recommended to unload pigs in small groups of 4-5 individuals, with at least two animals moving side by side [1].

Table 4. Effects of handling procedure during unloading on carcass lesions of market-weight pigs (n=30)

Handling procedure during unloading	Rough	Gentle	*P*-value	Significance
Number of pigs	15	15		
Carcass lesion score	1.20±0.20	0.67±0.16	0.0463	*
Carcass lesion type (%)				
Handling type-carcass bruises	80.00	40.00	0.0604	T
Fighting-type bruises	6.67	13.33	>0.9999	NS
Mounting-type bruises	13.33	6.67	>0.9999	NS
Carcass regions (%)				
Anterior part	80.00	60.00	0.4270	NS
Middle part	66.67	26.67	0.0656	T
Posterior part	40.00	13.33	0.2148	NS

Rough handling = pigs handled using the electric prods and sticks; Gentle handling = pigs handled in a slow and calm manner with a PVC sorting board and rattle paddle.
* Statistical significance at (P<0.05); T: tendency (0.05 < P < 0.10); NS: not significant (P>0.05)
[a, b] Different letters in the same row indicate a significant difference at P<0.05.

Effects of handling procedure during unloading on meat quality of market-weight pigs are depicted in Table 5. Meat obtained from pigs subjected to rough handling had higher temperature 45 minutes postmortem (P<0.0001), lower pH value 45 minutes (P=0.0100) and 24 hours (P<0.0001) after slaughter, increased drip (P=0.0013) and cooking loss (P=0.0167), higher L* (P=0.0104) and b* (P<0.0001) values and lower sensory colour score (P=0.0005). Consequently, pigs exposed to rough handling produced a higher percentage of PSE pork (P=0.0656).

Table 5. Effects of handling procedure during unloading on meat quality of market-weight pigs (n=30)

Handling procedure during unloading	Rough	Gentle	*P*-value	Significance
Number of pigs	15	15		
Pork quality parameters				
pH_{45min}	6.10±0.05[a]	6.28±0.04[b]	0.0100	*
T_{45min} (°C)	38.58±0.08[a]	37.25±0.22[b]	<0.0001	*
pH_{24h}	5.61±0,07[a]	5.99±0,03[b]	<0.0001	*
T_{24h} (°C)	1.87±0,03	1.84±0,02	0.4785	NS
Drip loss (%)	8.39±0.39[a]	6.35±0.41[b]	0.0013	*
Thawing loss (%)	4.65±0.47	4.61±0.40	0.9485	NS
Cooking loss (%)	24.94±1.02[a]	20.87±1.23[b]	0.0167	*
L* value	55,41±0,81[a]	51,95±0,96[b]	0.0104	*
a* value	11.12±0,31	11.17±0,41	0.9229	NS
b* value	9.63±0.20[a]	7.91±0.31[b]	<0.0001	*
Sensory colour score	1.76±0,11[a]	2.71±0.22[b]	0.0005	*
Pork quality classes (%)				
PSE meat	86.67[a]	40.00[b]	0.0209	*
RSE meat	13.33	26.67	0.6513	NS

RFN meat	0.00	6.67	>0.9999	NS
PFN meat	0.00	26.67	0.0996	T

Rough handling = pigs handled using the electric prods and sticks; Gentle handling = pigs handled in a slow and calm manner with a PVC sorting board and rattle paddle.
* Statistical significance at (P<0.05); T: tendency (0.05 < P < 0.10); NS: not significant (P>0.05)
[a, b] Different letters in the same row indicate a significant difference at P<0.05.

These results can be explained by the fact that rough handling during unloading leads to intensified metabolism in skeletal muscles ante and postmortem, resulting in increased meat acidification and temperature [8]. High meat acidification combined with high meat temperature induces denaturation of sarcoplasmic and myofibrillar proteins and reduction in their water holding capacity, thus causing the occurrence of PSE pork [8]. As rough handling during unloading compromises pig welfare and adversely affects the pork quality, most authors [6,9] agree that the use of electric prods should be strictly limited, while hitting, kicking and sticks must be strictly prohibited. Previous findings [6,9] reported that avoiding the use of the electric prods and sticks reduced the prevalence of carcass lesions and PSE pork by as much as 50%. This is confirmed in the present study, where the use of sorting boards and rattle paddles to move pigs during unloading instead of electric prods and sticks halved the prevalence of handling type-carcass lesions (rough handling: 80.00% vs. gentle handling: 40.00%) and PSE pork (rough handling: 86.67% vs. gentle handling: 40.00%) (Table 5). Also, gentle handling in pigs led to a slowdown of metabolic processes in skeletal muscles postmortem and only partial denaturation of meat proteins, so those individuals produced better pork quality, in terms of the lower prevalence of PSE pork and increased tendency towards pale, firm and non-exudative (PFN) pork (P=0.0656, Table 5).

4. Conclusion

The results showed that gentle handling of pigs during unloading resulted in improved animal welfare (lower frequency of slipping and falling and carcass lesion scores), decreased stress intensity (lower blood glucose level), as well as increased pork quality (lower prevalence of PSE meat). Education of abattoir personnel to better understand human-animal relationships, such as animal behaviour, practical aspects of animal handling and the influence of handling practice on the animal welfare and on the meat quality, as well as the use of alternative handling tools (sorting boards, rattle paddles and flags) would reduce the deleterious effects of rough handling during unloading. Education programs for abattoir personnel, which include training, evaluation of achieved knowledge and skills certification, should be repeated regularly so that acquired knowledge can be renewed and upgraded.

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Antioxidative properties and antigenotoxic potential of *Gentiana lutea* extracts against the heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine, PhIP

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Antioxidative properties and antigenotoxic potential of *Gentiana lutea* extracts against the heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine, PhIP

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Abstract. Lipid oxidation that occurs in different types of food can cause alterations in nutritional qualities, flavour, texture and shelf life of foods. Furthermore, high temperature cooking of protein-rich food can lead to formation of heterocyclic aromatic amines capable of compromising the integrity of DNA molecules. To reduce these harmful effects, research has been focused on investigating plants as a source of potential natural food additives and preservatives. Thus, the aim of this study was to estimate antioxidant and antigenotoxic activities of 50% ethanolic-aqueous root and leaf extracts of the medicinal plant, *Gentiana lutea*. Antioxidative effect was investigated using the DPPH assay, while antigenotoxicity against the mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was determined using *Salmonella* Typhimurium TA1535 in the SOS/*umuC* assay. Leaf extract showed high antioxidative effect with the ability to neutralize up to 87% of free radicals at 400 μg mL^{-1}. Antigenotoxicity testing revealed that both extracts exhibited remarkable genoprotective activity against PhIP-induced DNA damage, with the highest inhibition levels being 70% and 85% for root and leaf extracts, respectively. Results obtained are encouraging and suggest further research of *G. lutea* extracts as potential food preservatives and additives in improving food quality and human health.

1. Introduction

It is a known fact that diet and dietary habits can affect human health on different levels. One of the major problems when it comes to food safety is the free-radical mediated oxidation of foods that leads to quality deterioration of foods and consequently to impairing human health. Even though numerous synthetic antioxidants are used for food preservation, there are reports indicating the harmful effects of these additives [1]. Another problem comes from mutagens derived from food. One of the important classes of food mutagens are heterocyclic aromatic amines. These amines are formed during high temperature processing of protein-rich foods and require metabolic activation by liver enzymes in order to manifest their harmful effects. Their genotoxicity is manifested through forming covalent adducts with DNA molecules or generating reactive oxygen species, consequently leading to damage of biomolecules [2]. To counteract the negative effects of food oxidation and food mutagens, as well as to

satisfy consumer desire for natural and organic food preservatives, research is more focused on investigating different natural products as potential agents contributing to food safety**.**

It is well known that plants are an inexhaustible source of biologically active substances with various potential pharmacological activities. For that reason, medicinal and aromatic plants are being explored to a large degree as potential sources of effective, non-toxic and antioxidant compounds capable of improving food quality [3].

Gentiana lutea, yellow gentian, is a well known medicinal plant, widely used in folk medicine, as well as for the production of alcoholic and non-alcoholic beverages and pharmacological products [4]. Furthermore, rhizomes and roots of yellow gentian are used as an official drug by many pharmaceutical companies for the treatment of mild gastrointestinal ailments [5].

Taking into account the above, the aim of this study was to investigate the antioxidant activity of 50% ethanolic-aqueous root (GLR) and leaf (GLL) extracts of *G. lutea*. Furthermore, the antigenotoxic activity of extracts was estimated against 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) mutagen produced in thermally processed meat.

2. Materials and Methods

Plant material was obtained from the *Dr Josif Pančić* Institute for Medicinal Plant Research*,* Belgrade, Serbia, and extraction of plant material was done as previously described [6]. Antioxidant activity of extracts was measured through their ability to scavenge free radicals by applying the DPPH assay as described earlier [6]. Antigenotoxic effect of the extracts was investigated on the model organism *Salmonella* Typhimurium TA1535/pSK1002 in the SOS/*umuC* assay as previously described [7]. Prior to antigenotoxicity testing, a microdilution assay was done [7] and MIC values of the tested substances were obtained in order to avoid total inhibition of bacterial growth in the SOS/*umuC* assay. Furthermore, genotoxicity of extracts and PhIP mutagen was determined on *S.* Typhimurium in order to obtain non-genotoxic and genotoxic concentrations of the extracts and mutagen, respectively. Genotoxic potential was investigated without and with metabolic activation, provided by S9 fraction. After an appropriate incubation period, bacterial growth ratio (G) was evaluated by measuring optical densities (OD) at 600nm and using following formula: $G = sampleOD_{600}/controlOD_{600}$. Evaluation of G was additionally used to confirm lack of bacterial growth inhibition. A growth ratio less than 0.75 was selected as a marker of bacterial cytotoxicity. By measuring the absorbances (A) at 405nm, the DNA damage induction ratio (IR) was calculated using the formula: $IR = (sampleA_{405}/controlA_{405})/G$, and IR=2 was selected as the threshold of genotoxicity.

Antigenotoxic activity of the extracts against PhIP was tested using non-genotoxic doses of the extracts and in the presence of S9 fraction, since PhIP mutagen requires metabolic conversion. Percentage of inhibition of PhIP-induced damage was calculated using the following formula: $I\ (\%) = 1-(IR_{CT}/IR_M)\times 100$, where IR_{CT} and IR_M represent mean values of IR of co-treatment and sole mutagen treatment, respectively.

Statistical analysis was done using GraphPad Prism 6.01 software and one-way ANOVA with Dunnet's post hoc test was applied to test statistical significance ($p<0.05$).

3. Results and Discussion

The ability of the extracts to scavenge free radicals, determined in the DPPH assay, revealed stronger antioxidant activity of leaf compared to root extract (Figure 1). At the highest applied concentration (400 μg mL^{-1}), GLL extract scavenged up to 87% of DPPH radicals [6]. The high capacity of the gentian extracts to neutralize free radicals is in line with previously published data [8, 9], confirming the good antioxidant activity of *G. lutea*. In addition, the root extract of *G. lutea* successfully reduced lipid oxidation and colour changes in beef patties [10], suggesting its potential as a food preservative.

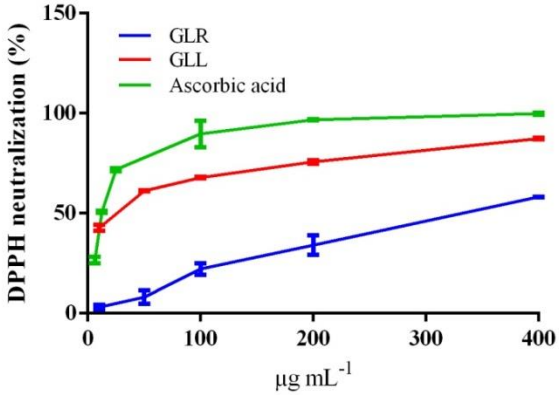

Figure 1. DPPH radical scavenging activity of *G. lutea* extracts

Results are expressed as mean values of neutralization (%)±standard deviations.
Ascorbic acid was used as positive control. GLR – root extract; GLL – leaf extract.

Furthermore, pre-screening of *S.* Typhimurium survival in the microdilution assay indicated that concentrations up to 2.5 mg mL^{-1} and 50 µg mL^{-1} for the extracts and PhIP, respectively, were non-cytotoxic, and could be used for further genotoxicity testing. The absence of cytotoxicity of tested substances was confirmed in SOS/*umuC* assay by determining the growth ratio (Figure 2).

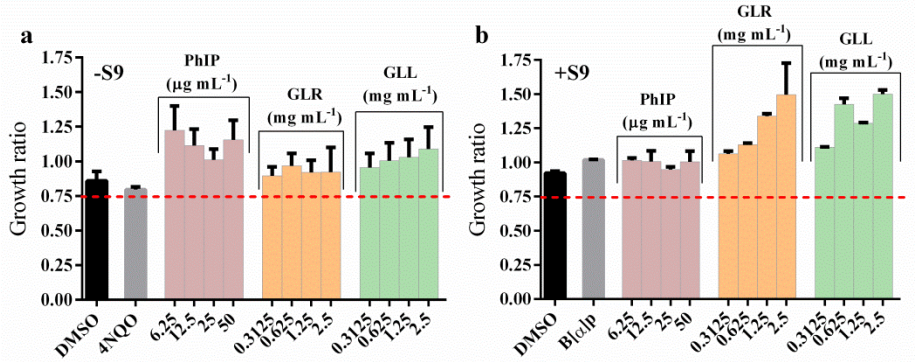

Figure 2. Effect of *G. lutea* extracts and PhIP on bacterial growth in SOS/*umuC* assay without (a) and with (b) metabolic activation (S9)

Results are expressed as mean values of growth ratio±standard deviations. DMSO (10%) was used as solvent control; 4NQO (0.5 µg mL^{-1}) and B[*a*]p (50 µg mL^{-1}) were used as positive controls. Red line positioned on 0.75 represents a threshold, below which the effect is considered cytotoxic. GLR – root extract; GLL – leaf extract.

Results of genotoxicity testing in the SOS/*umuC* assay showed that none of the extracts exhibited genotoxic potential in the absence of metabolic activation (S9) (Figure 3a). On the other hand, after testing with the addition of S9 fraction, both extracts showed genotoxic potential at certain concentrations (Figure 3b), indicating that they act as promutagens, i.e. they require metabolic activation in order to manifest their mutagenicity. As will be discussed below, the fact that the antigenotoxic agents can act in certain conditions and high concentrations as mutagens is not rare. Considering PhIP, as expected, it was only genotoxic in the presence of S9 fraction.

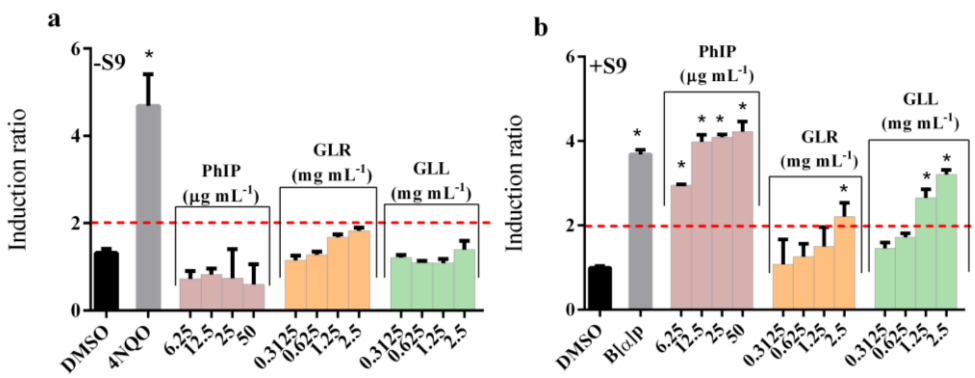

Figure 3. Effect of G. lutea extracts and PhIP on induction ratio in SOS/*umuC* assay without (a) and with (b) metabolic activation (S9).

Results are expressed as mean values of induction ratio±standard deviations. DMSO (10%) was used as solvent control; 4NQO (0.5 µg mL^{-1}) and B[*a*]p (50 µg mL^{-1}) were used as positive controls. Red line positioned on 2 represents a threshold, above which the effect is considered genotoxic. Statistical significance in regard to DMSO was tested using one-way ANOVA with Dunnet's post hoc test (*p<0.05). GLR – root extract; GLL – leaf extract.

In further work, only non-genotoxic doses of the extracts were used for antigenotoxicity testing against PhIP, applied at a concentration of 12.5 µg mL^{-1}. Results of antigenotoxicity revealed that both extracts exhibited strong genoprotective activity (Figure 4). The highest inhibition of PhIP-induced DNA damage was 70% and 85% for GLR and GLL, respectively. Similar results, in terms of genoprotective effect, were recorded for the extracts of *Gentiana asclepiadea* and *Gentiana cruciata* [11, 12]. It was shown that these extracts are capable of reducing DNA damage caused by hydrogen peroxide and the mutagen ethyl methanesulfonate.

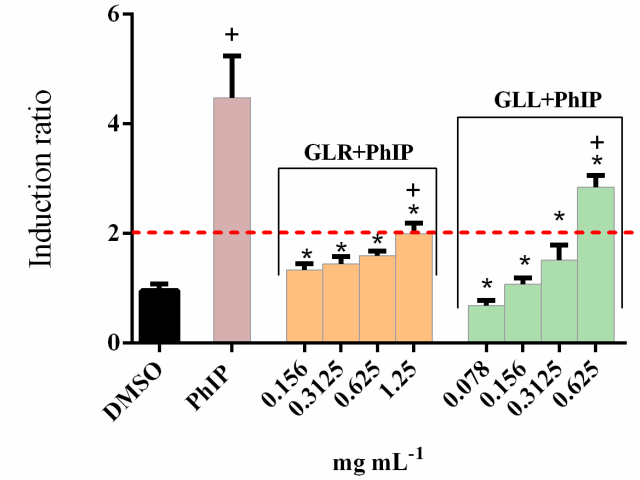

Figure 4. Antigenotoxic potential of *G. lutea* extracts against PhIP (12.5 µg mL^{-1}) in the SOS/*umuC* assay.

Results are expressed as mean values of induction ratio±standard deviations. DMSO (10%) was used as solvent control. Red line positioned on 2 represents a threshold, above which the effect is considered genotoxic. Statistical significance was tested using one-way ANOVA with Dunnet's post hoc test; p<0.05, + statistical significance in regard to DMSO; * statistical significance in regard to PhIP. GLR – root extract; GLL – leaf extract.

In our study, interestingly, both extracts were more efficient in reducing DNA damage at lower concentrations. Furthermore, we detected dual genotoxic/antigenotoxic nature of extracts. Generally, the extracts showed genotoxic effect when applied in higher doses, but at lower concentrations provided strong protection against PhIP mutagen. This kind of result can be explained by the hormesis phenomenon, which refers to situation where toxic substances provoke beneficial biological responses

when applied in low doses [13]. In addition, the response curve in the form of the letter "J", obtained in our study, is not uncommon for antimutagenesis studies [14].

The strong genoprotective activity demonstrated in our study can be partially explained by free radical scavenging activity of the extracts. Taking into account that PhIP can induce oxidative damage to DNA [15], the observed antioxidant activity of the extracts surely contributed to the detected antigenotoxicity. However, one should bear in mind that different secondary metabolites and interactions between them, as well as other mechanisms, can be responsible for antigenotoxic potential, which requires additional investigations.

4. Conclusion

Data obtained in this study indicate that 50% ethanolic-aqueous root and leaf extracts of *G. lutea* possess good antioxidative activity and strong capacity to reduce DNA damage induced by the thermally produced heterocyclic aromatic amine, PhIP. These results are promising and recommend further research of yellow gentian as a potential natural food preservative. However, the observed dual genotoxic/antigenotoxic effect of the extracts cannot be excluded and requires additional investigation in order to ensure the safe usage of *G. lutea* in the food industry.

Acknowledgement

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Meat quality of poultry fed with diets supplemented with insects: A review

Meat quality of poultry fed with diets supplemented with insects: A review

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Abstract. The development of sustainable feed ingredients for monogastric livestock is now also considering insect products. Although the regulation on the use of insect products differs among countries, resulting in restrictions on use in poultry diets, global research is exploring all the strengths and weaknesses of their inclusion. The scientific literature has extensively studied the relationship between insect-containing diet and effects on *ante-mortem* factors in fish and poultry, however the relationship between insect-containing diet and meat quality has only recently been considered. This review aims to collect the results of the studies that have related the dietary use of some insect species, such as the black soldier fly (*Hermetia illucens*), the yellow mealworm (*Tenebrio molitor*) and the silkworm (*Bombyx mori*), on the physicochemical and sensory traits of poultry meat. The insect source in poultry diets rarely changed the related physicochemical variables or the sensory profile of the meat, whereas the fatty acid (FA) profile was the variable that was most affected, and inclusion of black soldier fly always resulted in meats with a more saturated FA profile, yellow mealworm in a more monounsaturated FA profile, whereas silkworm produced meat with a more unsaturated FA profile, rich in valuable omega-3 FA.

1. Introduction

Statistics about demographic trends depict that by 2050 the world population should reach 9.1 billion people. This scenario is putting pressure on the search for alternative and sustainable feed resources, and among them insects, for the livestock sector [1]. In addition to the above reason, the use of insects in avian species feeding is supported by the fact that about 80% of birds are reported to include insects in their diets [2]; such birds include chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), guinea fowl (*Numida meleagris*), quail (*Coturnix coturnix*), and ostrich (*Struthio camelus*), which are species of interest for food production in different regions of the world [3,4,5,6].

To exploit their maximum potential as feed ingredients, insects are processed to obtain whole insect meal (full-fat), protein meal (PAPs) which can be defatted or contain some proportion of lipids according to the extraction method, and fat/oil. The fatty acid (FA) profile of insect lipids can be very extreme: two examples are the black soldier fly (*Hermetia illucens*; BSF) and the silkworm (*Bombyx mori*; SW). The fat extracted from BSF larvae contains 60-79% saturated fatty acid (SFA). Conversely, the SW chrysalis is very rich in omega-3 PUFA [7], and its oil presents a favourable n-6/n-3 ratio of 0.17 (personal communication). Thus, combining full-fat or partially defatted insect meal from different species could help ensure the best FA profile for animal feed [8]. The amino acid profile also differs between the different species of insects; in the case of the yellow mealworm (*Tenebrio molitor*; TM) larva, the content of all individual amino acids was found to be higher than that of barley, fish, brewer's yeast, beef/veal, and crustaceans, except for lysine, which was slightly higher in brewer's yeast [9].

Research studies conducted so far have tested a wide range of levels of substitution (5-100%, mainly substitution of fishmeal or soybean meal) or inclusion (0.75-60%) of insect meal, to find the best level, to cover nutrient requirements and to maximise growth and health performance, and product quality from farmed aquatic and terrestrial animals [10].

2. Insects in poultry feeds and meat quality

The effects of including dietary insect products (larva meal, pre-pupa meal, oil) on the quality of food-producing terrestrial animals have been studied mainly in poultry. The purpose of this review is to provide updated literature on the use of insect-based products as feed for poultry broilers, detailing the effects on the physico-chemical-sensory quality of the meat obtained. The review will consider the BSF (*Hermetia illucens*), the TM (*Tenebrio molitor*), and the SW (*Bombyx mori*), the first two for the greatest commercial interest in the EU, the third because it is potentially interesting in improving the dietary-nutritional value of meat.

*2.1 Dietary inclusion of black soldier fly (*Hermetia illucens*) and meat quality*

The BSF larva is one of the most commonly used organisms for aquaculture and one of the best studied for both aquaculture and poultry feeding. The BSF larva averagely contains 43.1±5.05 g protein/100 g dry matter (DM), and the amino acid profile is rich in leucine (6.72 g/100 g protein), lysine (6.22±1.08 g/100 g protein), and valine (5.38±0.82 g/100 g protein). Nutritionally important is also its contribution in calcium (24.1±12.8 g/kg DM) and phosphorus (6.01±1.77 g/kg DM) [11]. As aforementioned, the amount of larva fat and its FA profile are extremely variable and depend on the type of substrate. A description of the results obtained on the meat quality of poultry is provided below. Based on numerous studies (Table 1), the BSF as meal or fat in poultry diets has no [12,13,14,15,16,17,18,19,20,21] or limited influence [14,15,20,21,22,23] on physical meat quality (pH, colour, water holding capacity (WHC), shear force) of broiler chicken, quail, Barbary partridge, and Muscovy duck. Similarly, the poultry meat proximate composition also showed variable results, and they do not seem related to the insect meal inclusion level. Differences in meats' nutrient composition were mainly observed as more protein content [15,23], lowered [18] or increased [16,24] essential amino acids, and for enrichment in minerals, like calcium [24], sulphur [22], and copper [13]. In general, the sensory evaluation of poultry meat derived from animals fed diets supplemented with BSF did not differ from that obtained from control diets [14,17,24]. Instead, the BSF inclusion as meal or fat had a major contribution in modifying the FA profile of the lipids in the poultry meat [13,14,15,16,18,20,22,23,24,26,27]. The FA profile of the meat of monogastric animals is in line with the pattern of that of their diet, and the BSF (whatever its form) is rich in SFA, which make up approximately 70% of the total FAME, of which 43% constitutes C12:0 [21]. Therefore, it follows that the proportion of SFA in meat increases as a function of the dietary inclusion level of the BSF. In the majority of cases, this implies a worsening of the n-6/n-3 ratio [16], but either insect defatting or defatting their food substrate might not change [22] or could improve [18] the omega-6/omega-3 ratio. If the FA profile of the meat is changed by the inclusion of the BSF in the poultry diet, changes in the lipid oxidation of the meat is also expected. However, most of the studies did not observe changes in the oxidation of meat lipids in animals fed BSF [13,24,26]. However it is interesting to note that [12] observed a significantly low TBARS value in fresh meat after 7 days of refrigerated storage, and authors attributed it to the improved antioxidant activity (measured through the DPPH radical scavenging activity) of the meat due to the inclusion of the BSF in the diet.

*2.2. Dietary inclusion of yellow mealworm (*Tenebrio molitor*) and meat quality*

The yellow mealworm (TM) larva meal is considered a nutritionally suitable substitute for fishmeal and soybean in aquaculture and poultry diets, although its cost is currently too high and it cannot financially compete with standard feed sources. It should be emphasised that the strength of this feedstuff, therefore, lies in the high protein content (Nx6.25: 56-61%), characterised by a high biological value, as it includes all the essential amino acids in favourable proportions. Furthermore, it is a rich source of phosphorus [11] and potassium [9]. The fat (25-30% of TM meal) contains approximately 24% saturated FA, 24% polyunsaturated FA, and 50% monounsaturated FA, resulting in an omega-6/omega-3 ratio of 24 [9].

Contrasting results were obtained for physicochemical traits of meat from chicken broilers, apparently not depending on the inclusion level of TM meal (Table 2). However, the majority of the studies did not observe change in the meat pH, colour, moisture loss, shear force or fatty acid profile [28,29,30]. Instead, [31] observed that WHC, lipid and ash contents, TVB-N and sensory attributes worsened as the level of TM in the diet increased, and the authors partly attributed this trend to the possible presence of oxidised fat in dried insect meal. On the other hand, no other studies, testing higher TM inclusion levels, found adverse effects on sensory traits; on the contrary, [32] observed an improvement in meat juiciness and tenderness. When other poultry species were considered, such as Barbary partridge [20] and quail [33], no substantial differences in the meat physicochemical traits were observed due to the use of moderate to high levels of TM in the diet. Only meat colour changed in both bird species, although with an unclear pattern. The FA profile of the Barbary partridge meat was significantly affected by the dietary TM inclusion, particularly at the higher substitution level (50% of the soybean meal) [20]. FA changes resulted in the reduction of the C18:0 and omega-6/omega-3 ratio and the increase of C14:0, C15:0, C16:1, and C18:1 ($P<0.05$).

*2.3. Dietary inclusion of silkworm (*Bombyx mori*) and meat quality*

The silkworm pupa is characterised by high protein content (53.9% in the full-fat meal, 66.7% in the defatted meal), by a variable amount of lipids (29% in the full-fat meal, 9.5% in the defatted meal), the latter able to provide an extremely healthy FA profile (omega-3 FA: 29.5% and 31.5% total FAME, in the full-fat and defatted meal, respectively), suggesting that it is a valuable nutritional ingredient for feed of different monogastric livestock species [7]. Silkworm pupa meal has been successfully included in chicken broiler diet (Table 3), as it produced no effect on colour values, lipid content [34] or on sensory attributes [28,32] of the meat. A slight effect was observed in lipid content (3.56 vs. 4.48% for leg meat of control and treated broilers, respectively) in the study of [28] and in protein and ash contents (however, differences were not coherent with the inclusion level; [34]). Meat pH increased with the SW meal inclusion level, but it did not impair meat colour [34]. The best result for the dietary inclusion of SW pupa meal in chicken diets concerns the FA profile of the meat lipids: the PUFA n-3 increased, and the omega-6/omega-3 ratio decreased with the increase of the dietary SW meal substitution level ($P<0.01$). The C18:3 n-3 content in breast meat ranged from 6.75 to 15.0 to 28.4 mg/100 g meat, for control, 25% and 50% SW meal inclusion levels, respectively ($P<0.05$; [34]).

Table 1. Effect of dietary inclusion of black soldier fly on broiler meat quality.

Item	Avian species	Insect form	Substitution level range (%)	Inclusion level range (%)	Impact	References
pH, cooking loss[1], shear force[1]	chicken	meal		0.5-1.0	NS	[12]
			50-75		NS	[1][14]
				5-15	NS	[15]
Colour[1; 2], TBARS[a], DPPH radical scavenging	chicken	meal		0.5-1.0	P<0.05	[12]
			25-50		P<0.05	[1][22]
				5-15	P<0.05	[2][15]
pH[1; 2], colour[1;2], lipid oxidation, cooking loss, shear force	chicken	meal		5-20	NS	[16]
				5	P<0.05	[1][23]
			50-75		P<0.05	[2][14]
Amino acid, FA[b] profiles	chicken	meal		5-20	P<0.05	[16]
FA profile	chicken	meal	25-50		NS	[22]
				5	P<0.05	[23]
			50-75		P<0.05	[14]
				5-15	P<0.05	[15]
Mineral profile	chicken	meal	25-50		P<0.05	[22]
Proximate composition	chicken	meal		5	P<0.05	[23]
			50-75		NS	[14]
				5-15	P<0.05	[15]
Sensory evaluation	chicken	meal	50-75		NS	[14]
pH, colour, WHC[c], proximate composition, amino acid, FA and mineral profiles, sensory evaluation	chicken	meal		5-15	NS	[17]
FA profile	chicken	fat	100		P<0.05	[25]
			50-100		P<0.05	[26]
			50-100		P<0.05	[27]
pH[1], thaw loss, proximate composition[1], TBARS	chicken	fat	50-100		NS	[26]
					NS	[1][27]
Cholesterol	chicken	fat	50-100		P<0.05/NS	[26]
Sensory evaluation	chicken	fat	50-100		NS	[26]
pH, colour, total moisture loss, shear force, heme iron, shelf life	quail	meal		10	NS	[18]
Proximate composition, cholesterol, amino acid, FA profiles, sensory evaluation	quail	meal		10	P<0.05	[18]
pH, colour, WHC, shear force	quail	meal	25-100		NS	[19]
Proximate composition, sensory evaluation, cholesterol, TBARS	quail	meal		10-15	NS	[24]
Mineral, FA, amino acid profiles	quail	meal		10-15	P<0.05	[24]
pH, cooking loss	quail	meal		10-15	P<0.05	[21]
Colour, shear force, amino acid profile	quail	meal		10-15	NS	[21]
pH, shear force, cook loss, proximate composition	Barbary partridge	meal	25-50		NS	[20]
Colour, FA profile	Barbary partridge	meal	25-50		P<0.05	[20]
pH, colour, proximate composition, TBARS	Muscovy duck	meal		3-9	NS	[13]
FA profile, mineral profile	Muscovy duck	meal		3-9	P<0.05	[13]

[a]TBARS=Thiobarbituric acid reactive substances; [b]FA= fatty acids; [c]WHC=water holding capacity

Table 2. Effect of dietary inclusion of mealworm on broiler meat quality.

Item	Avian species	Insect form	Substitution level range (%)	Inclusion level range (%)	Impact	References
Proximate composition, sensory evaluation	chicken	meal		17	NS	[28]
pH, colour, WHC[a], proximate composition, TVBN[b], sensory evaluation	chicken	meal		1-3	P<0.05	[31]
pH, colour, drip loss, proximate composition, FA[c] profile	chicken	meal		7.5	NS	[29]
pH, colour, WHC, shear force	chicken	meal		2-8	NS	[30]
Sensory evaluation	chicken	meal		8.1	P<0.05	[32]
pH, shear force, cook loss, proximate composition, cholesterol	Barbary partridge	meal	25-50		NS	[19]
Colour, FA profile	Barbary partridge	meal	25-50		P<0.05	[19]
WHC	quail	meal		7.5-30	NS	[33]
Colour	quail	meal		7.5-30	P<0.05	[33]

[a]WHC=water holding capacity; [b]TVBN=Total volatile basic nitrogen; [c]FA= fatty acids

Table 3. Effect of dietary inclusion of silkworm on broiler meat quality.

Item	Avian species	Insect form	Substitution level range (%)	Inclusion level range (%)	Impact	References
Proximate composition, sensory evaluation	chicken	meal		17	NS	[28]
Colour	chicken	meal	25-50	7-14	NS	[34]
pH, proximate composition, FA[a] profile	chicken	meal	25-50	7-14	P<0.05	[34]
Sensory evaluation	chicken	meal		7.8	NS	[32]

[a]FA= fatty acids

3. Conclusions

The great economic impulse towards the use of insects as food and feed for ecological-environmental sustainability purposes has generated new companies producing insect meal and derivatives. A flywheel of interest has therefore been generated on several fronts, and the use of these products increasingly requires confirmation of safety and efficacy. In the last five years, much research has been conducted relating to the use of insects for alimentary use. Many of these studies aimed at the feed sector, which, however, has mainly considered the effect of their use on animal *ante-mortem* variables, whereas the study of the effects on nutritional, rheological and sensory quality of the meat has only intensified significantly in the last two years. This review focused on collecting and describing the results of research conducted so far on the effect of insects as feed on the meat quality of avian species. The results showed different effects, more depending on the insect species used than on the avian species that benefited from them. Overall, no adverse effects were observed on meat quality. Only the meat's FA profile was affected by the insect species included in the diet, suggesting its improvement through manipulation of the insect substrate, or the use of mixtures of insect meal or oil from different insect species.

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Textural properties of extruded snack products formulated with deboned poultry meat and brewer's spent grain

Textural properties of extruded snack products formulated with deboned poultry meat and brewer's spent grain

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Abstract. The incorporation of protein- and fibre-rich ingredients into starch-based extruded snacks is of interest for obtaining healthy products. However, development of this type of product has been limited, since protein and dietary fibre have negative effect on textural properties of extruded snacks. In the present study, response surface methodology was used in order to evaluate the effect of different ratios of mechanically deboned poultry meat (MDPM) and brewer's spent grain (BSG), as well as screw speeds, on hardness, firmness and crispiness of the extrudates produced. Regression analysis showed that BSG and screw speed had significant effects on all textural parameters, while MDPM had a significant effect only on the crispiness of snack products.

1. Introduction

Extrusion is a complex process consisting of continuous mixing, kneading, cooking, shearing, forming and/or puffing of the material [1]. Numerous products for the snack industry have been obtained by low moisture extrusion processes. Typically, commercial snack products are made of cereal flours and starches, and further seasoned with oil slurries. Thus, the products obtained usually have high fat and carbohydrate contents, and low percentages of proteins and fibres, i.e. are of low nutritional quality [2]. Moreover, in recent years, the prevalence of common diseases associated with poor nutrition, such as obesity, diabetes and cardiovascular disease, is rising, and hence, demand for healthier and nutritionally richer snack products has been evolving [3]. Extruded snack products are often consumed, especially by children and adolescents [4,5], and improvement of their nutritional quality could bring healthy diets closer to these younger populations. Since extrusion is flexible technology, different ingredients could be incorporated in order to improve protein, fibre and mineral contents.

Ingredients that could be used for protein fortification of extruded blends are whey protein, soy concentrate, pea protein, meat, and even insects [6–9]. To increase fibre content, different authors utilized different fibre-rich ingredients, such as wheat bran, rice bran, corn bran, wheat fibre, cauliflower by-products, apple and tomato pomace, etc. [10–12]. However, addition of protein and fibre sources has an influence on textural properties of extruded snack products. Increase of these two components lowers the expansion, and smaller sized pores are formed. Beside taste, the texture is the most important

parameter regarding consumer acceptability of snack products; hence it is crucial to obtain the desired texture of snacks [13]. Nevertheless, there are limited data explaining the influence of mechanically deboned poultry meat (MDPM) and brewer's spent grain (BSG) levels on textural properties of corn-based extruded products. MDPM is a low-cost turkey and/or chicken product, produced by mechanical separation of bone and attached skeletal muscle [14]. BSG is a nutritionally valuable by-product of the brewing industry, rich in protein (20%) and fibre (70%) [15]. Furthermore, the texture of snack products is also influenced by processing conditions. It has been suggested that screw speed is the process parameter with the most significant influence on the textural properties [16].

Having in mind the above, the aim of this study was to investigate the influence of different percentages of BSG and MDPM and different screw speeds on the texture of extruded snack products.

2. Materials and Methods

2.1. Raw materials

For the production of extruded snack products, the following four ingredients were used: cornmeal (6.5% protein; 0.59% fat; 0.31% ash; 79.6% carbohydrate; 5.73% dietary fibre), MDPM (15.8% protein; 14.98% fat; 0.95% ash; 0.1% carbohydrate; 0% dietary fibre), BSG and salt. BSG was dried in a convection dryer D-018 Solaris + (Dryer d.o.o. Belgrade Serbia) to meet the target moisture level of around 5.0% and milled in a hammer mill (ABC Inženjering Serbia) equipped with a 1mm sieve. The proximate composition of BSG after drying was: 19.1% protein; 5.58% fat; 4.38% ash; 65.94% carbohydrate; 45.59% dietary fibre.

2.2 Experimental design and data analysis

The independent variables selected for the study were: MDPM content (X1), 4, 8 and 12%; BSG content (X2), 10, 20 and 30%, and; screw speed (X3), 500, 700 and 900 rpm. The actual values are presented in Table 1. The response variables were: hardness, firmness and crispiness. Using Response Surface Methodology (RSM), based on a Box-Behnken design, a second-order polynomial regression model (Equation 1) was established to fit the experimental data ($P < 0.05$). In Equation 1, b_0 represents the intercept (constant), b_i the linear, b_{ii} the quadratic and b_{ij} the interaction effect of the factors, and Y represents response. From the fitted equations, response surfaces were generated.

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_{ii}^2 + \sum\sum b_{ij} X_i X_j \quad (1)$$

2.3 Sample preparation and extrusion process

The blends were prepared following experimental design (Table 1) by mixing cornmeal, MDPM and BSG in appropriate proportions in a bowl cutter SMZ 20/82 (Alexanderwerk, Germany). Each mixture contained 2% salt, and moisture content was set to 18%. The extrusion process was performed using a co-rotating twin-screw extruder of 880 mm length (model BTSK30, Buhler, Uzwil, Switzerland) of L/D ratio 28:1. The temperature profile was set at 100°C (zone 2-4) and 120°C (zone 6-7). Extrusion was conducted at a constant feed rate of 50 kg/h, with the three different screw speeds. At the end of the extruder, a circular die with an aperture diameter of 4 mm and six knives rotating with a constant speed of 350 rpm were mounted. After extrusion, snacks were dried for half an hour at room temperature, sealed in paper and plastic bags and stored.

2.4 Textural properties

Textural properties (hardness, firmness and crispiness) were analysed using a TA.XT2 Texture Analyser (Texture Technologies Corp., Scarsdale, NY/Stable MicroSystems, Godalming UK) equipped with 250 kg load cell. Hardness and firmness of the extrudates were measured in 15 probes according to methods described by Paula and Conti-Silva [17] – a compression test (aluminium cylinder probe of diameter 25 mm) and a cut test (Warner-Bratzler V-shaped cutting blade). Measurements of crispiness were repeated five times, using 5-blade Kramer shear cell [18].

Table 1. Experimental design for extrusion experiments with coded and actual variable levels

Run	Process variables		
	X1 MDPM (%)	X2 BSG (%)	X3 Screw speed (rpm)
1	4 (-1)	10 (-1)	700 (0)
2	12 (+1)	10 (-1)	700 (0)
3	4 (-1)	30 (+1)	700 (0)
4	12 (+1)	30 (+1)	700 (0)
5	4 (-1)	20 (0)	500 (-1)
6	12 (+1)	20 (0)	500 (-1)
7	4 (-1)	20 (0)	900 (+1)
8	12 (+1)	20 (0)	900 (+1)
9	8 (0)	10 (-1)	500 (-1)
10	8 (0)	30 (+1)	500 (-1)
11	8 (0)	10 (-1)	900 (+1)
12	8 (0)	30 (+1)	900 (+1)
13	8 (0)	20 (0)	700 (0)
14	8 (0)	20 (0)	700 (0)
15	8 (0)	20 (0)	700 (0)

3. Results and Discussion

The regression coefficients and analysis of variance for the analysed responses in hardness, firmness and crispiness are presented in Table 2. All the evaluated parameters showed an adequate adjustment with values of R^2 in range of 0.91 to 0.99, and coefficients of variation between 4.71 and 9.08. None of the models showed significant lack of fit, indicating that all the second order polynomial models correlated well with the measured data.

Table 2. Regression coefficient and analysis of variance for textural properties of extruded snack products

Coefficient	Hardness	Firmness	Crispiness
Intercept	13.71*	67.45*	169.42*
b_1	< -0.01	2.25	-5.01*
b_2	0.23*	2.70*	-6.19*
b_3	-0.01*	-0.04*	-0.01*
b_{12}	< 0.01	< -0.01	0.11
b_{13}	< 0.01	< 0.01	< 0.01
b_{23}	< 0.01	< 0.01	< -0.01*
b_{11}	0.03	-0.32	0.10
b_{22}	< -0.01	-0.06*	0.14*
b_{33}	< 0.01	< -0.01	< 0.01
P of F (model)	0.03	< 0.01	< 0.01
Lack of fit	0.60	0.33	0.05
R^2	0.91	0.97	0.99
CV (%)	9.08	6.96	4.71

The effects of the process variables on hardness, firmness and crispiness are shown in Figure 1 (A, B, C, respectively). Hardness and firmness showed similar response plots. The values of hardness and firmness ranged from 5.82 to 10.76 kg and from 38.28 to 78.24 N, respectively. These textural parameters did not change with MDPM increase, while increase of the percentage of BSG in the snacks promoted hardness. These results were in accordance with results of Stojceska et al. [12], showing that inclusion of BSG, a fibre-rich material, caused a rise of extrudates' hardness. It has been shown that

increase of fibre content results in less expanded and more compact structure, thus increased hardness and firmness [7]. Furthermore, some authors suggested that proteins also cause less expansion of extruded products [19], while others showed that addition of proteins in certain amounts can actually increase expansion of extrudates [20]. In the present research, MDPM as a source of protein did not have any significant influence on hardness and firmness, but only on crispiness. Hardness and firmness were negatively influenced by screw speed, i.e. they decreased as screw speed rose, similar to reports published earlier [9,12]. The crispiness of our extrudates varied between 64.25 and 135.1 (peak counts), and it was also slightly influenced by the percentage of MDPM in the snacks. Crispiness was negatively correlated with BSG, especially observed at high screw speeds. These results confirm the suggestion that fibre and protein often lead to less crispy products [7]. Furthermore, addition of fibre and proteins results in less starch in the mixture, which is the ingredient most responsible for the puffing of the snack [21], and more expanded products are characterized by lower hardness and higher crispiness [11]. Screw speed had a mild positive effect on crispiness of extrudates, which became more intensive when the percentage of BSG was low.

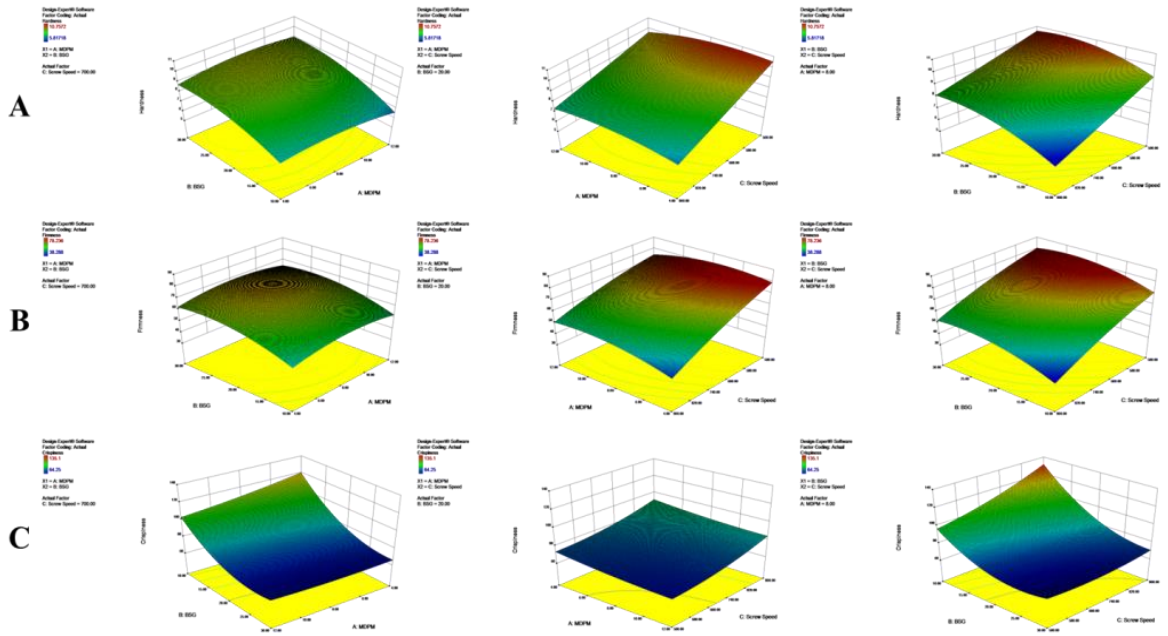

Figure 1. Response surface plots for the effect of MDPM, BSG and screw speed on the hardness (A), firmness (B), crispiness (C)

4. Conclusion

Response surface plots showed that hardness and firmness were highly influenced by BSG and screw speed, while hardness and firmness did not change significantly with the percentage of MDPM in the snack mixtures. Hardness and firmness increased with addition of BSG, but decreased with rising screw speed. On the other hand, crispiness was affected by all three independent variables. BSG had the most intensive effect on crispiness. A greater percentage of BSG in snack mixtures led to less crispy product, while increase of screw speed, when snack mixture contained 10% BSG, had a positive effect on crispiness. The presented study is encouraging for the development of healthy alternatives to the currently available commercial snack products that have poor nutritional value.

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How do food safety tools support the animal origin food supply chain?

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How do food safety tools support the animal origin food supply chain?

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Abstract. This study gives on overview of food safety tools that have been developed recently through the perspective of the animal origin food supply chain. It introduced some expected food safety legal issues, new technological outbreaks, food safety culture outlined in latest versions of food safety management system standards and tools applicable for the entire supply chains. Finally, the paper briefly shows some incentives associated with food safety and Covid-19 as well as the role of UN Sustainable Development Goals in animal origin food.

1. Introduction

Food safety as a concept intertwines with two ends of the pipeline – safety of food (as a product) and fulfillment / management of various food safety requirements from the farm to the fork. General food law outlines that "in order to ensure the safety of food, it is necessary to consider all aspects of the food production chain as a continuum from and including primary production and the production of animal feed up to and including sale or supply of food to the consumer because each element may have a potential impact on food safety." [1]. On the other hand, international food safety management system (FSMS) standards define food safety as assurance that food will not cause any (health) harm to the consumer when prepared and/or consumed according to its intended use [2, 3]. Based on the above, four main dimensions of food safety upscaled from food to the food supply chain can be recognized, Figure 1.

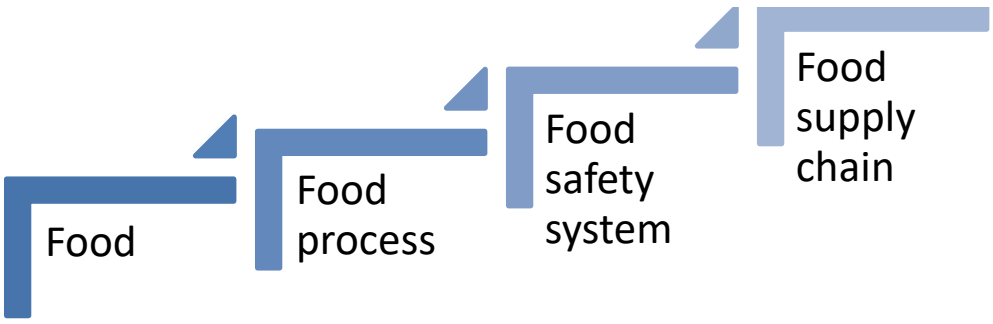

Figure 1. Four food safety dimensions

The aim of this paper is to provide an insight into food safety tools associated with each of the four dimensions that have been developed recently through the perspective of the animal origin food supply chain. In parallel, it provides an "out of the box" perspective of food safety in terms of the on-going pandemic caused by the coronavirus SARS-Cov-2, Covid-19 and UN Sustainable Development Goals.

2. Animal origin food

It is well-known that production of animal origin food is subject to high levels of (self)control and (self) inspection, as animals and animal origin food can transmit a variety of food borne diseases to consumers [4]. In that sense, a vast set of regulations is put into force to control safety of meat and dairy products, mainly focused on controlling maximal allowable values of potential chemical and microbial hazards, including the presence of toxic elements in food packaging. The microbiological criteria providing guidance on the acceptability of foodstuffs, is a good example [5]. Another issue of interest mainly associated with meat is its cancerogenic risk as outlined by the International Agency for Research on Cancer that analyzed 800+ epidemiological studies associating 15+ types of cancer with consumption of meat and meat products [6]. This caused many pro and con campaigns related to meat in human diets, where the cancerogenic and environmental issues were opposed to nutritional advantages of meat.

As healthy diets are in focus during the last decade, the pursuit of dietary health benefits led to development of new/modified food labels covering various nutritional and health claims. However, this raised another challenge as investigated in a study from 28 countries revealing three main variations: different labeling of various nutrition/health claims; modifications in authorization procedures and different levels of using scientific opinion [7]. Finally, an interesting response to the aflatoxin crisis that affects not only maize but also the milk supply chain was to implement an aflatoxin-safe certification to enable consumers' healthy food choices [8].

3. Food processes in animal origin food companies

When it comes to technological issues associated with the companies producing animal origin food, two development directions arise: (i) usage of non-thermal food processing technologies and (ii) implementation of Food Industry 4.0.

Non-thermal food processing technologies have different types of action, depending of the source of energy transfer and can be divided as: (a) technologies based on mechanical action; (b) electro-magnetic field-based technologies and (c) pressure-based technologies [9]. The main advantage of these new processing technologies is assurance of safety of the products [10]. Research is mainly focused on microbial inactivation, food safety and preservation while retaining quality of the obtained products. Use of these technologies, besides achieving microbial inactivation [11-13], provides some nutritional improvements [14-16] due to decreased processing time and prevention of negative effects of heat.

Examples are the use of ultrasonic power in an attempt to examine the oxidation of beef proteins [17] or the use of ultrasound in reducing the microbial load in milk [18]. However, the use of different types of non-thermal food processing technologies due to their novelty and different technological readiness levels, meaning they are not fully covered by (food) legislation. An example of change is from Health Canada, which analyzed a great number of ready-to-eat meats, raw meats, and egg products processed with high pressure processing and concluded that such foods should no longer be considered as novel foods [19].

(Food) Industry 4.0 is recognized as a combination of internet-oriented technologies and smart systems enabling advanced communication between each part of the system and improved human-machine interaction systems [20]. Further applications are expected in terms of implementing smart sensors, artificial intelligence and big data into food industry [21]. There are cases of partial implementation of Food Industry 4.0, like in maintaining the cold chain using IoT temperature sensors [22]. It is well known that the cold chain is vital in keeping meat safe, as low temperatures inhibit growth of microorganisms [23].

4. Food safety systems in animal origin food companies

Although there are many FSMS standards, the ones recognized by the Global Food Safety Initiative as most appropriate for the animal origin food supply chain are FSSC 22000, BRC and IFS [24]. They consist of three basic parts: prerequisite programs (PRPs), hazard analysis and critical control points (HACCP) and management requirements needed to upscale HACCP to a food safety management tool. Serbian meat and dairy companies prefer ISO 22000 as opposed to IFS/BRC [25, 26]. Their outcome in implemented FSMS is overseen in increased safety of food and higher levels of hygiene in establishments with an operative HACCP in place. Once HACCP became mandatory in meat establishments, it directly improved all process hygiene indicators, outlined in significant reductions of hygiene indicator organisms on all types of meat contact surfaces [27]. Another interesting perspective has been observed in animal origin food companies regarding quality management systems, where most of the companies interpret food quality as having the same importance as food safety [28].

Food safety culture is a new perspective of FSMS, where BRC is one of the international standards that promotes this new requirement. It is defined as attitudes, values and/or beliefs at site associated with the importance of food safety [3]. In parallel, scholars outlined five components necessary to asses this food safety perspective as follows: leadership, communication, commitment, resources and risk awareness [29]. A study performed in Central and Eastern Europe shows that bigger companies have higher levels of food safety culture as opposed to small companies [30, 31]. The same study confirmed that country of origin (EU *vs*. non-EU) is the greatest influencer in adopting food safety culture, mainly because the EU is putting into force more extensive FS legislation. Finally, this study identified that companies operating in the animal origin sector have a lower level of FSMS in place, as opposed to similar studies in Europe where FSMS in this sector, due to food safety risks, were on a higher level [32].

5. Animal origin food supply chains

When food safety is elevated to a supply chain perspective, different tools are developed in analyzing various food safety risks. One tool is the concept of food safety objectives, developed to prevent or minimize exposure of the consumer to any type of food hazard [33]. It employs techniques, or models applied from the farm to the fork considering all kinds of variations and changes associated with ingredients, process steps, distribution, and final food preparation (at home or in food service establishments). It provides a value, such as the appropriate level of health protection, whereby it calculates the maximum frequency and/or concentration of a food safety hazard in food at time of consumption with the aim of achieving public health goal(s) [34].

A second tool is to perform an exposure assessment. This is a quantitative measurement of health risks associated with a food hazard, whereby it is necessary to calculate occurrence of a specified hazard in food on one hand and to analyze consumption of food in a population based on consumption surveys on the other hand [35]. This has been used in the case of aflatoxin in milk and dairy products, where the results obtained become usable for all stakeholders in the dairy chain continuum for their decision making and/or developing risk mitigation strategies [36, 37].

6. Out of the box

Since the World Health Organization (WHO) announced that Covid-19 is a public health emergency of international concern [38], all aspects of life, including food supplies have been affected. To help food companies, the WHO developed a guidance document supporting scientific belief at the time of publishing [39]. As coronaviruses do not have the potential to survive on food products or food packaging [40], the European Food Safety Authority specified that food should not be considered as a risk and/or transmission route [41]. In order to challenge food safety during the pandemic, an international survey assessed food companies' responses to the pandemic [42]. Results of interest highlight that maturity of a FSMS was correlated with the response to the pandemic, where companies operating in the animal origin food companies were identified as companies having only basic but still effective food safety, with staff awareness and hygiene as the most important PRPs.

Another dimension of interest encompasses the 17 Sustainable Development Goals (SDGs) adopted by the United Nations within its 2030 Agenda [43]. Eight out of 17 SDGs are correlated with food and food systems [44], where SDG12 'Responsible consumption and production' and SDG 13 'Climate action' are goals highly associated with animal origin food. The livestock sector with its environmental impacts uses natural resources and causes environmental pollution [45] but also affects climate change [46, 47]. Upscaling to the animal origin supply chain, climate change effects occur at farms [48], affect meat and dairy production [49, 50] and end at households [51, 52].

As a result of these environmental impacts, the Food and Agriculture Organization defined sustainable diets as "diets with low environmental impacts which contribute to food and nutrition security and to healthy life for present and future generations" [53]. However, the pressure on changing dietary habits as a response to climate change affected by meat and other animal origin food, is still more theoretical that observed in every-day life. This kind of research focuses on analyzing effects of meat/dairy replacements, with plant-based substitutes. Such models reveal the potential of changing eating patterns and decreasing environmental impacts [54]. Although there are diets that exclude consumption of animal origin food such as veganism, raw foodism or fruitarianism, still a large majority of the human population eat (safe) animal origin food regularly or occasionally [48].

7. Conclusion

The entire animal origin supply chain is recognized as a sector directly or indirectly involved in the majority of food safety issues. Great efforts and developments are observed in all parts of the supply chain, downscaled to meat/dairy products and/or upscaled to the entire supply chain. In spite of more demanding requirements, required by new challenges such as Covid-19 and UN Sustainable Development Goals, this sector still manages to deliver safe food to consumers all around the world.

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Use of engineering tools in modelling first bite–case study with grilled pork meat

Use of engineering tools in modelling first bite – case study with grilled pork meat

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Abstract. This study provides an engineering approach in modelling the first bite. Based on the mechanical properties of grilled pork meat obtained by applying compression and Warner Bratzler tests and using the Finite element method, a 3D model of cubic pieces has been created. It was then used for simulating the first bite of pork meat grilled at two temperatures and three positions of the jaws. Stress distribution during impact of upper and lower jaw shows growth of internal stress in the direction of jaw movement, leading to crack development and breaking of meat.

1. Introduction

Meat is a postmortem skeletal muscle tissue of animals [1] and is subject to a variety of physiological and biochemical changes after slaughter [2]. It is a combination of muscle fibres, intramuscular connective tissue and intramuscular fat [3]. The complexity of meat depends on various parameters, such as species/breed, age and muscle position in the carcass [4]. Meat and meat products are complex systems and can be referred to as matrices of interacting components that can be determined by processes and forces operating at the micro-scale [5].

The finite element method (FEM) is an engineering tool, increasingly used in food science. As such, it is capable of analysing and modelling the deformation behaviour of food by solving complex mechanical problems [6]. In meat science, it has been used mainly for analysing mass/heat transfer [7-9], with no studies simulating the first bite. The aim of this research was to measure mechanical properties of grilled pork meat and, based on the results, perform a first-bite modelling simulation using the FEM.

2. Materials and methods

Pork meat (m. *longissimus dorsi*) was purchased locally in Zemun, Serbia, and grilled using two pre-defined grilling regimes (T1 - medium, T2 - long) in a Tefal grill (OptiGrill+). To control the processes, two temperatures were monitored: (i) in meat centres using a digital thermometer (Trotec GmbH - Model BT20, Germany) and (ii) on the surfaces of meat using an infrared thermometer (TES-1327KUSB). To calculate density for both grilling temperatures, 30 samples of grilled meat were cut into cubes of exact measured length, width, and thickness (measured with a digital vernier calliper) as well as mass (measured using an analytical balance (OHAUS Adventurer – Model AR2140, USA) [10].

2.1. Mechanical properties

Mechanical properties of the grilled meat cubes were determined by performing compression and Warner-Bratzler shear tests. All samples had cubic dimensions (20×20×20 mm), and both tests were performed in 15 replicates. Grilled pork meat was cubed using thin-bladed sharp knives to minimize damage to the fibres and taking into account their direction [11].

Compression test was conducted on a Brookfield CT3 Texture Analyser using the following settings: test speed - 1 mm/s, trigger load - 10g, target mode - 30%, cylindrical probe - 50.8 mm diameter. Warner-Bratzler shear test was conducted on a TA.XT plus Texture Analyser, under the following parameters: test speed - 1 mm/sec, target mode - distance (21 mm), sample shape - rectangular, selected probe - HDP/WBV, load cell - 50 kg. Both tests were performed on all three planes of each cubic meat sample (Figure 1).

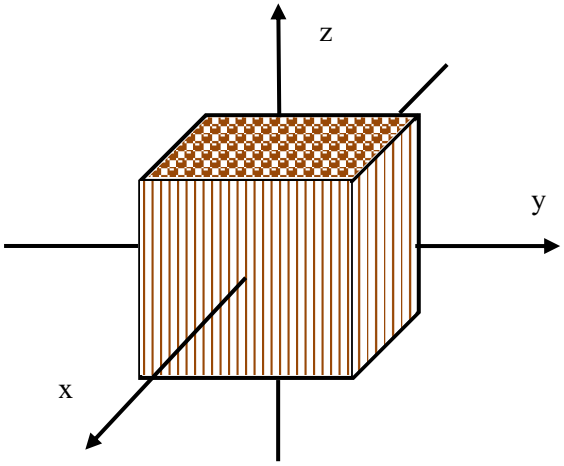

Figure 1. Orthogonal isometric view of a cubic meat sample used for 3D modelling - grilling surface (xOy plane); direction of fibres parallel with the z-axis

Using previous works of Vallespir, Rodríguez, Eim, Rosselló and Simal [12] and Nieto, Vicente, Hodara, Castro and Alzamora [13], true stress and strain were calculated (Equations 1 and 2, respectively). Rupture stress (σ_R) and strain (ε_R) were extracted from the first peak of the stress-strain curve, while Young's modulus (Ed) was calculated for the common linear part of the stress-strain curve, as presented by Djekic, Ilic, Guiné and Tomasevic [14], Equation 3.

$$\sigma_R = \frac{F(t) * (H_o - H(t))}{A_o * H_o} \quad (1)$$

$$\varepsilon_R = ln\frac{H_o}{H_o - \Delta H} \quad (2)$$

$$E_d = \frac{\sigma_R}{\varepsilon_R} \quad (3)$$

Legend: F(t) – force at time t; H_o – initial sample height; ΔH – height difference; H(t) – height at time t; A_o – sample area.

Although meat could be considered as an anisotropic material, for the purpose of this study, the authors assumed the following: (i) grilled meat is an orthotropic material, with three perpendicular planes of material property symmetry (Figure 1); (ii) during compression tests, expansion of meat in the direction perpendicular to the specific loading direction is equal in the other two planes with constant volume before and after loading; (iii) the Poisson's ratio is the ratio between the transversal (lateral) strain and the longitudinal strain ($\nu_{ij} = -\varepsilon_j/\varepsilon_i$), where ν_{ij} corresponds to an expansion in direction "when compression is applied in direction ".

2.2. First bite simulation

Cubic 3D solid models of grilled meat were created using SolidWorks Simulation FEM code. For mesh construction, tetrahedral solid element type was used with 50,406 elements and 71,869 nodes as proposed by Wang and Sun [15], who modelled roasted meat with four-node tetrahedral elements. Our simulation assumed the following: (i) 3D model is a 20×20×20 mm cube (ii) first bite is perpendicular to the longitudinal direction of fibres and parallel to the x-axis; (iii) first bite force was assumed as the value obtained from WB test divided by two, considering the upper and lower jaws.

Simulations were performed for three positions of the first bite calculated as line pressures 20 mm long and assuming tooth width of 1 mm. The positions were in the middle of the biting plane (10 mm from each edge); biting at one third (6.67 mm) from one edge, and at one quarter (5 mm) from one edge, Figure 2.

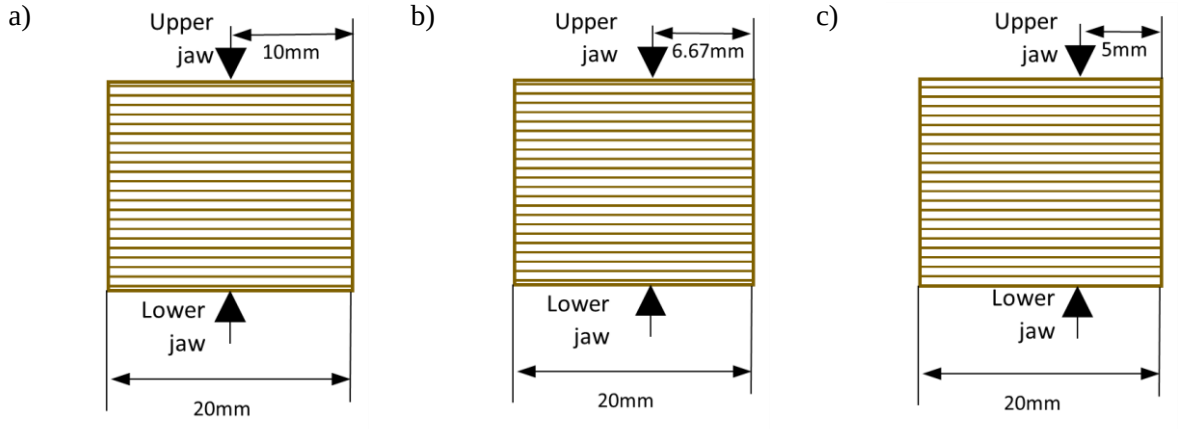

Figure 2. Front view of the positions of upper and lower jaws in the simulated 3D models

3. Results and discussion

From Figure 1, the meat's grilling surface was set on the plane perpendicular to fibres that were parallel to the z-axis. For grilling temperatures T_1 and T_2, values in the centre of the product were 74.6°C and 90.6°C, while on the surface, values were 263.4°C and 272.1°C, respectively. The density of meat grilled at the lower temperature was 1.23 g/cm^3 compared to 1.17 g/cm^3 obtained for the higher grilling temperature.

Firmness values obtained by the Warner-Bratzler Shear Force test show increase with the increase of grilling temperature (64.5 N compared to 79.3 N). This test measures the maximum force needed to cut off a meat sample [16], so the authors used these values as the biting force for first-bite modelling. Rupture stress, ε, was between 0.06 kPa and 0.08 kPa for T_1 and between 0.06 kPa and 0.07 kPa for T_2, calculated for all three directions. Rupture strain, σ, in the three directions ranged between 3.4 and 6.7 for T_1 and 5.4 and 7.3 for T_2.

Values of Young's modulus in the three directions also increased with the increase of grilling temperature, so the obtained values were between 54.3 and 75.3 kPa (T_1) and 111.1 and 148.8 kPa (T_2). Finally, Poisson's ratio was between 0.319 and 0.491. This concurs with some previous studies that reported Poisson's ratio of meat and meat products between 0.2 and 0.49 [17, 18]. As most soft tissues are roughly incompressible (Poisson's ratio up to 0.49), this justifies our assumption that when a material is compressed axially (in one direction), it expands laterally in the other two directions [19, 20].

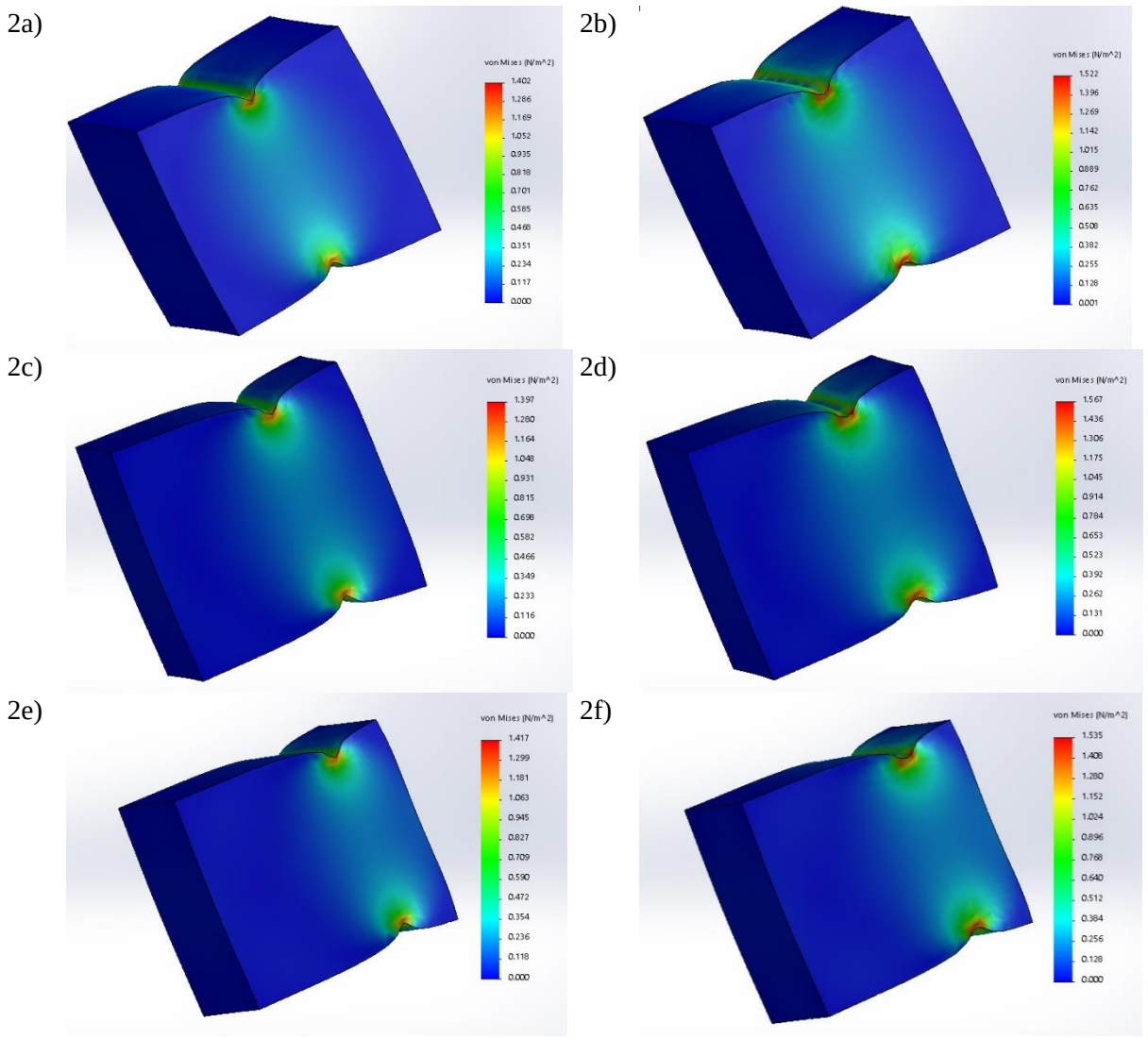

Figure 2. Stress distribution during impact of upper and lower jaw shown for the six models: a) pork meat T_1 – x/2; b) pork meat T_2 – x/2; c) pork meat T_1 – x/3; d) pork meat T_2 – x/3; e) pork meat T_1 – x/4; f) pork meat T_2 – x/4. Colour scale bar indicates gradient areas (from maximum to minimum) of von Mises stress (N/mm^2) in the direction of pressure.

Based on the Warner-Bratzler values, the pressures applied at first bite were 1.61 N/mm^2 for T_1 and 1.98 N/mm^2 for T_2. According to the FEM simulation, higher values for von Mises stress were obtained for the higher grilling temperatures (1.522 N/mm^2 – 1.567 N/mm^2 for T_2 as opposed to 1.397 N/mm^2 – 1.417 N/mm^2 for T_1), showing slight differences regarding the position of the teeth during biting.

Based on the results, this study shows potential in predicting deformations during the first bite [21]. This is notable, since grilled meat has different particle size fragmentation distributions during mastication and before swallowing [22]. The mechanical characteristics of meat directly influence behaviour during oral processing, such as the number of chews, consumption time per bite, chewing cycle, average bite size and/or eating rate [23].

4. Conclusion

This research highlights the potential of using FEM to simulate the first bite of meat and is one of the first of its kind that has tried to connect mechanical properties in modelling the first bite. Results show growth of internal stress in the direction of jaw movement, leading to crack development and breaking of meat. The highest values are in the area of teeth pressure, and as such, lead to the conclusion that upon biting, the meat structure will suffer irreversible damage dividing the grilled meat in two, as happens during the first bite.

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Evaluation of trace element levels in beef cuts available to the consumers in Serbia

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Evaluation of trace element levels in beef cuts available to the consumers in Serbia

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Abstract. The present study evaluated the levels of Mn, Se, Cu and Zn in beef cuts available at markets in Serbia. We also assessed the risk associated with the consumption of these foods based on the estimated daily intake (EDI) of these elements. Thirty-six beef meat cuts were analysed using inductively coupled plasma mass spectrometry (ICP-MS). The EDI of all the studied elements was estimated on the basis of a calculation of the amount of beef consumed by Serbian households (mean beef consumption of 17.2 g/person/day). The studied beef cuts pose no risk with respect to the EDIs of Mn, Se, Cu and Zn. Among the four studied beef cuts, shoulder had the highest content of dietary zinc (68.2 mg/kg).

1. Introduction

The chemical hazards in food of animal origin (meat and meat products) are different [1] and they are severe public health concern all over the world. The concentrations of elements in animal tissues depend on the age and sex of animals, chemical composition of meat, sampling season (hunting), and nutrition [2-4]. In recent years, concern has arisen in the EU regarding adjustment of mineral supplementation to actual physiological needs of animals, as large loads of trace elements (mainly Cu and Zn) are occurring in the environment [5].

On the other hand, it is common knowledge that consumption of meat has been an important component of the human diet for a long time, as it provides highly bioavailable elements required for normal development and health [6, 7]. In this context, meat is an important source of energy and nutrients, including proteins, minerals (manganese (Mn), zinc (Zn), copper (Cu), iron (Fe) etc.) and vitamins (B12, folic acid). Minerals contained in meat, in comparison with those present in plants, are more easily absorbed [8]. Consequently, the determination of essential elements in all types of food, including meat and meat products, is necessary for quality assurance and consumer health protection.

Meat consumption is based largely on availability, price and tradition. The consumption data of meat and meat products worldwide are presented in Table 1 [9]. In Serbia, poultry is the most commonly consumed meat type per capita (18.7 kg), followed by pork (18.5 kg) and beef (6.3 kg) in 2019 [10].

Mn, selenium (Se), Cu, Zn and other major elements are essential micronutrients that need to be consumed in adequate amounts to maintain normal physiological function [11]. Small quantities of these elements are vital for growth and development and they can be obtained through a balanced diet. [12]. Mn is a cofactor for many enzymes and it is involved in amino acid, cholesterol, and glucose and other carbohydrate metabolism [13]. Se is a constituent of more than two dozen selenoproteins that play critical roles in reproduction, thyroid hormone metabolism, DNA synthesis and protection from

oxidative damage and infection [14]. Cu is a cofactor for several enzymes (known as cuproenzymes) involved in energy production, iron metabolism, neuropeptide activation, connective tissue synthesis and neurotransmitter synthesis [15]. Zn is involved in numerous aspects of cellular metabolism, supports normal growth and development during pregnancy, childhood, and adolescence and is required for proper sense of taste and smell [16].

Table 1. Meat consumption worldwide and by continent in 2017 [9]

	Meat Consumption (kg/per capita/year)*				
Type of Meat	World	Africa	America	Asia	Europe
Bovine	9	5.63	27.83	4.68	14
Mutton and goat	1.86	2.49	0.62	1.93	1.75
Pork	15.7	1.48	18.65	15.18	35.75
Poultry	15.18	6.21	41.94	9.71	24.59
Others	0.84	1.43	0.65	0.55	1.84
Total	42.58	17.24	89.69	32.05	77.93

The lack of information on the concentrations of trace elements in beef cuts in Serbia was the main reason to carry out the present study. In this context, this work evaluated the levels of Mn, Se, Cu and Zn in such food. The obtained results were compared with the literature data from other countries. In order to assess exposure to trace elements through the consumption of beef cuts, the daily and weekly intakes were calculated and compared with the nutritional limits.

2. Materials and Methods

2.1. Sample collection

A total of 36 different beef cuts (beef steak, hind leg, shoulder and neck) commercially available in Serbia were collected during 2020. After collection, meats were labelled and stored in polyethylene bags and frozen at -18°C prior to analysis.

After acid mineralization of homogenized beef cuts, microwave digestion (Digestion System: Milestone, Sorisole, Italy) was used for sample preparation. Analysis of Mn, Se, Cu and Zn was performed by inductively coupled plasma mass spectrometry (ICP-MS), (iCap Q mass spectrometer, Thermo Scientific, Bremen, Germany). The most abundant isotopes were used for quantification. The accuracy of the analysis was verified by analysing the certified reference material NIST SRM 1577c (bovine liver, Gaithersburg, MD, USA).

2.2. Estimated daily intake

Nutritional exposure to the examined trace elements was calculated by estimation of the daily intake (EDI). The daily intake (DI) of each measured element in beef cuts was calculated used the following equation:

$$DI\ (mg) = [D\ (g) \times C_{element}\ (mg\ kg^{-1})]/1000$$

where DI is the daily intake (mg), D is the average Serbian per capita daily consumption of beef (17.2 g) [10] and $C_{element}$ is the concentration of element (mg kg^{-1}) detected in beef cut.

EDIs were expressed in mg/kg bw day was calculated according to the following equation:

EDI = DI (mg) / kg bw = mg/kg bw day

where bw is individual's body weight (bw; assumed to be 70 kg).

The calculation of the EDI is the same as the provisional maximum tolerable daily intake (PMTDI). The PMTDI value is the permissible human exposure of a substance in food and drinking water [17]. Hazard quotients (HQs) for Mn, Se, Cu and Zn in the analysed samples were calculated according to the following equation:

$$HQ\ [\%] = [EDI_{calc} / PMTDI] \times 100$$

where EDI_{calc} is the EDI found in this study, and PMTDI is the established provisional maximum tolerable daily intake (PMTDI) (Table 2)

Table 2. Provisional tolerable weekly intake (PTWI) and provisional maximum tolerable daily intake (PMTDI) of essential elements (Mn, Se, Cu and Zn)

Elements	PTWI	PMTDI	References
Mn	49.7 µg/kg bw/week	500 µg/day (7.1 µg/kg bw/day)*	NRC, 1989 [18]
Se	30.1 µg/kg bw/week	300 µg/day (4.3 µg/kg bw/day)	EFSA, 2006 [19]
Cu	3.5 mg/kg bw/week	0.5 mg/kg bw/day	WHO, JECFA, 26 (1982) [20]
Zn	7 mg/kg bw/week	1 mg/kg bw/day	WHO, JECFA, 26 (1982) [20]

*bw – assumed to be 70 kg

3. Results and discussion

3.1. Levels of Mn, Se, Cu and Zn in four beef cuts

The results obtained for Mn, Se, Cu and Zn levels in four different beef cuts are presented in Table 3.

Table 3. Mn, Se, Cu and Zn levels (mean ± SD*) in selected pork cuts

			Levels (mg/kg)			
	Beef cut	n**	Mn	Se	Cu	Zn
1	Beef steak	7	0.110±0.049	0.088±0.052	0.783±0.206	46.068±17.335
2	Hind leg	16	0.131±0.054	0.179±0.054	0.743±0.310	42.286±14.616
3	Shoulder	6	0.126±0.061	0.081±0.051	0.826±0.164	68.165±15.119
4	Neck	7	0.146±0.050	0.088±0.045	0.829±0.282	58.055±16.016

*SD – standard deviation; **n – number of samples

The lowest and the highest Mn levels, respectively, in beef cuts were 0.110 mg kg^{-1} in beef steak and 0.146 mg kg^{-1} in neck. Mn contents in the literature have been reported in the range of 0.076-0.15 mg kg^{-1} in beef cuts [21] and 0.10-1.6 mg kg^{-1} in beef, beef with vegetables and beef breakfast samples [22].

The level of Se in beef cuts from this study was in the range 0.081-0.179 mg kg^{-1}. Se contents in the literature have been reported in similar ranges. Bilandžić at al. [21] established the Se level as between

0.087 mg kg^{-1} (neck) and 0.21 mg kg^{-1} (shoulder) in different beef cuts. Se contents in beef meat products have been reported in the range of 0.08-0.8 mg kg^{-1} [22, 23].

The Cu levels in beef cuts (0.743-0.829 mg kg^{-1}) in this study were close to Cu levels in beef cuts available to the population in the Croatian capital (0.61-0.84 mg kg^{-1}) [21]. De Sousa Ramos et al. [24] established the Cu content was 0.883 mg kg^{-1} in beef hamburger. Similar Cu levels were established in beef with vegetables (0.89 mg kg^{-1}) and beef breakfast (0.71 mg kg^{-1}) [22].

Beef is considered to be a source of Zn with very high bioavailabity [25], although the zinc level depends on the meat cut. This study showed that the lowest and the highest Zn levels, respectively, in beef cuts were 42.286 mg kg^{-1} in hind leg and 68.165 mg kg^{-1} in shoulder. In the literature, Zn levels in beef cuts varied between 38.8 and 56.3 mg kg^{-1} [21, 22, 24, 26].

3.2. Daily intake (DI) of trace elements

The average DI (µg/day) of Mn, Se, Cu and Zn estimated for the four beef cuts for an adult in Serbia are reported in Table 4.

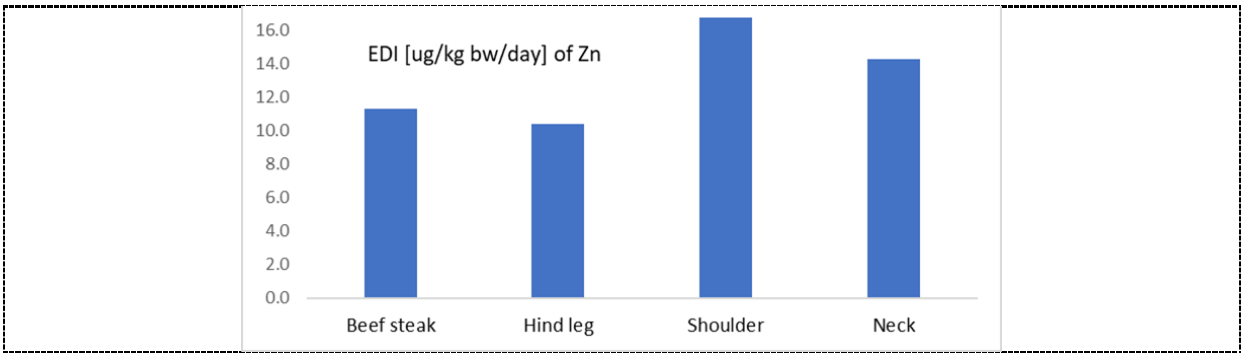
Table 4. Dietary intake, DI (µg/day) of the trace elements Mn, Se, Cu and Zn in beef cuts

	Dietary intake, DI (µg/day)			
Beef cuts	Mn	Se	Cu	Zn
Beef steak	1.892	1.514	13.468	792.370
Hind leg	2.253	3.079	12.780	727.319
Shoulder	2.167	1.393	14.207	1172.438
Neck	2.511	1.514	14.259	998.546

The mean average DI was 2.21, 1.88, 13.68 and 922.67 µg/day for Mn, Se, Cu and Zn, respectively. In the case of Zn, beef shoulder provides the highest intake of this element out of the four selected beef cuts (1172.438 µg/day). However, the DI of Se was the lowest from shoulder (1.393 µg/day), whereas the highest DIs of Mn and Cu were from neck.

3.3. Estimated daily intake (EDI) and hazard quotients (HQs) of trace elements

Figure 1 shows the EDI of Mn, Se, Cu and Zn, and their comparisons, considering the average Serbian per capita consumption of beef (17.2 g/person/day) [10].



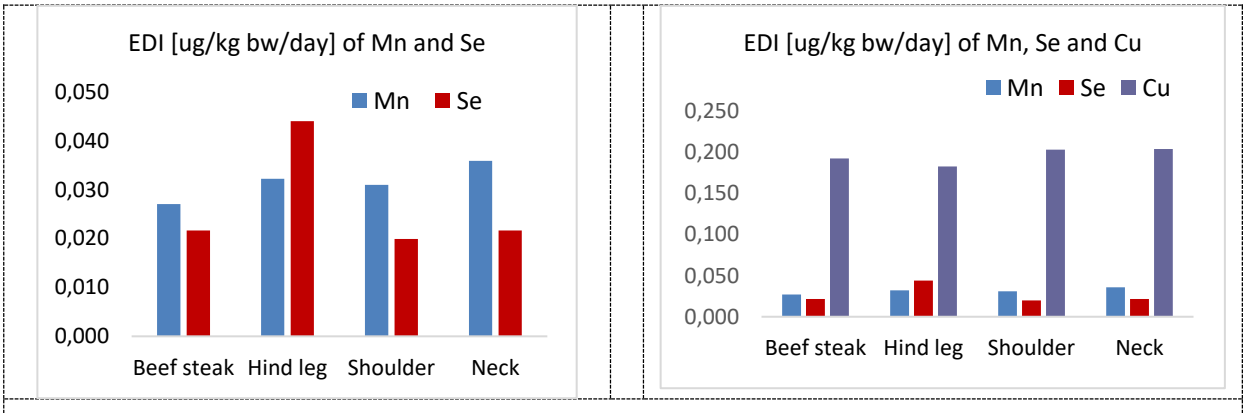


Figure 1. Estimated daily intake (EDI) of Mn, Se, Cu and Zn and their comparisons.

The findings of this study show that the EDIs (µg/kg bw/day) of the analysed trace elements differ widely. It is not possible to directly compare our EDI to those reported in the literature since our study was based on the intake from only one type of food. Among the four beef cuts in this study, shoulder had the highest content of dietary Zn (68.2 mg/kg) and, consequently, the highest EDI (Figure 1). EDIs for Mn and Se fell in a similar range, while the EDI of Cu was from six- to ten-fold higher than EDIs for Mn and Se.

Table 5 shows the hazard quotients (HQs) of Mn, Se, Cu and Zn, expressed as % of the calculated EDI in this study out of the PMTDI, recommended values set by NRC, EFSA and WHO (Table 2).

Table 5. Hazard quotients (HQs) of the trace elements Mn, Se, Cu and Zn

	Hazard quotient, HQ (%)			
Beef cuts	Mn	Se	Cu	Zn
Beef steak	0.38	0.50	0.04	1.13
Hind leg	0.45	1.02	0.04	1.04
Shoulder	0.44	0.46	0.04	1.67
Neck	0.51	0.50	0.04	1.43

The HQs obtained were between 0.04 % (in all four beef cuts) and 1.67% (Zn, shoulder). This shows that the obtained EDIs of Mn, Se, Cu and Zn, which refer to beef consumption by the Serbian population, are much lower than the provisional maximum tolerable daily intakes (PMTDI). Hence, element levels in examined four beef cuts were within safe limits.

4. Conclusion

This is a rare study to have determined the intake of trace elements (Mn, Se, Cu and Zn) from beef in the population in Serbia. The levels of the four elements in commonly consumed beef cuts (beef steak, hind leg, shoulder and neck) were determined. The results obtained show trace element ingestion from beef sources was acceptable for human consumption at nutritional levels.

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Potential of *Frangula alnus* to contribute to food safety: antibiofilm effect against *Staphylococcus aureus*

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Potential of *Frangula alnus* to contribute to food safety: antibiofilm effect against *Staphylococcus aureus*

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Abstract. Contamination by numerous food-borne pathogens is a major challenge facing the food industry daily. Even though there are many strategies in the fight against contamination, pathogens able to attach to different surfaces and form biofilms are the biggest concern. *Staphylococcus aureus* is a common food-borne pathogen capable of forming biofilms on foods and food contact surfaces. The prevalence of multidrug resistant *S. aureus* is high in raw products, high-protein foods and processed products. Bearing in mind *S. aureus* resistance to numerous antibacterial agents, the aim of this study was to investigate antibiofilm activity of an ethyl-acetate extract of the medicinal plant, *Frangula alnus*, against *S. aureus* ATCC 25923 and *S. aureus* ATCC 43300. It was demonstrated that extract reduced survival of both tested strains by up to 67%. Furthermore, quantification of biofilm biomass showed that extract possesses the extraordinary ability to inhibit biofilm formation of both tested strains (up to 91%). On the other hand, the effect on preformed biofilm was less pronounced and measured only for *S. aureus* ATCC 43300, wherein about 28% of preformed biofilm was eradicated. The results obtained in this study encourage further investigation of *F. alnus* as a novel antibiofilm agent or preservative in the food industry.

1. Introduction

Microbial contamination is the major problem in the food industry that leads to spoilage or contamination of food and, consequently, affects human health. This kind of contamination is unavoidable, since products in any step of processing could be in contact with microorganisms [1]. Pathogenic bacteria could exist and grow on equipment used in the food industry, which allows it to enter food, causing problems in food processing, packaging and consumption.

Despite all preventive measures, good hygiene and disinfection procedures, formation of biofilm by food-borne pathogens is a serious obstacle to safe food. Biofilms are dynamic microbial communities bound to biotic and abiotic surfaces and embedded in self produced extracellular polymeric substances (EPS). The biofilm formation is a complex process that begins with adhesion of planktonic cells to surfaces, followed by their growth and EPS production, resulting in mature biofilms [2]. The biggest issues with biofilms are that they are difficult to eradicate and bacteria in this form are very resistant to adverse events and environments. Furthermore, biofilms could serve as a source for cross-contamination of food, reducing the effectiveness of food processing and quality of food [3].

Among many bacteria present in food and able to form biofilms, *Staphylococcus aureus* is a very important food-borne pathogen and a leading causative of food-borne diseases around the globe. It can easily contaminate high-protein food such as eggs, milk, raw and cooked meat and soybean products [4]. In addition, the ability of *S. aureus* to form biofilms improves its survival on food-contact surfaces and the environment. Furthermore, *S. aureus* in biofilm is chronic source of contamination, since the dispersed cells from the biofilm can continue contaminating food and, thus, interfere with further food chain processes [5]. *S. aureus* has the main advantages of forming multilayered biofilm and being highly resistant to numerous antimicrobial agents.

In order to keep food being contaminated and to extend its expiration date, chemical disinfectants and preservatives are widely used in the food industry. However, constant use of these agents contributes to rapid increase of bacterial resistance and to the agents' harmful effects [6]. Natural products, such as extracts obtained from medicinal plants, could be safe alternatives to synthetic antimicrobial agents. Since plants have a wide diversity of secondary metabolites, plant extracts possess a number of biological activities that potentially could be exploited [7]. *Frangula alnus* is an interesting, traditionally used medicinal plant with various biological activities, among which its antibiofilm potential is poorly investigated [8].

Taking the above into account, the aim of this study was to investigate the antibiofilm activity of an ethyl-acetate extract of *F. alnus* against the food-borne pathogen *S. aureus*.

2. Methods

In this study two bacterial strains were used: methicillin sensitive (MSSA) *S. aureus* ATCC 25923 and methicillin resistant (MRSA) *S. aureus* ATCC 43300. Firstly, in order to investigate the effect of ethyl-acetate extract of *F. alnus* on bacterial survival, extract was two-fold diluted in Mueller Hinton Broth (MHB) in 96-well microtitre plates and bacterial inoculum (10^4 CFU/mL) was added. After 24h incubation, optical density was measured at 600nm (OD_{600nm}) on a microplate reader (Multiskan FC, Thermo Scientific, Shanghai, China). Tested concentrations of extract were in the range 0.031 mg/mL to 2 mg/mL. Antibiofilm testing included the effect of extract on biofilm formation and on preformed biofilms. The effect was quantified by crystal violet (CV) staining of biofilm biomass as described earlier [9]. Concentrations that were tested were selected based on previously published minimal inhibitory concentrations (MICs) of extract and were in range 1/16×MIC to MIC for biofilm formation and 1/2×MIC to 4×MIC for biofilm disruption [9]. Statistical analysis of data was done by applying GraphPad Prism 6.01 Software (Software, Inc) using one-way ANOVA with Dunnett's post hoc test. Data of antibiofilm testing are presented as mean value±standard deviation of three independent experiments done in hexaplicate.

3. Results and Discussion

The results obtained by measuring the OD_{600nm} showed that *F. alnus* extract had a strong effect on bacterial survival of both tested strains (Figure 1). The highest inhibition for *S. aureus* ATCC 25923 was at concentration 0.5 mg/mL (up to 60% inhibition), while for *S. aureus* ATCC 43300 the highest inhibition was 67% (0.0625 mg/mL). These results are in accordance with some studies that demonstrated antibacterial activity of *F. alnus* extracts at slightly higher concentrations [8, 10]. Differences in reported results could be attributed to extraction procedures, the use of different solvents as well as to growth conditions of plant which could interfere with its chemical composition. In addition, a potentially hormetic dose response was observed in both *S. aureus* strains. The hormesis response is known as a response of cells to different agents that can be either beneficial or detrimental, and which have specific, U-shaped dose dependence [11].

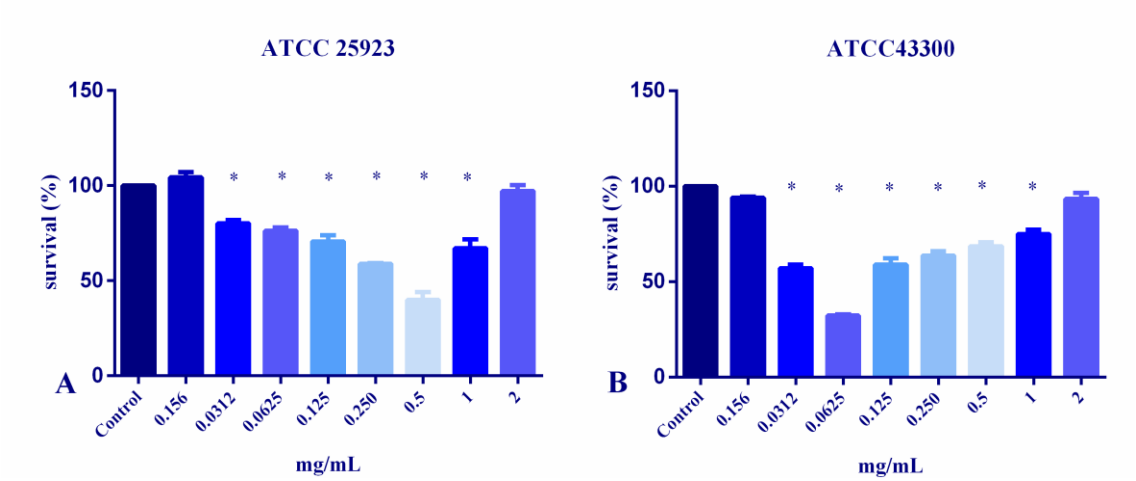

Figure 1. The effect of *F. alnus* extract on *S. aureus* A) ATCC 25923 and B) ATCC 43300 survival. * statistical significance $p \leq 0.05$

Furthermore, quantification of biofilm biomass revealed that *F. alnus* extract possesses an extraordinary ability to inhibit biofilm formation of both tested *F. alnus* strains in a dose-dependent manner. Biofilm formation of *S. aureus* ATCC 25923 was inhibited by 34.2 to 91.6 % at all tested concentrations, while for *S. aureus* ATCC 43300, inhibition was in range 32.8 to 73.6% (Table 1). It is well known that *F. alnus* is traditionally used because of its high amount of anthraquinones, and Lee et al. (2016) showed the inhibitory activity of this group of secondary metabolites against *S. aureus* biofilms [12]. Thus, the demonstrated antibiofilm activity of ethyl-acetate *F. alnus* extract could be attributed to these compounds. Moreover, it is interesting to note that subinhibitory concentrations of extract were successful in inhibiting biofilm formation, which is in line with a study describing this phenomenon for natural products [13].

Table1. The effect of *F. alnus* extract on biofilm formation by *S. aureus* strains

S. aureus strain	% of inhibition				
	1/16×MIC	**1/8×MIC**	**1/4×MIC**	**1/2×MIC**	**MIC**
ATCC 25923	34.2±0.04	56±0.13*	50.7±0.05*	84.1±0.14*	91.6±0.24*
ATCC 43300	32.8±0.06	34.3±0.09	35.5±0.07*	71.6±0.09*	73.6±0.11*

*statistical significance $p \leq 0.05$

On the other hand, the effect on preformed biofilm was less pronounced. Eradication potential was observed for *S. aureus* ATCC 43300, with eradication up to 28% at the MIC (Table 2). In contrast, for *S. aureus* ATCC 25923, a significant increase in biofilm biomass was observed at 2×MIC and 4×MIC (Table 2). Such result could be explained by the fact that some substances could trigger a stress response in biofilms, which leads to the production of extracellular polymeric substances, resulting in increased biomass [14]. Since that extracellular matrix is a means of biofilm resistance, increase of biofilm biomass could be considered as an adaptive response to the *F. alnus* extract [15].

Table 2. The effect of *F. alnus* extract on preformed biofilms of *S. aureus* strains

S. aureus strain	% of total biofilm biomass			
	1/2×MIC	**MIC**	**2×MIC**	**4×MIC**
ATCC 25923	106.8±0.07	107.2±0.3	135.2±0.5*	143±0.09*
ATCC 43300	85.5±0.17*	71.7±0.16*	74.9±0.15*	74.5±0.4*

*statistical significance $p \leq 0.05$

4. Conclusion

According to the results obtained in this study, ethyl-acetate extract of *F. alnus* showed strong ability to prevent biofilm formation by both tested *S. aureus* strains. Concerning the results, it is obvious that the inhibitory effect is strain and dose specific. Bearing in mind the problem of food contamination that food industry faces, this biofilm inhibitory activity is of a great importance. However, additional studies are needed in order to apply *F. alnus* as a natural disinfectant or preservative in the food industry and to improve food safety.

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Lactic acid bacteria: from food preservation to active packaging

Lactic acid bacteria: from food preservation to active packaging

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Abstract. Lactic acid bacteria (LAB) have acted in food fermentations through the ages due to their safety and resilience to specific harsh conditions of high salinity or low pH present in food and gut where they live. Their interaction with human technological development started in food but goes beyond, as some LAB contribute to the health of humans and animals as probiotics. The stress tolerance of LAB also makes them excellent, robust industrial microorganisms for production of lactic acid and other chemicals. The lactic acid market has had a high growth rate in the last decade mainly due to expansion of poly-lactide production. Poly-lactides are biocompatible, thermostable and biodegradable polymers of lactic acid, suitable for use in food packaging or in medicine, as scaffolds, implants or delivery systems. The ability of LAB to grow on complex waste substrates but efficiently produce selected isomers of lactic acid has positioned them at the core of bio-based packaging production, and this field is expected to grow in the future. Therefore, LAB are important for food – for preservation, flavour and packaging, but also beyond food – as probiotics, paraprobiotics and postbiotics. Recent trends in these fields of LAB application are analysed in this work.

1. Introduction

Fermentation was a turning point in food consumption and, consequently, in human history. It prevents food spoilage, human hunger and undernourishment and decreases food wastage [1]. This spontaneous process was the first application of biotechnology for safer, healthier and longer available nutritious food, which contributed to the change from hunter-gathering human communities towards agricultural society [2]. Today, fermented food consumption is rising, as is interest from the scientific community in new ways to apply fermentation as a processing method [3].

Lactic acid bacteria (LAB) are largely responsible for many traditional food fermentations since the dawn of consumption of these foods. All LAB have the common metabolic characteristic of producing lactic acid, and that has been the criterion to categorise a very diverse group of microorganisms as LAB. Taxonomically, *Lactobacillus* and 22 new related genera [4], *Lactococcus,*

Enterococcus, Streptococcus, Pediococcus, Leuconostoc and *Weissella* genera are most often considered LAB [5].

Representatives of LAB are used as starters, surrogates, probiotics, paraprobiotics and postbiotics in food research and technology, but LAB as industrial microorganisms are also used to produce lactic acid in biorefineries. LAB can use variety of substrates rich in fermentable sugars to grow and stereoselectively produce L(+) or D(-) lactic acid isomer for synthesis of FDA-approved polymers – poly-lactides [6]. This puts LAB in a unique place to be used as live or inactivated microorganisms in food and food supplements but also, as industrial microorganisms that can convert food waste and similar complex substrates into important compounds for food, chemical and other industries. As end products, poly-lactides are used for packaging, from nanocapsulated ingredients to disposable caps in the food industry, and from stents to scaffolds in medicine, because of their biocompatibility and thermostability [7]. Thus LAB are highly important microorganisms for the circular economy processes that are expected to dominate production in the next decades.

2. Lactic acid bacteria in food

Lactic acid bacteria in food are present as starters to provide acidity, preserve food and contribute to flavour. When applied as starters, production of acids and other antimicrobial compounds (peroxide, bacteriocins, etc.) plays a key role in food preservation [8,9] while proteolysis and exopolysaccharide production mainly affect food texture [10–12]. Very comprehensive reviews on LAB as starters [5,13], particularly in the dairy [8] and meat [14] industries are available, and commercial formulations contain mostly *Lactobacillus*, *Lactococcus* and *Streptococcus* species. However, artisanal and traditional production of fermented food depend on much wider microbial communities of LAB called non-starter LAB [15,16]. Recent advances in molecular diagnostics equipped scientists to study more deeply the microbiota of fermented food and feed. Processing of feed, through ensiling for example, also depends on naturally occurring microbiota and often results in the variable quality of the final silage [17]. In depth studies are expected to give new strains soon to be applied in industry, particularly for vegetable based food [18], which is increasing in market share. The need to adequately classify fermented food and feed is recognised by relevant bodies with the aim to stimulate and regulate the fermented food market [3].

Metabolic pathways in LAB are the result of an interplay between the abundance of nutrients present in their natural habitat – food or human gut – and stressors naturally existing in these environments, for example high salinity, very low pH or bile salts. The ability of LAB to survive microbial stress involves the "stressome" [19], which makes these bacteria robust enough for industrial application, but also makes them suitable as surrogates in challenge tests. With rising application of non-thermal technologies, LAB are often used to test and optimise treatment conditions, for example food preservation by pulsed electric field [20,21]. With generally recognised as safe (GRAS) status, LAB can be used without hampering or compromising food safety during product development.

3. Lactic acid bacteria beyond food

One subgroup of LAB has positive effects that go beyond safety or flavouring of food – these are probiotic bacteria. Probiotics are: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [3,22]. Not all LAB are probiotics, but probiotic LAB should potentiate food functionality, being a valuable contribution even in the treatment of some diseases. The main requirements for probiotic bacteria are to apply them live and to provide evidence about their health benefit for the host, while fermented food does not need to show any effect over the nutritional value of the food matrix. This is the main difference between fermented food and probiotic fermented food. However, evidence is accumulating that even cell components of probiotics or inactivated whole bacteria could provide health benefits to the host [23,24]. These findings open space for new types of products derived from probiotic bacteria – postbiotics and paraprobiotics. Postbiotics are defined as soluble compounds that can be produced by probiotic bacteria or released after the inactivation and lysis of bacteria [25,26] – enzymes, bacteriocins, organic acids, peptides,

exopolysaccharides etc. Paraprobiotics are inactivated probiotic bacteria which still affect human health in positive manner [27], but also can have some technological advantages over live bacteria – like lower susceptibility to contamination and better process control [28]. These are new concepts in food supplementation introduced in the last five years, but exploited previously in traditional food production, and all rely, firstly, on efficient biotechnological production of LAB biomass.

4. Lactic acid bacteria for food

Selected strains of LAB can produce L(+) or D(-) lactic acid with very high stereo-specificity of above 95% [29] on complex substrates like food wastes, by-products or residues, and which cannot be achieved in chemical processes. This is particularly important for production of poly-lactides. Physicochemical characteristics of poly-lactides are determined by the proportion of L(+) or D(-) lactic acid in the polymer [30], so the ability to produce one selected isomer over a racemic mixture has pushed LAB fermentation to dominate lactic acid's production routes.

Poly-lactide production will consume approximately 50% of all lactic acid produced until 2025, as the main driver of lactic acid demand [31]. This creates pressure to provide sufficient amounts of substrates for LAB fermentation without competing with food production. LAB can effectively use complex waste substrates and by-products rich in fermentable sugars like food waste or agricultural waste. When LAB are used for lactic acid production, the biomass remaining after fermentation could be valorised as high value feed additive [32]. However, to achieve high lactic acid yields on lignocellulose-rich substrates, which are by far the most abundant in nature, it is necessary to use genetically engineered strains or to perform a variety of treatments to release fermentable sugars into the fermentation media. Technological challenges limit faster adoption of novel substrates for lactic acid fermentation, but also, environmental impacts have to be thoroughly examined in order to select the best approach. For assessment of overall sustainability, chemical routes for poly-lactide production and processing also play an important role, but a very limited number of studies have addressed this issue. Integrative studies on lactic acid production from alternative substrates and consecutive poly-lactide production and processing are limited [30], but it is evident that new value chains for bio-based plastics are in the making. Recent legislation, like the Circular Economy Action Plan 2020 [33], will drive the field forward and clarify the path for best practices, more sustainable production and better end-of-life solutions in future.

5. Conclusions

LAB as safe microorganisms have found many roles in supporting human societies for millennia. They enabled some degree of food safety, contributed to metabolism of food and supported our health mainly through the gut. In the future, LAB will be further exploited, particularly in the production of functional fermented food, with our extended knowledge on microbial communities present in traditional production. Randomised clinical studies are needed for analysis of health benefits and more evidence-based applications of probiotics, paraprobiotics and postbiotics.

In biorefinery, the current challenge is to provide enough substrate for lactic acid fermentation by LAB due to the high demand for lactic acid. New legislation stimulates the circular economy and supports creation of new value chains, which can expand the contribution of bio-based polymers beyond their current market share of just 2%. Poly-lactides will play significant roles in packaging production in the future, especially for high end applications, as poly-lactides are FDA-approved. LAB-based processes will have to be soon expanded to more abundant substrates like lignocellulose, with technological advancements including substrate treatments, strain adaptions and innovations in process design.

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The possibilities of alternative protein use in animal nutrition

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The possibilities of alternative protein use in animal nutrition

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Abstract. The Food and Agriculture Organization (FAO) predicts there will not be enough food for human and animal nutrition until 2050. Global demand for animal protein for human consumption is increasing, and this consequently increases the price of these ingredients. This will open several challenges to provide enough animal feed. In the European Union, the use of processed animal proteins in pig and poultry diets is prohibited due to the bovine spongiform encephalopathy (BSE) legislation, while globally, the land availability for soy cultivation is limited. The European food market is dependent on huge import of soybean, which is the main source of valuable proteins and one of the main ingredients in feeds. Feed ingredients must not contain antinutritive factors that would adversely affect animal production and must have an acceptable price. Some of the alternative plant sources of protein are fava beans, peas, lentils, hemp, different grain seeds, etc. To find alternative sources of protein of animal origin scientists are increasingly investigating the use of worm, snail or grasshopper meals and also marine organisms, usually algae, shells or starfish. Single cell proteins are a specific kind of protein from different microbial sources, including microalgae, yeast, fungi, and bacteria.

1. Introduction

The Food and Agriculture Organization (FAO) predicts there will not be enough food for human and animal nutrition in the world until 2050. According to prediction, the production of meat (poultry, swine, and beef) will increase almost two times [1]. However, global demand for animal protein for human consumption is increasing, and this consequently increases the price of these ingredients. This will open several challenges to the global capacity to provide enough animal feed. However, in the European Union, the use of processed animal proteins in pig and poultry diets is prohibited due to the bovine spongiform encephalopathy (BSE) legislation, while globally, the land availability for soy cultivation is limited. The European food market is dependent on the huge import of soybean, which is the main source of valuable proteins and one of the main feed ingredients [2]. Researchers over the world are investigating the possibility of using other sources of protein for animal nutrition.

The most widely used protein sources in livestock production (monogastric animals) are corn, soybean meal, sunflower meal (cake) and rapeseed cake. From this point of view, plant species characteristic of an area can significantly reduce production costs, especially for small producers, and could be a source of alternative protein of plant origin. They must not contain anti-nutritive factors that would adversely affect animal production and must have an acceptable price. Some of the alternative plant sources of protein are fava beans, peas, lentils, hemp and different grain seeds. To find alternative sources of protein of animal origin for animal nutrition, especially in organic production systems,

scientists are increasingly investigating use of worm, snail, or grasshopper meal and also marine organisms, usually algae, shells, or starfish. Animal protein meals are good sources of essential amino acids, especially lysine and methionine, energy and minerals (calcium and available phosphorus) [3]. Insects can be sustainable alternative sources of protein, particularly if they are grown on biological waste substrates. In that way, they can effectively convert low-quality wastes into high-quality proteins. The main obstacles to animal-based protein use are consumer acceptance, regulatory issues and cost-effectiveness (competitiveness) [4].

2. Plant-based proteins

Plant-based protein consumption is increasing very fast globally. It has reached an annual consumption rate of about 7% [5]. Frequently used protein-rich plants are soybean, different legumes and oilseeds. Comparing their raw states, plant-based proteins have similar protein content to meat. On the other hand, they are rich in fibre and contain less saturated fat than meat [6].

At this moment, food producers and consumer society promote the reduction of animal-based protein consumption. It seems that plant-based proteins are a more sustainable and healthier option [7]. There is an extensive diversity of sources, and one of them is legumes [8]. Legumes (Family Leguminosae) can be divided into pulses (fava beans and peas) and oilseeds (soybean). They differ in carbohydrate and lipid contents [9] (Table 1). Pulses are low in fat and are valuable sources of vitamins (i.e. B12), minerals (i.e. iron, zinc, magnesium, calcium), and phytochemicals, positive for both human and animal health [10].

2.1. Legumes and oilseeds

A valuable but not enough exploited sustainable protein source is fava bean (*Vicia faba L.*). It has a low fat level (1-2%), and is rich in fibre (7-9%) and polyphenols. Fava bean is also a good source of lysine. [9]. From the agricultural point of view, fava bean has many environmental advantages, because it can fix atmospheric nitrogen and grow and develop at extreme climatic conditions [9,11].

Another possible source of protein is lentil (*Lens culinaris*). Lentil grain contains 40% starch, 25% crude protein and 15% neutral detergent fibre (calculated on dry matter basis), making lentil a nutritious ingredient for pig feed [12]. Lentil not used for human consumption can be an alternative feedstuff. It can substitute soybean meal in swine diets to reduce feed cost [12, 13]. The main obstacle to using lentil in pig nutrition is the presence of anti-nutritional factors in raw lentils. Adverse effects of feeding lentils to swine include reduced protein utilization and impaired meat quality and taste [14].

Table.1 Nutritional value of some legumes and oil cultures [15, 16]

	Soybean	Fava bean	Lentil	Pumpkin	Pea
Protein (g)*	41.00	27.99	28.60	57.76	25.70
Fat (g)	19.60	1.57	1.6	15.06	1.4
Carbohydrates (g)	7.60	54.70	57.60	6.84	53.70

* Calculated on 100g of dry matter

Pumpkin seed cake, which remains from the oil extraction process, could be a valuable protein-based ingredient in animal nutrition, especially to meet the protein requirements of ruminants [17]. It contains almost 60% protein, more than many commonly used oilseed-based feed ingredients [18], such as soybean meal [19]. Pumpkin cake is rich in amino acids lysine (3.2%) and methionine (1.8%), and improves the palatability of ruminant feed [20]. Substitution of soybean meal with pumpkin seed cake in the diet of dairy goats does not decrease milk production or change the fatty acid profile of milk [17].

2.2. Microalgae

To mitigate environmental issues due to the expansion of agriculture, use of land, and carbon emissions, microalgae could be a sustainable food source both for humans and animals. Compared to other alternative sources of proteins, microalgae contain valuable nutrients, such as omega-3 and omega -6 polyunsaturated fatty acids, and could compare to marine fish. The nutritional benefits of fish, such as essential omega-3 fatty acids and protein, often come directly from their consumption of marine algae [21]. Microalgae are also low in chemical contamination and have great purity.

Pigments are valuable components of microalgae that can act as antioxidants. They can improve animal health and be a natural colorant. The addition of algae into animal feed provides many benefits, such as improvement of growth and body weight, lowering feed intake, improve immune response, act as antibacterial and antiviral components (replacement of antibiotics), and microalgae enrich animal origin products with bioactive compounds, i.e., peptides and antioxidants. Recently, algae became a "cell factory" in the food industry and showed the rapid growth of the bio-economy in the feed industry. Microalgae are one of the few vegetable sources of vitamin B12 and iodine [6]. Microalgae's bioactive compounds could improve biological defences in the body against inflammatory diseases [22, 23]. However, the nutritional value of microalgae varies depending on the species, growth conditions, harvest location and season [6] (Table 2).

Table 2. Nutrient compositions in different microalgae species [24, 25, 26]

Microalgae species	Composition (%)		
	Lipids	Protein	Carbohydrates
Botryococcus braunii	33	39.61	2.38
Chlorella vulgaris	14-22	51-58	12-17
Spirulina maxima	6-7	60-71	13-16

3. Animal-based proteins

Contemporary aquaculture uses ingredients that are not suitable for human consumption to produce valuable proteins, which contributes to food security and upgrades the nutritional value of proteins as well. Due to the increase in global population, and the increase of the proportion of animal proteins in human diets, demands for valuable proteins are also increasing. Consequently, global aquaculture is facing continuing growth, followed by growth in global aqua feed production, which is expected to grow up to 73.15 million tons by 2025 [27]. In the past, fishmeal was the main protein source in aqua feed diets due to its nutritional profile that corresponds to the requirements of most aquatic species. Intensive use of this valuable ingredient together with limited sources has led to a drastic increase in its price. This was the driving force for scientists and feed producers to seek alternative protein sources and contribute to sustainable aquaculture.

The goal of sustainable aquaculture was to find alternatives by utilizing agro-industrial by-products, food leftovers, former foodstuffs, new by-products (e.g. from green biotechnologies), new protein-rich feeds produced by recycling unused biomasses (earthworms, insects) and aquatic resources (algae). Insect and worm meals had already consumed in many countries of South Asia, Africa, and Latin America. Several years ago, they started to be present on the global market. They are considered as a sustainable protein source and a possible solution for the replacement of animal-based protein [28]. Regarding nutritional quality, insects are good sources of protein and fat (Table 3). They also contain all essential amino acids necessary for human and animal health. They are rich in polyunsaturated fatty acids, vitamins and minerals. However, insects contain the antinutritional factor, chitin, which can reduce protein digestibility and can cause allergy. The nutritional value of insect meal differs depending on the species, phase of development, and type of feed.

The most frequently used insects in animal nutrition are yellow mealworm (*Tenebrio molitor*), black soldier fly (*Hermetia illucens*) and house cricket (*Acheta domestica*). The amino acid profile of *T.*

molitor meal generally meets fish requirements, and, in some cases even surpasses these requirements. In comparison to fishmeal, insects are generally low in omega-3 fatty acids that are required for optimal fish quality. However, it is possible to influence insect meal composition by rearing insects on substrates rich in omega-3 fatty acids. For example, if *T. molitor* is fed on bio wastes containing linseed as a source of omega-3 fatty acids, the fatty acid composition of the insect meal will be changed. Apart from the nutritive value of insect meal, safety should be also taken into consideration when using bio waste as a rearing substrate. Microbiological safety, heavy metal content and screening of pesticides residues in insect meal are very important in evaluation. Insects are also recognized as a valuable source of antimicrobial peptides, which do not contribute to the development of natural bacterial resistance. When used in animal nutrition, insect antimicrobial peptides can inhibit the growth of potentially pathogenic intestinal bacteria. Larvae of *T. molitor* are recognized as a source of protein with antimicrobial activity, which is active against Gram-positive bacteria and fungi [29, 30].

Table 3. Nutritional value of animal-based protein raw materials [31, 32]

	Fish meal (III quality)	Meat meal (III quality)	Worm meal
Protein, g	60	50	49-69
Fat, g	10	15	10-27
Ash, g	20	30	5-8

4. Single-cell proteins

Single-cell protein is the biomass or protein extract from pure or mixed cultures of algae, yeasts, fungi or bacteria. It can be used as an ingredient or a substitute for protein-rich foods and is suitable for human consumption or as animal feeds [33].

Single-cell protein was first mentioned in the 1960s to describe protein-rich foods produced from yeasts as dietary supplements for livestock and humans. Single-cell protein was considered as an alternative protein source that might fill a gap in food and feed production [34]. The growth of microorganisms, more rapid than that of the higher plants, makes them very attractive as high-protein crops; whereas only one or two grain crops can be grown per year, a crop of yeasts or moulds can be harvested weekly, and bacteria can be harvested daily [35]. Single-cell protein-based meal has the potential to provide the animal feed industry with a sustainable, renewable feed ingredient to make up for the deficiencies of plant-based meal and reduce the need for fishmeal in diets [36]. Single-cell protein is currently produced from a limited number of microbial species, particularly when considering human consumption. The range of sources for Single-cell protein used in animal feed is broader than that approved for human consumption and is expanding. Bacterial single-cell protein generally contains 50-80% protein on a dry weight basis and the essential amino acids [38].

5. Conclusions

Alternative proteins, plant or animal origin, could be used as substitutes for traditional feed ingredients. It requires research attention and interest in the media and wider forums as a way to fulfil the nutritional needs and food demands of the growing population. Some results show that numerous protein alternatives can have important environmental and health benefits. Most protein alternatives have resulted from the fourth industrial revolution and promise other advantages such as greenhouse gas reduction. Alternative proteins also promote food security by decreasing land usage to grow animal feed for the production of human food.

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Selected physicochemical, technological, and hygienic characteristics of artisanal and sausages produced with functional starter culture

Selected physicochemical, technological, and hygienic characteristics of artisanal and sausages produced with functional starter culture

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Abstract. Fast acidification is one of the main factors of microbial stability of dry fermented sausages. Development of functional starter cultures for improving safety of sausages without altering their quality is under way. This study compared aspects of physicochemical, technological, hygienic and instrumental quality of sausages produced with or without functional starter culture. Finished sausages with starter had lower water activity and lower levels of enterobacteria and lactic acid bacteria, compared to artisanal ones. During most of the ripening, pH was lower in sausages with starter, but in the final products, the same pH was observed in both groups of sausages. In sausages with starter lower redness was determined than in artisanal sausages, while other parameters, colour and chewiness, did not differ significantly. Use of starter culture improved physicochemical, technological and hygienic characteristics of the final products.

1. Introduction

The antimicrobial factors acting in dry fermented sausages (DFS) can prevent growth and reduce the counts of unwanted microbiota. Fast acidification induced by starter cultures and drying in air-conditioned rooms are the two most important of many factors in the hurdle concept on which is based safety of industrially produced dry fermented sausages. Rapid growth of technologically useful bacteria and their main metabolic activity in the presence of sugar induce reduction of pH and at the same time, they are competitive to undesirable microorganisms. Development of a third generation of functional starter cultures is under way and presents the use of well adapted strains that occur in traditional artisanal foods and which do not negatively affect technological or sensory properties of sausages [1]. As another main safety factor, low water activity (a_w) suppresses growth of all microbiota and values of $a_w \leq 0.9$ defines the microbial stability of finished DFS [2]. So, the main aims of this study were to assess dynamics of physicochemical factors (pH, a_w) during ripening, as well as levels of undesirable (enterobacteria) and technologically useful microorganisms (lactic acid bacteria) in sausages produced with (S) or without (A) functional starter culture. The final DFS were also evaluated in terms of colour and texture quality. These preliminary results should provide the basis for further investigation of

technological and hygienic improvement of dry fermented sausages by use of selected starter culture of the third generation.

2. Material and Methods

Chorizo sausages were produced in a local meat plant from pork meat. A traditional recipe was used without nitrite as an additive. Half of each batter was produced with the starter Flora Italia from Christian Hansen Company (*Pediococcus acidilactici*, *Lactobacillus sakei*, *Staphylococcus carnosus*) and another half as artisanal DFS (without starter). The production process for both subgroups of sausages lasted 5 weeks at 12°C - 14°C.

Microbiological analysis: enterobacteria count (EBC) was determined on *Enterobacteriaceae* Count Plate Petrifilms incubated at 37 °C for 24h, lactic acid bacteria (LAB) on Lactic Acid Bacteria Count Plates Petrifilms at 30 °C for 48h.

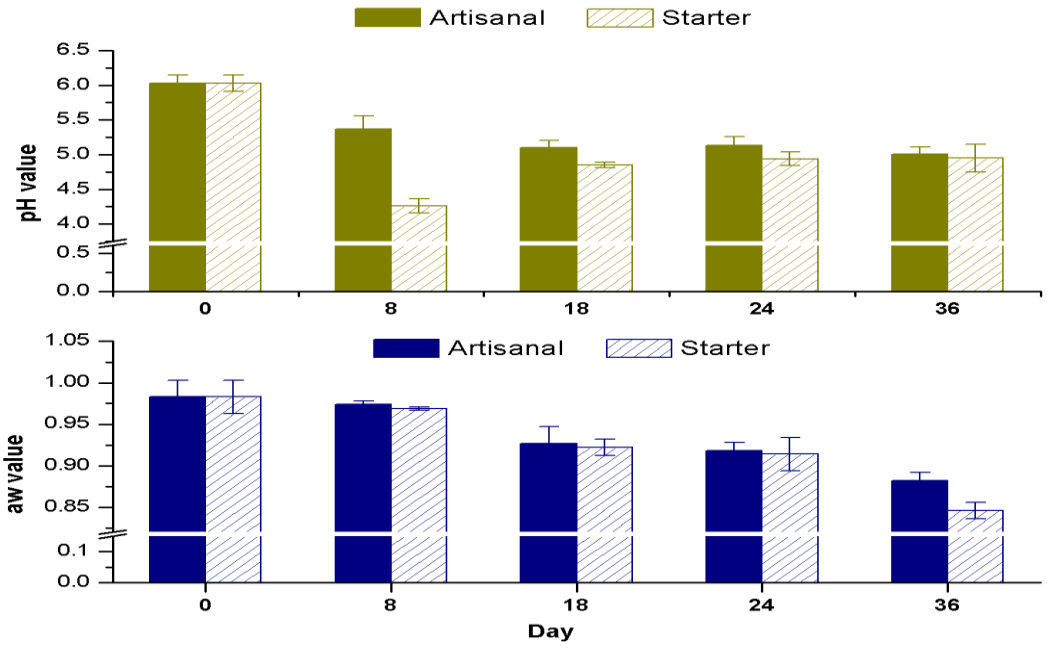
Physico-chemical analysis: examination of physico-chemical parameters in sausages comprised of determination of a_w and pH using LABswift-aw set equipment and pH meter, respectively.

Colour analysis was described in detail in [3].

For texture analysis (chewiness) the procedure of Marcos et al. was adopted, with small modifications [4].

3. Results

Figure 1 pH and a_w in sausages without or with starter during ripening



Sausages with starter had significantly lower pH during ripening ($p < 0.05$), compared to artisanal sausages while in the final products values became approximately the same.

Lowering of water activity during the production process was similar for both groups of sausages. However, in the final stage sausages with starter had significantly lower a_w ($p < 0.05$) compared to artisanal ones.

Table 1 Levels of *Enterobacteriaceae* and lactic acid bacteria in sausages without and with starter during ripening

Day	*Enterobacteriaceae* count log10 cfu/g ± SD		Lactic acid bacteria count log10 cfu/g ± SD	
	A	S	A	S
0	3.2 ± 0.4	3.2 ± 0.4	4.1 ± 0.2	4.1 ± 0.2 + starter (6 log 10 cfu/g)
8	6.5 ± 0.2[a]	4.3 ± 0.2[b]	8.9 ± 0.2[c]	9.5 ± 0.2[d]
18	5.5 ± 0.4[a]	1.5 ± 1.4[b]	9.3 ± 0.3[c]	9.2 ± 0.2[c]
36	2.6 ± 0.5[a]	< 1.0[b]	9.4 ± 0.3[c]	8.7 ± 0.4[d]

A – Artisanal sausages (without starter); S – sausages with starter; SD – standard deviation

Averages with different letters (for EBC - a or b; for LAB – c or d) in the same row differs significantly ($p < 0.05$)

Table 2 Colour and texture parameters of sausages without and with starter at the end of ripening

Colour parameters	A	S
Lightness	33.85 ± 4.37	33.82 ± 5.12
Redness	19.63 ± 2.29 [a]	17.86 ± 2.96[b]
Yellownes	16.47 ± 3.7	15.76 ± 3.55
Texture parameter (Chewiness)	9.39 ± 2.81	9.62 ± 3.7

Averages with different letters in the same row differs significantly ($p < 0.05$)
A – artisanal sausages (without starter); S – sausages with starter; SD – standard deviation

4. Discusion

The pH of artisanal sausages were within the frame considered as common for this type of DFS [5,6]. In sausages produced with starter culture, pH first dramatically dropped, probably due to the high metabolic activity of starter microorganisms, but then increased and reached the same level as in the artisanal sausages in the final stage of production.

Levels and changes of a_w in artisanal and sausages produced with starter culture were also within the frame considered common for this type of DFS [6,7]. Compared to artisanal, sausages with starter culture had similar or slightly lower a_w during most of the ripening, but reachhed significantly lower values in the final stage of production.

Patterns and levels of LAB during the production process were generally similar to those reported for this type of DFS [8,9]. Lactic acid bacteria in sausages with starter showed somewhat higher values at the beginning of production, but in the final products, LAB levels were considerably lower than in artisanal DFS. Higher susceptibility of starter culture strains to lowering of a_w and other stress factors may cause such results.

In artisanal sausages initial *Enterobacteriaceae* loads increased during the first part of the ripening, but then decreased to moderate numbers in the final products, mainly as a consequence of lowering of a_w. In sausages with starter culture levels of this group of bacteria were lower during the whole ripening process, compared to levels in artisanal sausages and reduced to under the detection limit in final products. Faster acidification of sausages with starter culture probably inhibits growth of *Enterobacteriaceae* during the first part of ripening and consequently is responsible for their lower number in the final products .

Regarding lightness, yellowness and chewiness no differences between artisanal DFS and sausages with starter were observed. However, significantly lower redness value in sausages with starter culture is probably because of faster acidification which intensifies partial or total denaturation of myoglobin.

5. Conlusion

Selected starter culture improved physicochemical and hygienic characteristics of the chorizo sausages. Moreover, in sausages with starter culture there are no changes of instrumental color and texture parameters, with the exception of redness.

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The path from protein profiling to biomarkers: The potential of proteomics and data integration in beef quality research

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The path from protein profiling to biomarkers: The potential of proteomics and data integration in beef quality research

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Abstract. This study aimed to provide an overview of the strategy of meat quality biomarkers identification from protein profiling to the establishment of putative protein biomarkers with a focus on beef tenderness and colour traits. Further, the current knowledge gained by data-integration, also known as integromics, of published meat proteomics studies during the last decade is briefly discussed in terms of the current list of protein candidate biomarkers revealed using different proteomics platforms and evaluated by proteomics-based approaches. The main biochemical pathways underlying the determination of tenderness and colour traits as important beef eating qualities revealed by bioinformatics analyses such as Gene Ontology annotations, pathway and process enrichments are further considered. This paper also addresses the potential of integromics and data-mining, in the era of big data and data analytics, to broaden our knowledge on the biochemical mechanisms underlying the conversion of muscle into meat and the consequences on beef sensory quality traits (tenderness and colour). Finally, the emerging interest of using such gathered and shortlisted protein biomarkers for first validation and then early *post-mortem* prediction of the potential quality of beef carcasses is highlighted.

1. Introduction

The production of meat, mainly beef, with consistent high quality is an ongoing challenge for farmers, meat industry stakeholders and meat research centres. In fact, the consistency of beef eating quality traits such as the appearance firstly judged at the point of sale by visual colour and degree of marbling and after cooking mainly evaluated by the three important beef palatability traits tenderness, juiciness and flavour intensity, directly influence the marketability of beef products and the re-purchase decisions by consumers [1-3]. However, several factors ranging from the farm-to-fork continuum [4, 5], including the interactions between the biological identities of the animal and the myriad biochemical muscle-to-meat conversion processes taking place after slaughter are known to affect the final beef sensory qualities [6, 7]. Many studies have revealed by means of conventional scientific methods some reasons of the variation in meat quality (for review: [7]) and certain of them proposed how to control during pre- or post-slaughter periods the biochemical processes occurring in the conversion of muscle to meat.

At the beginning of the 21st century, tremendous progress in high-throughput techniques (data-driven research), mainly OMICs known also as 'Foodomics' in the field of food sciences, for genes, proteins and metabolites analyses along with a considerable development of sophisticated statistical algorithms and bioinformatics tools allowed to explore meat quality and its development in ever greater detail and in a holistic manner [8, 9]. Therefore, OMICs techniques has the ability to generate large quantities of biological data about the genome, transcriptome, proteome, metabolome, etc. [10] from important

number of animals using tissue biopsies or fluids, to relate them with the important beef quality phenotypes including tenderness and colour to decipher the reasons of their quality variation. Among the OMICs approaches used in the field of meat science, proteomics seemed to be an effective quantitative analysis approach through the large-scale and systematic characterization of the global or partial analysis of the proteome (muscle tissue or fluids such as plasma) at given moment and environmental conditions to propose explanatory mechanisms at the origin of the variability of beef eating quality traits. Proteome analysis depends on five major steps; protein separation, identification, characterization, quantification and functional characterization, allowing the study of interactions between the proteins [11, 12]. The importance of proteomics in the field of meat science is illustrated by a large number of papers and reviews concerning its applications to characterize the cellular and molecular mechanisms behind most meat quality traits from all species, skeletal muscle in the context of livestock production, or biological traits and diseases in farm animals. Indeed, proteomics by means of gel-based and gel-free approaches coupled with mass spectrometry has emerged as a well-defined strategy in the field of meat science [13] which, through its in-depth characterization of the whole bovine muscle proteome (**Figure 1**), allowed to provide substantial data on the biochemical mechanisms underpinning important meat quality traits [8, 13, 14] including tenderness [12, 15-17], colour [18, 19], marbling [20], water-holding capacity [21, 22], and pH decline [23, 24], etc. In addition, proteomics was used to investigate the dynamic changes and modifications occurring in *post-mortem* muscle proteome [25, 26] and most importantly for the identification of protein biomarkers [6, 13].

In the following sections, the main steps of protein biomarkers discovery and current list of protein biomarkers and underlying pathways revealed by proteomics for the two major beef quality traits (tenderness and colour) are briefly summarised. Further, the potential of integromics in beef quality research to broaden our knowledge on the biochemical mechanisms behind the determination of beef tenderness and colour qualities is covered. The emerging interest in gathering published proteomics datasets on these two traits and the currently identified lists of putative protein biomarkers with the aim of creating unique repertoires is further discussed.

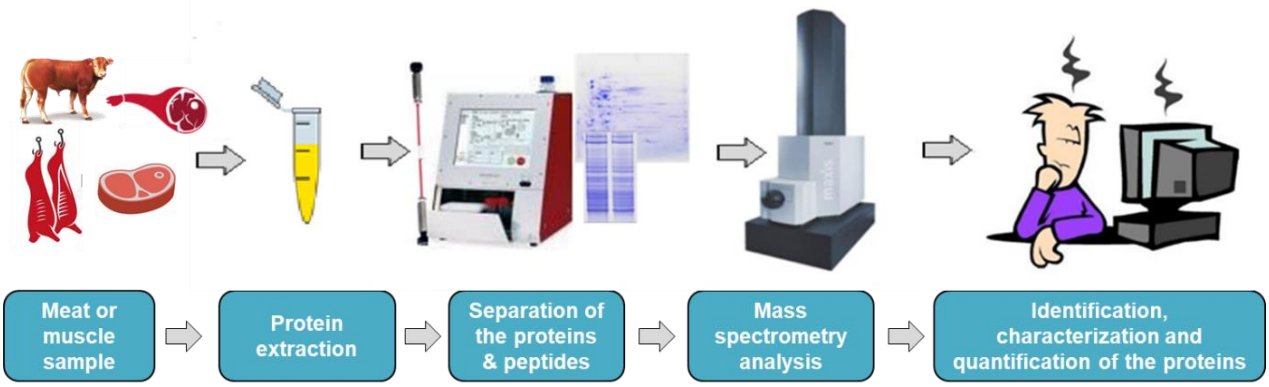

Figure 1. Main steps of proteomics analysis: from biological sample to identified proteins.
Schematically, proteomics analysis begins with the extraction and fractionation of proteins from a biological sample (biopsy of muscle taken early post-mortem or aged meat), followed by protein separation by electrophoresis using mono- or bi-dimensional gels and revelation (staining) of the separated proteins, then recovery of the bands or spots of interest and their identification by mass spectrometry after tryptic digestion. The peptides obtained are used for the identification, characterization and/or quantification of proteins by means of bioinformatics tools (in silico) as well as for statistical analyses to relate them with the phenotypes of interest.

2. The path from protein profiling (expressed proteins) to biomarkers: the input of proteomics

The main research area where beef research proteomics has been hugely applied is in the field of protein biomarkers discovery for which the pipeline and main phases are based on the strategy and path developed for biomarkers in medicine as described in several comprehensive reviews [27].

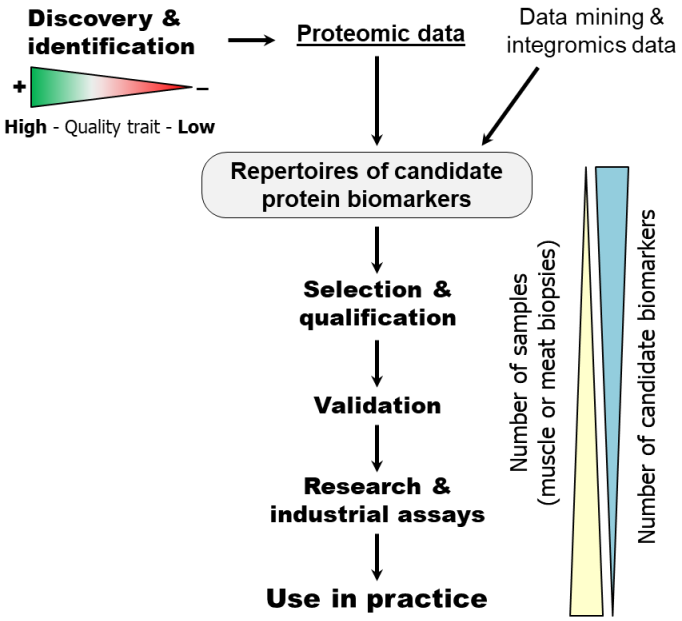

Figure 2. Brief description of the main phases of the meat quality protein biomarkers discovery path: from proteomic data generated on divergent quality traits (high quality *versus* low quality: tender *vs.* tough beef) to validation and development in practice for the carcasses and meat quality potential management/prediction.

During the discovery and identification phase, lists of 10 to 100 proteins with different abundances between two compared conditions (i.e., tender vs. tough) are identified before their qualification and validation to less than 10 biomarkers at the end of the path before practical use. Data mining of published proteomics play an important role in the preparation of the repertoires of candidate protein biomarkers to identify the robust ones (identified several times in independent meat proteomics experiments) that can be selected for qualification in the next steps.

The pipeline of biomarkers discovery was adapted to animal production and meat quality (**Figure 2**), more importantly in cattle for beef tenderness, and composed of six major phases including (i) discovery/identification, (ii) qualification, (iii) verification, (iv) research assay optimization, (v) industrial validation and (vi) commercialization [12, 15]. In all these phases, the quantification of the abundance of the proteins and their analyses using appropriate statistical algorithms along with bioinformatics tools is a pivotal step. Indeed, most research that is conducted on biological samples attempts to maximize the detection of differential proteins, although this is related to the sensitivity and precision of the instruments, while minimizing the number of samples required for analysis (**Figure 2**).

Therefore, the first phase consists in a comparison of groups of samples (mainly muscle biopsies and recently other biological sources such as plasma or meat exudates are tested) divergent for one phenotype, for example beef tenderness with high (tender) and low (tough) quality evaluated by sensory panellists or instrumental methods such as Warner-Bratzler shear force [12, 16]. The samples are mostly selected using specific thresholds that correspond to a consumer's acceptance or satisfaction with the quality of a meat product [28]. During the discovery or identification phase, the main objective remains an increase as much as possible of the number of candidates related to the variability of the beef trait of interest. Thus, the nature of the sample should be carefully selected to ensure an acceptable yield of candidate biomarkers on a maximum number of samples. The identification of new biomarkers thus consists in achieving a trade-off between the need to use a particular tissue or bio-fluid, and the need to identify the most exhaustive list of candidate protein biomarkers possible on a large number of samples. Accordingly, the samples are in general of the same conditions such as the same muscle (especially M. *longissimus thoracis*), same breed or gender, animals reared under the same production system, to analyse only the trait of interest. This allows reduce confused factors and bias, leading to accurate identification of proteins differentially abundant between the divergent meat quality groups [17].

In terms of methodology, the first discovery phase is composed of several steps from sampling and categorization of the samples based on the phenotype of interest at a specific threshold, protein extraction (using total protein extracts; sarcoplasmic proteins or myofibrillar proteins depending on the objective) till the identification of the differential proteins by mass spectrometry and their quantification. The current separation technologies are playing an important role in this respect by ensuring high reproducibility and sensitivity of the identification. In fact, the identification can be done either at the proteins or peptides levels after protein digestion of each sample mainly using trypsin, and is referred to as 'top-down' or 'bottom-up' approach, respectively. In the former approach, one- or two-dimensional electrophoresis coupled to mass spectrometry is the most common and successful method for analysis, separation and identification of the proteins. Although electrophoresis-based methods have been extensively used in the first years, the most suitable modern technology for protein biomarker discovery remains mass spectrometry in gel-free or label free manner [29]. Therefore, in the second approach and as an alternative to gel electrophoresis, the novel mass spectrometry platforms with very high sensitivity and resolution are used for the bottom-up approach allowing by means of shotgun proteomics better repeatability and complete analysis of the proteome or the targeted sub-proteomes (*e.g.* mitochondria).

When the candidate protein biomarkers have been identified and confirmed using several statistical and chemometrics analyses (*i.e.*, significant correlations with the phenotypes of interest; good splitters in decision trees; high importance in projection (VIP) scores in partial least squares; good separation of quality classes in clustering analyses…etc.), the next step is their evaluation (qualification) and validation in large scale. This phase requires high-throughput tools and if possible those that could be used in practice by the industry to manage meat quality of an animal or a carcass [12, 30]. The input of meat scientist will play another important role to shortlist a subset of biomarkers based on the biological knowledge of each protein, the ease of quantification and other technical aspects mainly tested in the qualification step. Overall, the qualification phase follows the same optimised steps of the discovery one, therefore ensuring that the differential expression of the protein is detectable by the assay that will be used for its evaluation. This consists for example of assaying fast techniques for the qualification of biomarkers in acceptable number of animals or carcasses, allowing the confirmation of the differential abundance of the protein using a method generally different from the one used for the identification of the candidate biomarker. In this context, the candidate protein biomarkers can be tested using immune-based techniques such as western blotting [31, 32], Dot-Blot [23, 33, 34] and Reverse Phase Protein Array [15, 35, 36]; or label-free gel mass spectrometry tools, namely Selected Reaction Monitoring (SRM), Sequential Window Acquisition of all Theoretical spectra (SWATH) [37] or Parallel Reaction Monitoring (PRM) [38]. These steps along with those of integromics based on previously published studies [12, 16, 18] will allow to shortlist a small number of highly qualified candidate biomarkers to move forward in the pipeline of biomarkers discovery through an external validation phase.

Finally and very briefly, during the validation and development phases, qualified and validated protein biomarkers are mainly measured on an important number of animals (thousands of samples) representative of the slaughtering routine in the context where the protein biomarkers should be validated. During the validation phase, a modification of the detection method can be necessary, mainly because mass spectrometry is not accepted as a validation assay method. At this level, the objective will consist of determining the reliability and robustness of the targeted biomarker, as well as of its internal validation on a larger number of animals of high heterogeneity and preferably under industrial conditions. For example, during this step one can perform an evaluation of the specificity and sensitivity of the biomarker by means of appropriate methods using receiver operating characteristic curves among others [39]. To conclude, the advances on analytical methods and bioinformatics tools will enable in the future further access to detailed information on enzymatic events, physiological responses or metabolic status that would allow a better optimisation of the robustness and accuracy of the biomarkers.

3. Proteomics and beef tenderness biomarkers

Tenderness is considered as one of the most important palatability traits of cooked beef and a known driving factor that affects its acceptability along with the buying decisions by consumers who are willing

to pay more for tender cuts [40, 41]. However, variability in beef tenderness still occur and is related to several factors ranging from farm-to-fork [5, 9, 28], also described to be a result of complex interconnected molecular pathways [16]. In fact, the inherent variability of tenderness among beef cuts is mostly understood by consumers who associate it for example to the cooking methods or to the price of the meat cuts. However, the variability from the same meat cut, especially the valuable pieces such as ribeye, are a serious concern for both consumers and meat industry. Moreover, there is currently no consistent conclusion about the mechanisms of beef tenderization, but several studies have recognized that the meat tenderizing process involves myriad pathways such as the degradation of structural proteins, energy metabolism pathways, response to stress and oxidative stress, apoptosis and signalling pathways [6, 14] as confirmed recently by the integromics study of Gagaoua and co-workers [16].

In our quest for beef tenderness biomarkers, more than 100 proteomics studies were conducted in the last 20 years for several objectives. It is beyond the scope of this short paper to review the entire range of proteomics studies on beef quality and the reviews that were conducted. However, it is important to mention the recent ground-breaking integromics meta-analysis performed by our group on 28 eligible beef tenderness proteomics experiments (22 papers) from the literature. This analysis allowed to propose the first comprehensive list of 124 biomarkers (**Figure 3**) from which 64 were found in a minimum of two studies, allowing then to shortlist for future validation a robust panel of 33 biomarkers (bold and italics gene names in **Figure 3**) that were identified in at least four independent experiments [16].

ACTA1	**TNNT1**	MYL6B	DES	*VCL*	**CKM**	**TPI1**	IDH1	*OGDH*	**PARK7**
CAPZB	**TNNC1**	*ACTB*	*WDR1*	*COL1A1*	**ENO1**	LDHA	PYGM	*SLC25A11*	**SOD1**
FHL1	**TNNI2**	*CAPZA3*	MYOZ1	*COL1A2*	**ENO3**	*GPI*	UQCRC1	NDUFS1	**PRDX6**
MYH7	**MYLPF**	TPM3	*MYOZ3*		**GAPDH**	PGAM2	GPD1	ALDH1A1	*SOD2*
MYH1	*FLNC*	ACTN3	*PDLIM7*		**ALDOA**	*SDHA*	*AKR1B1*	*GOT1*	*PRDX1*
MYBPH	KLHL41	CSRP3	*PDLIM1*		**MDH1**	*PDHB*	*DLST*		*PRDX2*
MYL1	MYL3	*ACTN2*	*SMTNL1*		**PKM**	AK1	ATP5B		GSTP1
TNNT3	MYL2	MYH2	*TMOD1*	**A**	**PGM1**	*ACBD6*	*NNT*	**B**	MSRA
CA3	*RABGGTA*	EEF1A2	*VDAC1*	*HPX*	*STBD1*	*CALM2*	**HSPB1**	**HSPA9**	*PDIA3*
GDI2	HINT1	HBB	VDAC2	*CAVIN1*	*ANKRD2*	*FABP3*	**HSPB6**	**YWHAE**	*SH3BGRL3*
ALB	*AAMDC*	*NUTF2*	*TF*	*ADSS1*	*KCNJ15*	*SHC4*	**CRYAB**	*HSPA2*	*CSN2*
RAB21	*ZNF197*	*HIP1R*	*POR*	HIST2H2AA3	*ANXA6*	TP53	**HSPA1A**	*CCT8*	
PARP1	*ZFHX4*	*MPHOSPH9*	*MB*	LGALS1	TRIM72	*AHCY*	**HSPA8**	*STIP1*	
ITPR1	**E**			*PSMB2*	*PSMC2*	**F**	**HSPA1B**	**C**	**D**

A. n = 35	Muscle contraction, structure and associated proteins	**D.** n = 11	Oxidative stress proteins
B. n = 29	Energy metabolism	**E.** n = 36	Regulation of cellular processes, binding and transport
C. n = 11	Heat shock proteins	**F.** n = 2	Proteolysis

Figure 3. List of the 124 putative protein biomarkers of beef tenderness identified in the integromics meta-analysis of Gagaoua *et al.* [16] based on 28 proteomics-based studies performed on M. *Longissimus thoracis*.

The proteins are organised and highlighted by different colours according to their functional pathways; blue = muscle contraction, structure and associated proteins; orange = energy metabolism proteins; red = heat shock proteins; green = oxidative stress proteins; yellow = proteolysis; and black/grey = regulation of cellular processes, binding, apoptosis and transport proteins. The full protein names are given in detail in [16]. The proteins (gene names) in bold character are robust candidate protein biomarkers identified in a minimum of 4 independent proteomics studies. Those in italic were identified in one study only. The others with normal characters were identified 2 or 3 times.

Briefly, this meta-analysis allowed to identify in a robust manner the importance of the changing integrity of muscle contractile and structure proteins, energy metabolism enzymes, response to stress and oxidative stress proteins in the determination of beef tenderness, in that order of importance. Moreover, protein networks analysis delivered a functional annotation of the 124 proteins from M. *longissimus thoracis* and provided key insights into the interconnectedness among various pathways and processes in the muscle which are pivotal in producing high quality beef. Therefore, six interconnected pathways were identified to play a pivotal role in the determination of beef tenderness these being: (i)

Muscle contraction and structure development; (ii) Energy metabolism; (iii) Cellular responses to stress; (iv) Response to oxidative stress; (v) Proteolysis and (vi) Regulation of cellular processes, binding, apoptosis and transport. In addition, it seemed from the protein-protein interactomics that these six pathways and most of the proteins directly or indirectly impinge on apoptosis onset in *post-mortem* muscle confirming the importance of this early *post-mortem* phenomenon [6] in muscle to meat conversion and consequences on beef palatability traits, likely tenderness. The meta-analysis study further revealed the importance of mitochondria, thus suggesting to conduct more studies in the future to understand the specific role of mitochondrial metabolism *post-mortem* on tenderness development, and how this is related to differences between muscles with different proportions of fibre types [42], where mitochondrial metabolism differs.

On another hand, similar integromics meta-analysis of published proteomics studies were recently conducted by our group to create new biomarkers repertoires for beef colour [18] and the quality defect of dark, firm, and dry (DFD) beef, otherwise termed dark-cutting beef [43]. From our data integration experience, it seemed that meta-analysis and data-mining of published proteomics studies are very useful tools to gather and compare meat eating quality OMICs studies conducted under various platforms [16, 18, 44] or even from a single laboratory using the same proteomic platform [12] in the frame of integrated animal systems biology to draw robust conclusions. These analyses allowed also to shortlist protein biomarkers for validation along with broadening our knowledge on the underlying mechanisms compared to conventional (traditional) studies.

Further, in the era of big data and data analytics, and in a time when OMICs data is easier than ever to collect, an important limitation on fully elucidating molecular signatures of relevance relates to the 'shape' of data that is commonly generated. Within proteomics datasets, and OMICs data in general, the number of data recorded for each sample being vastly greater than the number of samples / phenotypes in a traditional (conventional) study and this challenges statistical inference. For the future, multi-OMICs approaches and sophisticated statistical and bioinformatics would allow further development in this topic to allow further progress in the pipeline of biomarkers discovery as well that of a better understanding of the mechanisms behind beef tenderization.

4. Proteomics and beef colour biomarkers

Lean beef colour is critical to fresh meat marketability as it often influences consumer purchasing decisions (indicator of freshness and wholesomeness) and attractiveness at the point of sale. Historically, the role of muscle proteins in meat colour have been identified including the major role of muscle fibre type [42], glycolysis and sarcoplasmic proteins [19], oxidation and myofibrillar structure [7, 45]. Proteomics has only recently been used to study the muscle proteome associated with beef colour stability and/or variation [46], allowing new insights about the underlying mechanisms. Indeed, proteomics was mainly used to investigate the biochemical basis of pre- and post-harvest aspects affecting colour and to identify predictive candidate protein biomarkers for colour stability [3]. Currently, there exist around 13 proteomics studies that aimed to study beef colour with the goal of identifying biomarkers that are correlated with one of the colour parameters [18]. Using an integromics data-mining approach, these studies were gathered by Gagaoua and co-workers in a unique repertoire of 79 protein biomarkers for 5 different muscles (**Figure 4**) with a major number of them (n = 59) from M. *longissimus thoracis* [18]. This protein list was also subjected to bioinformatics and pathway process enrichments, therefore allowing to confirm several biological pathways previously known to be involved in meat colour development, including energy metabolism (mainly glycolysis), contractile and associated proteins, proteolysis, chaperones and heat shock proteins, oxidative stress and cell redox homeostasis, and binding, cofactor and transport proteins, including signalling or apoptosis (**Figure 4**).

The analysis of the pathways governing colour stability showed that similar mechanisms are shared with tenderness, however glycolysis and other associated energy metabolism functional pathways are predominant for colour while muscle structure and contractile proteins were for beef tenderness determination [16, 18]. For beef colour proteomics and with a cut-off level ≥3 studies that identified a given protein, 27 potential biomarkers were shortlisted as robust from which β-enolase (ENO3),

peroxiredoxin 6 (PRDX6), HSP27 (HSPB1), phosphoglucomutase 1 (PGM1), superoxide dismutase (SOD1) and μ-calpain (CAPN1) were consistently reported (cut-off level ≥5) by multiple studies as being differentially expressed and having a pivotal role in beef colour.

The two integromics allowed to confirm the central role of energy metabolism in determining beef colour and tenderness [16, 18], as well as its role in determining *post-mortem* pH [43]. Apart from the role of energy metabolism in determining meat colour, the study highlighted the potential of integromics to reveal the importance of oxidative stress, cell redox and contraction pathways, and particularly their interactions, in beef colour. In fact, the changes in muscle structure and contractile proteins were proposed to have their role through light scattering from structural elements and the paleness of the meat surface. The oxidative/redox proteins were proposed to have a role in the onset of oxidation *post-mortem*, hence impacting beef colour, and importantly, colour stability during storage and retail display.

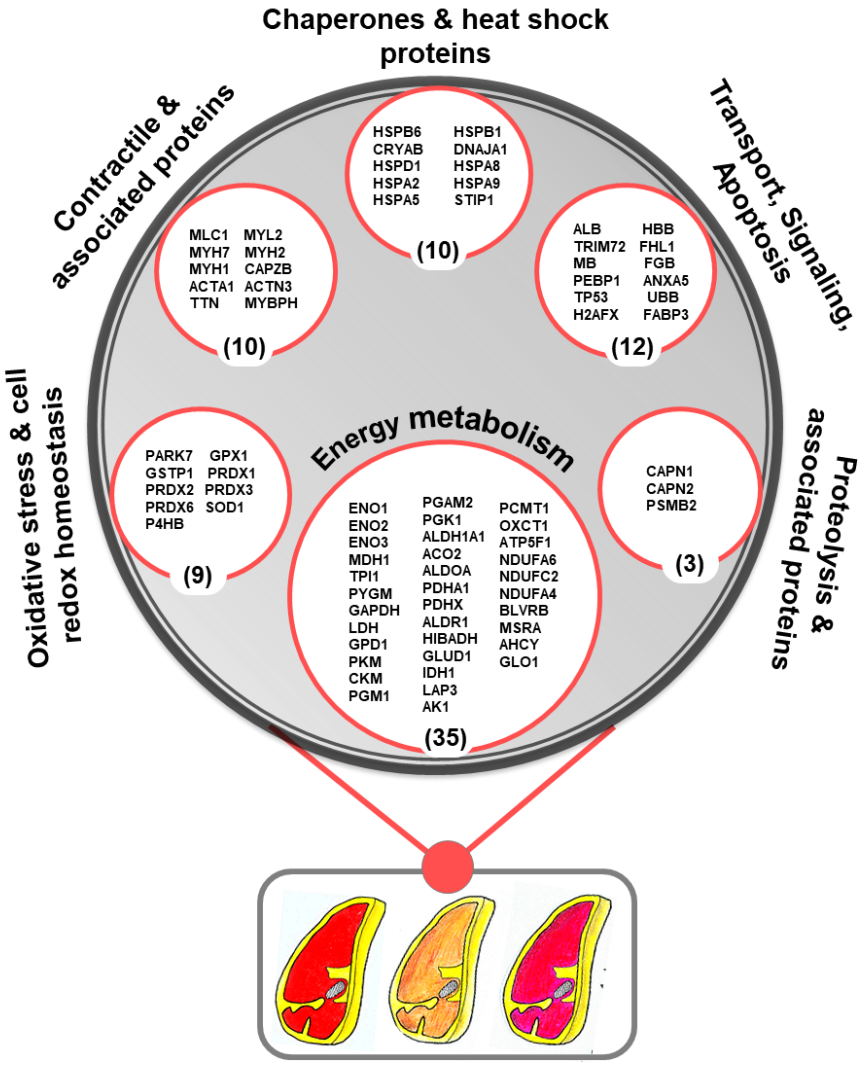

Figure 4. List of the protein biomarkers candidates (n = 79) of beef colour across five bovine muscles, but mainly *Longissimus thoracis* (n = 59), gathered in the integromics meta-analysis of Gagaoua *et al.* [18] to be correlated with colour parameters (*L**, *a**, *b**, *C**, *h**, MRA, OCR, RCR and R630/580). *Proteins were detected by gel-based and/or gel-free approaches coupled to mass spectrometry and organised by biological pathways and molecular functions to which they belong. The number of protein biomarkers for each biological pathway is shown in brackets. The full names of each protein (gene name) can be found in the original paper [18]. The graph is reproduced with permission from [18].*

5. Conclusion

To summarise, proteomics has provided during the last decade substantial data in terms of protein biomarkers related to or explaining the development of beef tenderness and colour stability. The recent integromics meta-analyses were valuable sources that synthesized the current knowledge and existing studies and beef proteomics datasets, hence allowing the building of reference databases (repertoires) of putative protein biomarkers of beef tenderness and colour traits. These repertoires allowed an in-depth description of the main biological pathways and mechanisms involved in the determination of these major sensory beef quality traits. In fact, such protein biomarkers repertoires may be enriched with newly identified proteins in future proteomics work, to allow further insights into the biological pathways involved in each meat quality trait and also to shortlist robust and generic biomarkers for validation and use for routine. For the future research, multi-OMICs will further increase our knowledge about these two quality traits by producing robust datasets to perform new types of analyses such as OMICs multi-layered networks. Also, more applications for data integration and analysis in the field of beef research would be demonstrated.

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Cellulose hydrocolloids in meat products: current status and challenges in developing functional food

Cellulose hydrocolloids in meat products: current status and challenges in developing functional food

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Abstract. Due to the growing health problems associated with the increased intake of saturated and *trans* fats, and the unbalanced n-6/n-3 ratio in the diet, in recent years numerous studies have focused on finding adequate substitutes for fat in meat products, while the meat industry made additional efforts to implement the obtained formulations (oleogels) in the standard production processes. Insoluble cellulose fibre in the form of microcrystalline cellulose has proven to be a promising ingredient in reduced fat, fibre-enriched functional food development, since it has been safely used as a food additive for many years with a known beneficial effect on human health. This review will discuss the recent advances of MCC application associated with alternative cellulose sources and processing technology, functional physico-chemical properties and potential as organogelator in fat mimetics. Finally, recently published data concerning its practical application in meat products as fat or starch substitutes will be presented.

1. Introduction

Daily activities, habits and diet can significantly improve health and quality of life. With the modern lifestyle, meat products have become one of the most important foods due to their nutritive value (biologically high value proteins, essential fats, soluble vitamins and minerals), convenience and taste. Therefore, they are extremely popular with consumers and the domestic meat industry produces them in a large quantity and assortment [1].

Meat products are made with high fat content and are deemed unhealthy. Some coarse and fine ground cooked products on the market, such as breakfast sausages or patties, depending on the local regulations, contain over 30% animal fat [2]. In meat products, the lipid phase is commonly present in the crystallized (solid) form, and so serves as a structure modifier, affecting the texture. The widespread consumption of *trans* and saturated fats, and unbalanced omega 6/omega 3 (n-6/n-3) ratio intake have been associated with number of adverse consequences on human health that led to higher incidence of coronary heart disease, inflammation, oxidative stress, endothelial dysfunction, several types of cancer, metabolic syndrome and obesity [3]. Thus, it is important to, ideally, replace animal fat in the meat batter with another lipid phase based on mono/polyunsaturated fats without negatively impacting processing, functionality and shelf life (stability during storage) of the product [4]. This replacement is an extremely difficult task, not only from the technological point of view but because of consumer

reaction to changes in textural and sensorial characteristics of meat products. Most of the pioneer studies focused on the fundamental understanding of the structuring principles and rheological characterization of fat mimetics [5]. However, in the last two decades, this issue has encouraged the meat industry to develop some new, applicable approaches and invest a lot of resources and effort in order to produce reformulated, healthier functional meat products [6].

There has been extensive research in the field of meat processing, with significant progress in understanding the interaction between raw ingredients and processing parameters. Some innovative options in research have opened due to new ingredients available on the market and because consumers' preferences and demands have changed, in terms of less processed, organic food, with reduced cholesterol, saturated fatty acid (SFA) and sodium contents, and food enriched with fibre or biologically active components of plant origin [7].

2. Developing of reduced/low-fat meat products

The International Food Information Council Foundation reported that at the moment of purchase, 64% of consumers consider health, 67% of consumers check for saturated fat in labels and about 50% of all consumers are likely to purchase a product with a "no saturated fat" claim [8]. The meat product can be labelled as "reduced-fat" if the fat amount is decreased by 30% compared to the standard product. Nutritional organizations indicated recommendations for an optimal fatty acid intake profile where the focus is on limiting the consumption of saturated fats to under 10% of caloric intake [9]. Therefore, due to the appealing aspect, convenience and versatility, consumers are very interested in using low-fat meat products as part of the healthy diet, as long as appearance and taste of the final product is not changed above the acceptable limit [8].

Two main strategies in producing reduced/low-fat meat products are developed. The first one considers the use of low-calorie ingredients, while the second implies the replacement of the animal fat with highly unsaturated vegetable and marine oils or lipid-rich raw materials of plant origin [1]. Since fats are one of the ingredients with major flavour, mouth-feel and overall eating-pleasure capabilities in meat products, while interesterified fats are less appreciated by consumers, the approach related to oleogelation has shown to be advantageous. Moreover, approximately 50 patents concerning different fat mimetics have been filed in the last 10 years, suggesting the potential commercial value of its application in the food industry [5].

2.1. Application of oleogels

Structuring of oil media and designing of self-standing thermo-reversible viscoelastic gelator networks in the form of oleogels offers an interesting approach towards the development of nutritionally balanced meat products [10]. Incorporation of oils with oleogels in meat matrices as fat substitutes is a technological breakthrough, as this approach not only ensures the presence of polyunsaturated fatty acids (PUFA) in meat products but also the molecules that exert the gelling features could play an equally important role due to their possible direct or indirect bioactivity (e.g. dietary fibre) [3].

Dietary fibre, classified as a high-molecular weight oleogelator, is considered to be suitable for reformulated functional meat products because of the natural flavour and good water binding capabilities that prevent cooking loss [11]. Meaning, besides health benefit effects, it also provides economic profit to processors [12]. In addition to the importance of the oil phase, the nature of the gelling molecule (hydrophobicity, crystallinity, glass transition temperature) greatly affects the final oleogel's properties and efficiency [13]. Namely, the carbohydrate component determines the mechanical properties of oleogels, such as plasticity, cohesiveness and hardness (one of the most desirable characteristics of fat) [5]. Carbohydrates are especially appropriate for use in indirect dispersion methods where oil gelation is performed in the presence of water [3]. Oil-in-water emulsion stabilized by regenerated cellulose (RC) and carboxymethyl cellulose (CMC) has been shown to be extremely successful in the preparation of these oleogels [14]. Furthermore, ethyl cellulose also stands out among organogelators for meat product development because of the possibility of textural modifications similar to traditional formulations containing animal fat [15].

One of the newer promising organogelators is carboxylated nanofibre cellulose (cNF). Due to insufficient data related to the potential cytotoxicity and genotoxicity by cNF ingestion, previous research has mainly dealt with its application in edible and degradable food packaging. However, nano-size provides a high aspect ratio, specific surface area, high strength, stiffness and hydration, which is why cNF can produce a self-reassembled gel. Therefore, cNF has attracted great interest from the scientific public as a possible efficient filler for complex network matrices with high water content such as meat products [16].

3. Cellulose derived dietary fibre

The raw material of non-digestible dietary fibre is cellulose, a long chain carbohydrate polymer of glucose units linked by β-1,4-glycosidic linkages that build amorphous (paracrystalline) and crystalline regions [17]. The crystalline, rigid, linear part of the chain is of interest for the chemical, mechanical, or biological isolation of α-cellulose and production of functional ingredients that include microcrystalline cellulose (MCC), microfibrillated cellulose, nanocrystalline cellulose, nanofibrillated cellulose and bacterial cellulose [18]. CMC, methyl cellulose and hydroxyprophylmethyl cellulose (HPMC) are chemically modified cellulose. For example, CMC is obtained from alkali-cellulose after a reaction with monochloroacetic acid [18].

The most successfully commercialized form of crystalline cellulose is MCC (powder or colloid form) with well-established applications across diverse areas, especially in food and pharmaceutical industries [19]. It is estimated that the global market for the MCC, as a non-toxic, renewable and biodegradable material, will reach US$1360 million by 2024, with these two sectors as the biggest beneficiaries.

MCC has been widely investigated as a next-generation core material in various fields due to its excellent properties such as low density, high modulus, heat resistance, and transparency [20]. The physicochemical properties of MCC largely depend on its starting raw material and processing technology (extraction process) [18]. Namely, MCC that originates from non-wood sources, for example lignocellulosic materials from agricultural residues, is likely to have more impurities such as lignin, pectin and hemicellulose compared to MCC from wood and cotton sources. This indicates differences in surface area, molecular weight, particle size and shape, degree of crystallinity and polymerization, porous structure, moisture content and performance, and these are critical material attributes relevant for food applications [18,21].

3.1. The potential of MCC and CMC as hydrocolloids

Buttermilk powder, modified corn starch, wheat flour, soy protein concentrate, lupin protein and whey protein are commonly used extenders to replace meat[22]. However, many of these non meat proteins have allergen potential and this must be declared on the labels. To overcome this problem some alternative components can be used as functional ingredients to replace meat, including hydrocolloid carrageenan, xanthan, guar gum, konjac, MCC and CMC [17]. CMC and MCC have been used in many reduced-fat products as thickeners, suspendors, and gel and emulsion stabilisers, usually in combination with other gums. With the confirmed synergistic effect of these two hydrocolloids, and in order to obtain a more efficient fat substitute in the products, a commercially available product, Avicel, was formulated, which consists of co-dried MCC with about 10-15% CMC [23].

MCC and CMC are approved for use in foods as fibre additives and do not contribute to the caloric content of foods [24]. They have desirable functional physicochemical properties (gelation, water absorption, solubility, water holding capacity and pH) and can be used to improve stability and texture in particular in low-fat products [22]. Additionally, unlike soy protein, MCC use does not lead to changes in flavour. However, since a meat batter is a highly concentrated system, the functionality of novel ingredients, such as charged and uncharged fibre, largely depends on possible interactions between proteins and hydrocolloid that could lead to unexpected changes and affect quality attributes of final products [17,25].

3.2. MCC and CMC in meat products

Schuh *et al*. [17] investigated the effect of CMC and MCC at levels of 0.3-2.0% on structural/functional characteristics of emulsified sausages. They concluded that the addition of CMC (>0.7%) led to the destabilization of the sausage batter and a decrease in firmness and viscoelasticity. On the other hand, MCC was highly compatible with the meat matrix, and it improved firmness of the sausages, maintaining water-binding capacity at the level of the control sausages. Similarly, Gibis *et al*. [26] showed that replacing 10% of ground beef with a dispersion of CMC in concentrations higher then 1% led to destabilization of the microstructure, and poorer sensory quality and texture of fried beef patties. MCC could replace approximately 50% of fat in patties and improve the texture, with the best sensory scores obtained at 2% of MCC. Furthermore, these authors concluded that CMC at concentrations >0.5% is not suitable as a fat replacer in beef patties [26]. Oh *et al*. [27] investigated HPMC for the production of canola oil solidlike oleogels used as animal fat replacement in patty formulations at levels of 50 and 100%. HPMC oleogels significantly reduced cooking lost and made the texture of the patties much softer, with the highest overall acceptability at the 50% replacement level. Furthermore, HPMC oleogels showed good resistance against oxidation allowing, the formulation of healthier patties with SFA/PUFA ratio of 0.18 [27].

Recently, a new enhancement of meat product nutritional value is proposed and considers the replacement of starch with dietary fibre. Mejia *et al*. [28] evaluated the incorporation of a soluble (βG) and insoluble fibre (MCC), alone (1-3%) or in combination (1.5%), as a starch replacement in meat emulsions. They found that MCC did not change cooking loss, texture profile or colour parameters of beef emulsions, meaning that MCC or combined MCC/βG could be a good starch replacement that yields meat emulsions with fewer calories and greater insoluble fibre content.

In the United States (US), MCC has “generally recognized as safe” (GRAS) status and has gained recognition and applications within the food industry for over 40 years [18]. Regarding the constantly growing market of clean label products, meaning natural, minimally processed food, without artificial ingredients, and with ingredients that come from sustainable sources, MCC is on the list of unacceptable ingredients of major retailers. However, these chemical-sounding hydrocolloids are chemically modified polysaccharides derived from cellulose, the most abundant renewable resource in nature with a long history of safe use [5].

4. Nutritional and health benefits of insoluble dietary fibre

Mechanisms of MCC’s beneficial effects in humans are mainly indirect and include but are not limited to the control of gastric emptying and ileal brake (satiety effect), hypoglycaemic response (diabetes) and plasma cholesterol levels (cardiovascular disease) [18]. Additionally, insoluble dietary fibre defends the colonic epithelium from the harmful effects of ingested carcinogenic compounds [13,29]. It is suggested that daily intake greater than 50 g could exhibit these positive effects on human health, and this dose could be achieved by regular food or with supplements [13]. It is estimated, for example, that people in the US consume about 10 to 15 g fibre/day, while the US National Cancer Institute recommended a total daily fibre intake of 20 to 30 g/day, without exceeding 35 g/day [12]. In Europe, 3% fibre is required in a product before it can be labelled as a source of dietary fibre [26]. In any case, side effects such as flatulence, bloating, stomach pain, diarrhoea, and constipation must be considered when high amounts of fibre are included in the diet [30,31].

5. Conclusions and future work

So far, cellulosic insoluble fibre has been found to be of great importance in the production of new functional foods due to its low cost, abundant availability and effectiveness at low concentrations. In particular, cellulose fibre showed good potential in the formulation of alternatives to unhealthy fats in meat products. Nevertheless, oleogel technology has not yet been implemented in already established regular industrial processes, so extensive research is needed to provide additional insights into the structure, bioactive protection and bioactive delivery of oleogels as well as their interaction with the meat matrices and behaviour during processing, in order to standardize them and make it easier for practical applications in meat industry.

Promising results were obtained using MCC and CMC in the production of healthier cooked meat products. Therefore, further research should be directed towards the production of combinations of MCC and CMC, i.e. binary and ternary organogelator systems, and new forms of cellulose micro- or nano-structured lipid carrier. The possibility of using MCC oleogels in less minced meat products with mosaic cross section (e.g. fermented sausage), where the fermentation and ripening requires greater stability of oils in the gel structure, deserves study.

Regarding the proposed new strategies by the European Union in environmentally friendly and economically sustainable food production by 2030, further efforts should be focused on new directions in obtaining cellulose hydrocolloids and methodologies that would enable the exploitation of low-cost lignocellulosic raw materials (agricultural residues). This could be one way to reduce waste in the production chain and to limit the environmental impacts of intensive agriculture.

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Review of microbiological analysis of water in meat, milk and fish production in the Republic of Srpska (Bosnia & Herzegovina) in the period 2018-2020

Review of microbiological analysis of water in meat, milk and fish production in the Republic of Srpska (Bosnia & Herzegovina) in the period 2018-2020

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Abstract. Water is essential for life, and a satisfactory supply must be available to all. Improving access to safe drinking water can result in tangible benefits to health. This study analysed samples of water from meat, milk and fish production from Republic of Srpska (Bosnia & Herzegovina) sampled in the period 2018-2020. A total of 390 samples were examined. The aim of the study was to determine the microbiological status of water used in meat, milk and fish production in the republic, in order to identify the risks to food safety. Microbiological testing used methods BAS EN ISO 6222, BAS EN ISO 7899-2 and BAS EN ISO 9308-1/A1. The microbiological status of water used in the production of meat, milk and fish in the Republic of Srpska in the period 2018-2020 has significantly improved compared to previous years. It is important that the presence of pathogenic bacteria in the water is at a low level. However, there are concerns that almost one-fifth of the water comes from wells, which are not under constant surveillance. This is especially important given the possibility of well water contamination and consequent food contamination.

1. Introduction

The production and distribution of biologically stable drinking water should be a non-negotiable goal for water utilities, with the perspective of providing the same water quality to consumers as is produced at the treatment facility. This can only be achieved by adequate monitoring and control of microbial processes during water treatment and distribution [1]. Water supplies within food production premises should be subject to risk and hazard assessments to ensure that appropriate water quality is maintained throughout the production process [2, 3].

The presence of bacteria in drinking water *per se* is not an issue, as long as no pathogenic organisms are present: there are bacteria in drinking water, even in relatively high numbers (10^3 to 10^6 cells/mL), without consequences on human health [4, 5]. Water temperature is an essential factor influencing bacterial growth kinetics and competition processes. Drinking water temperatures typically range between 3 and 25°C in European countries [6], and fluctuate seasonally within this temperature range even within a single drinking water distribution system. Elevated water temperatures have often been associated with increased bacterial abundance in drinking water distribution systems [7, 8], and with higher numbers of indicator organisms such as coliforms or *Aeromonas*.

The microbiological quality of water is commonly defined as a maximum acceptable number or concentration of bacteria that do not constitute a health hazard [9]. *Escherichia coli (E. coli)*, intestinal

enterococci (EC), coliform bacteria (CB) and total colony count at 22°C are monitored in accordance with set monitoring frequencies. *E. coli* and intestinal enterococci are considered core parameters. To assess the quality of water intended for human consumption, the minimum level is 0 CFU/100 ml for *E. coli* and intestinal enterococci. In Republic of Srpska (Bosnia & Herzegovina), the limit value for total count at 22°C (TC 22°C) is 100 CFU/ml, and for total count at 37°C (TC 37°C), the limit is 20 CFU/ml. Also, CB, *E. coli* and intestinal EC must not be detectable in 100 ml sample of water [10].

Many infectious diseases of animals and humans are transmitted by water contaminated with human and animal excrement, which becomes a source of pathogenic bacteria, viruses and parasites (protozoa, parasite eggs) capable of surviving for different periods, and raises the health risk for many people throughout the world. In order to eliminate the risk related to disease transfer, water intended for mass consumption is treated and disinfected before use [11, 12]. On the basis of the results, adequate measures can be taken that include prevention of contamination and systemic disinfection. Indicator organisms are used to assess the microbiological quality of water. Many pathogens are present only under specific conditions and, when present, occur in low numbers compared with other micro-organisms. Whilst the presence of coliform bacteria does not always indicate a public health threat, their detection is a useful indication that treatment operations should be investigated [13].

The use of indicator bacteria, in particular *E. coli* and coliform bacteria, as a means of assessing the potential presence of water-borne pathogens has been paramount to protecting public health [14]. *E. coli* is a coliform bacterium and has historically been regarded as the primary indicator of faecal contamination of both treated and untreated water. *E. coli* occurs in the faeces of all mammals, often in high numbers (up to 10^9 per gram of faeces). This widespread occurrence in faeces, coupled with methods for the recovery and enumeration of *E. coli* that are relatively simple to conduct, has contributed to the detection of this bacteria being the cornerstone of microbiological water quality assessment for over 100 years. The presence of *E. coli* in a sample of drinking water may indicate the presence of intestinal pathogens. However, the absence of *E. coli* cannot be taken as an absolute indication that intestinal pathogens are also absent. *E. coli* are the only biotype of the family *Enterobacteriaceae* which can be considered as being exclusively faecal in origin [13, 15] and this species can make up to 95% of the *Enterobacteriaceae* found in faeces.

The water used during handling and processing of milk products can be potential sources of microbial contamination with possible negative consequences on food safety. Especially, the water used in maintaining the hygiene of milking and milk storage equipment is crucial to the quality and safety of the products. *E. coli* was isolated in 39.20% of samples of water [16]. Enterococci include a number of species that occur in the faeces of humans and warmblooded animals. The main reason for their enumeration is to assess the significance of the presence of coliform bacteria in the absence of *E. coli*, or to provide additional information when assessing the extent of possible faecal contamination. As such, they are regarded as secondary indicators of faecal pollution [15]. Routine basic microbiological analysis of drinking water should be carried out by assaying the presence of *E. coli* by culture methods [17].

In the period 2015-2017, 26.20% of 584 water samples were of unsatisfactory quality [18]. In a study by [19], it was determined that all water samples used in the production of milk and milk products were microbiologically safe.

The aim of this study was to determine the microbiological status of water used in meat, milk and fish production in the Republic of Srpska (Bosnia & Herzegovina), in order to identify the risks to food safety.

2. Materials and Methods

Samples of water from meat, milk and fish production were from the Republic of Srpska (Bosnia & Herzegovina), sampled in the period 2018-2020. A total of 390 samples were examined (122 in 2018, 127 in 2019 and 141 in 2020).

Laboratory testing of waters was performed at the Dr Vaso Butozan Public Veterinary Institute of the Republic of Srpska, Banja Luka. Microbiological examination was carried out according to the

Regulation [10]. This included enumeration of colony forming units (CFU) expressed as total count of bacteria cultivated at 22°C (TC 22°C) and 37°C (TC 37°C) according to BAS EN ISO 6222 [20], CB and *E. coli* according to BAS EN ISO 9308-1/A1 [21] and intestinal EC according to BAS EN ISO 7899-2 [22].

In our research and in the statistical analysis of the obtained results, we used, as basic statistical methods, descriptive statistical parameters. The research results are presented in tables and figures.

Results and DiscussionTable 1 shows the test results in relation to the total number of water samples (% satisfactory or unsatisfactory) for 2018-2020.

Table 1. Percentage of waters classified as satisfactory and unsatisfactory, 2018-2020

Year	Satisfactory	Unsatisfactory
2018	91.80	8.20
2019	81.89	18.11
2020	90.78	9.22

No source of water that is intended for human consumption can be assumed to be free from pollution. All sources have different microbiological qualities and could be subject to natural or manufactured sources of pollution that can result in the deterioration of water quality to the point where treatment is no longer effective in removing all of the contamination. Zero-probability level of microbiological contamination of drinking water does not exist [9]. It is incorrect to state that drinking water distribution and delivery systems should be sterile, but the active growth of microorganisms is considered indicative of failures in water processing units or distribution [23]. Water is used in various ways in milk production and dairy industry, thereby becoming part of the food intentionally, inevitably or accidentally. The contamination of the food by water-borne microorganisms occurs directly much more often, but indirectly too, after multiplication of these microorganisms on the cleaned surfaces of the equipment used [24].

The presence of pathogenic bacteria in the water supply system is a particularly worrying fact given that water must be microbiologically acceptable, which means that it must not contain pathogens [10]. A possible explanation for this is dilapidation and damage to water supply installations leading to water contamination. For this three-year period, there were on average 88.16±5.45% satisfactory and 11.84±5.45% unsatisfactory water samples. The results, compared to previous research [18], indicate the much better microbiological status of water in the republic. Use of contaminated water in the handling and processing of milk products can cause a higher potential health risk than the risk through direct drinking. This is due to the fact that multiplication of pathogenic micro-organisms can occur in milk and milk products with amplification of the load of the pathogens [25].

When it comes to water contamination in relation to the supply system, 82.41% of samples was from a public water supply system and 17.59% was from wells. Table 2 shows the test results (satisfactory/unsatisfactory categories) of water by supply system in relation to the total number of samples for the period 2018-2020.

Table 2. Percentage of waters classified as satisfactory or unsatisfactory by supply system in relation to the total number of samples, 2018-2020

Year	Water supply system		Well supply system	
	Satisfactory	Unsatisfactory	Satisfactory	Unsatisfactory
2018	94.78	5.22	42.86	57.14
2019	83.48	16.25	66.67	33.33
2020	92.05	7.95	88.68	11.32
$\bar{x}$±δ	90.10±5.90	9.81±5.75	66.07±22.92	33.93±22.92

Comparing the results of water testing in relation to the supply category, the significantly higher number of unsatisfactory samples of well water than reticulated water supply is noticeable, which is expected considering that the public water supply system is under daily control with regular chlorination. In contrast, well waters do not flow, stagnate and are not under constant control or are controlled very rarely, usually once a year as an official control.

Some studies revealed that wash water can be source of bacterial contamination for milk and further compromise the quality and safety of milk or milk products [26, 27]. Table 3 shows the average test results in % according to test parameter for the period 2018-2020.

Table 3. Percentage of wash water classified as unsatisfactory according to microbiological tests, 2018-2020

Year	TC 22°C	TC 37°C	EC	*E. coli*	CB
2018	5.74	5.74	1.64	1.64	1.64
2019	14.96	14.19	1.57	1.57	3.14
2020	8.51	7.80	3.55	3.55	4.26
$\bar{x}\pm\delta$	9.74±4.73	9.24±4.41	2.25±1.12	2.25±1.12	3.01±1.31

According to [15], *E. coli* are the only true indicators of faecal contamination; they are exclusively of intestinal origin and are found in faeces. Their presence indicates mostly fresh faecal contamination and, thus, points to serious shortcomings in protection of the specific water source, treatment of water and its hygienic safety. Faecal streptococci provide evidence of faecal contamination and tend to persist for longer in the environment than thermotolerant or total coliforms. Total colony counts are enumerations of the general population of heterotrophic bacteria present in water supplies. The enumerations can include bacteria with water environment as a natural habitat or those that have originated from soil or vegetation. Two incubation temperatures and times are used for total count, 37°C for 48 h to encourage the growth of bacteria of mammalian origin, and 22°C for 72 h to enumerate bacteria that are derived principally from environmental sources. In a study of testing water at milk collection points, 20.40% of unsatisfactory samples were found, of which *E. coli* and coliforms were detected in 10.20%, and faecal streptococci in 12.24% [28]. Analysis of water from milk collection points, originating from wells, showed 63.90% of samples were unsatisfactory [29]. The current results differ from these results of similar research and indicate the significantly more favourable microbiological status of water.

3. Conclusions

Research shows that the microbiological status of water used in the production of meat, milk and fish in the Republic of Srpska (Bosnia & Herzegovina) in the period 2018-2020 has significantly improved compared to previous years. It is especially important that the presence of pathogenic bacteria in the water is at a low level. However, there are concerns that almost one-fifth of the water comes from wells, which are not under constant microbiological surveillance. This is especially important given the possibility of well water contamination and consequent food contamination. For this reason, it is recommended that microbiological control of well water be performed more frequently for the purpose of timely disinfection, as well as consideration of the possibility of connecting facilities used for food production with water supply system.

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Occurrence of *Escherichia coli* in mussels (*Mytilus galloprovincialis*) from farms in Boka Kotorska Bay, Southern Adriatic Sea

Occurrence of *Escherichia coli* in mussels (*Mytilus galloprovincialis*) from farms in Boka Kotorska Bay, Southern Adriatic Sea

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Abstract. This study reports the occurrence of *Escherichia coli* in 243 mussel samples collected along the Boka Kotorska Bay (six harvesting areas), which is located in the Southern Adriatic Sea. Bivalve molluscs can concentrate contaminants from their water environment, so because of that, determination of *E. coli* levels is an important parameter for microbial pollution assessment in the investigated areas. The number of *E. coli* ranged between <18 MPN/100 g to 16 x 10^3 MPN/100 g of mussel soft tissues. In 243 bivalve mollusc samples, analysed during the period 2018-2019, 68.3% of them had low contamination levels, i.e. ≤ 230 MPN *E. coli*/100 g; 31.3% had between 230 and 4600 MPN *E. coli*/100 g, and 0.4% had > 4600 MPN *E. coli*/100 g. Statistical analysis of the number of *E. coli* in mussels established that the mussel farm vl. Duško Vlahović (M5) had the highest mean *E. coli* levels (949.00 ± 2541 MPN *E. coli*/100 g), while the lowest mean level was recorded in Boka mussels (M1) (149.20 ± 258.80 MPN *E. coli*/100 g). Boka Kotorska bay is classified as a Class B mussel production area because it has 32.9% of samples with *E. coli* MPN values between the 20-230 MPN/100g.

1. Introduction

Mussels are filter-feeding animals that process large volumes of water and small particles of phytoplankton, zooplankton, viruses, bacteria, and inorganic matter from the surrounding water they live in to obtain food. At the same time, various pathogens are stored in them, and therefore they can be indicators of pollution in the environment [1]. Consumption of raw or inadequately cooked mussels poses a potential risk for consumers because of many bacterial infections, especially *Escherichia coli* [2]. According to the current EU regulations (854/2004/EC, 2004) [5], mussel sampling sites have to be classified according to their suitability in terms of microbiological and chemical water quality [3]. Depending on the content of *E. coli* in mussels, all localities should be affiliated as Class A (<230 MPN *E. coli*/100 g mussel), B (<4600 MPN *E. coli*/100 g mussel), or C (4600–46,000 MPN *E. coli*/100 g mussel) areas [2].

The contamination of mussels is influenced by different factors, such as pollution sources, rainfall, salinity, temperature, and many others. In recent years, the Montenegrin coast, especially the coast of the Bay of Kotor, has been exposed to constant spills of waste from various industries, shipyards, hotels, and hospitals that are discharged into the sea and lead to pollution the aquatic environment [4]. This study aimed to examine the presence of *E. coli* in *Mytilus galloprovincialis* from farms in Boka Kotorska

Bay, Southern Adriatic Sea, during a one-year period to classify the production area and determine the existence of correlations between six sites from which samples were taken.

2. Materials and Methods

During the period 2018-2019, 243 samples (about 40 samples from each sampling site) of *Mytilus galloprovincialis* were collected from six harvesting areas: Boka mussels (M1), Cogi mar (M2), Goran Biga (M3), Slobo Vujovic (M4), Dusko Vlahovic (M5), Bosko Supica (M6). Each of these farms is located in Bay Kotorska Bay. Samples were taken randomly, packed in sterile bags, and sent to the laboratory. The sample used for analysis (10 g of flesh and intravalvular liquid) was obtained by homogenizing 10-15 mussels.

E. coli was quantified following ISO/TS 16649–3(2005) [7]. The most probable number (MPN) of *E. coli* was obtained using the 5-tubes and 3 dilutions method. From the whole mussel, we used flesh and intervalvular liquid (FIL), of which we measured a 75 g amount, diluted with the Tryptone salt water (1:3), and homogenized in Stomacher (2 min). Seventy mL of Tryptone salt water were added to 30 mL of this mix and were homogenized until 1:10 dilution. A 10 mL of the dilution was further added to 5 tubes of double-concentrated MMGB (Minerals Modified Glutamate Broth). Next, 1 mL of the 1:10 dilution was added in 5 tubes of single concentrated MMGB, while in the remaining 5 tubes a 1 mL of a 1:100 dilution per tube was added. All the tubes were incubated at 37°C for 24 h. Confirmation of *E. coli* was based on culturing 1 µL of positive tubes onto plates TBX agar (Tryptone Bile X-Glucuronide agar, Oxoid, Wesel, Germany) which were incubated at 44°C for 24 h. The presence of *E. coli* was confirmed by the growth of blue colonies. The number of *E. coli* in 100 g of sample was calculated based on the number of positive results in three dilutions, using MPN tables [7]. Statistical analysis of the results was elaborated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) and MS Excel. Descriptive statistics of each data set were computed. Statistical significance levels were set at 5 and 1%. All the results are presented in tables, figures, and graphs.

3. Results and Discussion

During the one-year study period, a total of 243 mussel samples were examined for *E. coli.* Results showed that the number of *E. coli* in 86 samples (35.4%) was < 18 MPN/100 g, in 80 samples (32.9%) was ≤ 230 MPN/100 g; (Class A areas), in 76 samples (31.3%) was between 230 and 4600 MPN/100 g (Class B areas) and in 1 sample (0.4%) was > 4600 MPN/100 g (Class C areas) (Fig 1.). The highest detected number of *E. coli* was 1.6×10^3 MPN/100g, found in one sample from Dusko Vlahovic (M5). If only one sample is out of range 20 - 230 MPN/100g, this harvesting area can not be classified as a Class A area (Reg. (EC) No 854/2004) [5]. Similar results were obtained in studies conducted in South Albania [8]. We recorded a slightly higher number of samples in this area where the *E. coli* MPN was > 4300/100g. In contrast, a study by Henigman et al. [9] reported much better results. On the Slovenian coast, during the tested period, 88.2% of samples did not exceed 230 MPN *E. coli*/100 g.

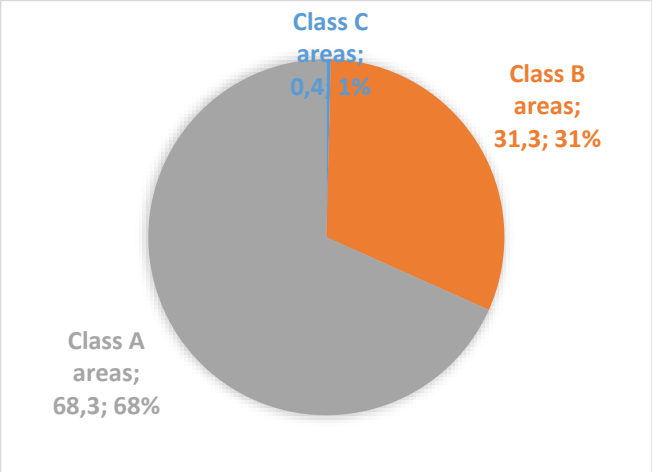

Figure 1. Classification of bivalve mollusks according to production areas

Numbers of *E. coli* in mussels from harvesting areas in Boka Kotorska Bay are shown in Table 1. Statistical analysis of the number of *E. coli* in mussels from six farms on the Montenegrin coast established that the mussel farm Dusko Vlahovic (M5) had the highest *E. coli* MPN values (949.00 ± 2541), while the lowest *E. coli* MPN value was recorded in Boka mussels (M1) (149.20 ± 258.80).

Table 1. Number of *E. coli* (MPN/100g) in Boka Kotorska Bay cultivated mussels

	n	$\bar{x}$	SD	SE	CV (%)	X max	X min
M1	41	149.20[a]	258.80	40.4200	173.42	1100	18
M2	41	149.40[b]	386.10	60.3000	258.43	2400	18
M3	41	238.00[c]	582.00	90.8900	244.48	3500	18
M4	40	658.50	1191	188.3000	180.85	5400	18
M5	40	949.00[abc]	2541	401.8000	267.80	16000	18
M6	40	294.90	447.70	70.7900	151.85	2400	18

The same letters indicate significant differences between groups: a, b, c, p<0,

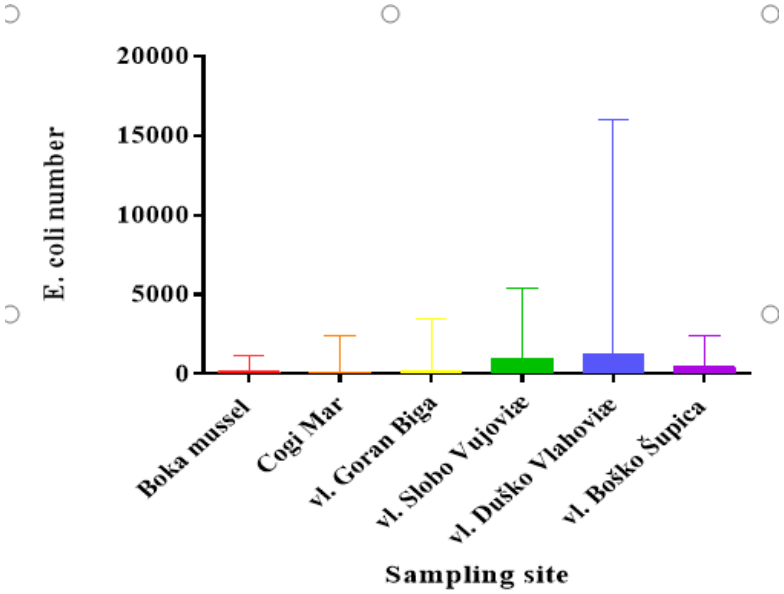

Figure 2. *E. coli* MPN in mussels by farms

Statistically significant differences were found between M1 and M5; M2 and M5; M3 and M5 ($p < 0.05$).

4. Conclusion

Montenegrin shellfish breeders have just recently begun to implement EU classification on shellfish harvesting areas. This research study demonstrated that the South Adriatic area of Boka Kotorska bay had been classified as a Class B mussel production area due to a high percentage of samples with *E. coli* MPN values less than 4600 MPN/100 g. Although the results obtained in six tested harvesting areas did not exceed upper limits laid down by the EU Regulation No 854/2004, all mussels caught in the area must undergo a depuration process before being placed on the market.

Since *E.coli* is a primary indicator of the microbiological quality of shellfish, this study will contribute to risk assessment in human consumption of this type of seafood originating from Montenegro.

Acknowledgment

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Establishing enhanced *Listeria* control: Coupling environmental monitoring with risk-based product testing

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Establishing enhanced *Listeria* control: Coupling environmental monitoring with risk-based product testing

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Abstract. Prevention and control of *Listeria monocytogenes* remains a challenge in food manufacturing facilities and methods adopted vary across different production systems and food categories. Regulatory policies also vary from region to region, although there is a convergence across the world towards risk-based approaches. Given these inconsistencies, the objective of this commentary is to reiterate two fundamentally critical components of *Listeria* control and prevention, and the potential benefits of actively coupling food contact surface testing and risk-based product testing programs.

1. Introduction

Foodborne illness associated with *Listeria monocytogenes* remain a significant global public health burden. Outbreaks of Listeriosis resulting from consumption of food contaminated with *L. monocytogenes* are related to a diverse range of products. Significantly, susceptible populations such as the elderly (>65 years), neonates and pregnant women, and immunocompromised individuals and those suffering from chronic disease conditions (cancer, diabetes, cardiovascular problems, etc.) account for majority of cases [1, 2, 3, 4]. Consequently, disease incidence is typified by high mortality rates of up to 30% [1,5]. Efforts to impact disease reduction have been targeted at various stages in the farm to fork supply chain, yet incidence of invasive listeriosis remains high across the world.

Unlike other pathogens, Listeria thrives in cool and moist environments and is problematic in foods stored at refrigeration temperatures. Unsurprisingly listeriosis outbreaks are predominantly associated with high-risk foods that are stored at these temperatures. Farber et al. noted major Listeriosis events over the past decade resulted predominantly due to contaminated ready-to-eat (RTE) foods considered to be high risk (which support growth of the pathogen) such as sliced delicatessen meats and soft cheeses [6]. More recently listeriosis cases and outbreaks have also been identified with low-risk foods (RTE foods which do not support growth of the pathogen), such as frozen foods (ice cream, frozen vegetables, frozen chicken products, etc.) [7, 8, 9, 10]. The term low-risk is ascribed to frozen foods for example due to their storage temperature state, conditions which do not support the growth of the pathogen. It should be emphasized invasive listeriosis is primarily problematic with the high-risk category of foods, quantitative modeling shows greater than 90% of these cases are caused by ingestion of RTE food containing >2000 colony forming units (CFU)/g [11]. Foods stored in vacuum or in modified atmospheric packaging with extended shelf life in refrigeration, allow growth of the pathogen to high levels during shelf life. The distinction of whether a food supports or does not support growth of the pathogen in RTE foods is thus at the center of developing a risk profile and importantly at the way both

the industry can implement management practices and how regulatory policies are framed and enforced across the world.

As consumer eating habits and diets evolve to comprise more RTE foods, there is also a growing concern of listeriosis risk with these foods and most global regulatory policies continue to target the presence of *L. monocytogenes* in RTE foods with zero tolerance for foods that support growth. Codex, European Union, Canada, Japan, New Zealand all employ a risk-based tolerance of 100 CFU/g with respect to RTE foods that do not support growth [11, 12, 13, 14, 15]. Current scientific information and data relative to the nature of the pathogen, potential growth in food, and public health impact (illnesses and outbreaks) indicate *L. monocytogenes* is a remote public health risk in low-risk foods such as frozen foods. Even still, U.S. regulatory policies (both U.S. Food and Drug Administration (FDA) and Food Safety Inspection Service (FSIS)) broadly enforce a 'zero' regulatory action limit for the presence of the hazard, *L. monocytogenes* across all foods without relying on a risk-based distinction [16, 17].

2. Current U.S. regulatory policies for meat and poultry products

FSIS, the agency under U.S. Department of Agriculture which oversees all food safety regulations relevant to meat and poultry products, enforces its *Listeria* rule across all RTE foods (reference) and provides specific recommendations to prevent cross contamination of post-lethality exposed RTE meats and poultry products. However, the rule also explicitly precludes Not-RTE food products with on-package validated cooking instructions.

Importantly, under FSIS regulations, RTE foods exposed to the post-lethality environment should implement robust sanitation programs, appropriate hygienic equipment design principles, pathogen testing regimen, and other pre-requisite programs including Good Manufacturing Practices (GMPs) as part of the food safety system. Adulteration is defined either by the presence of *L. monocytogenes* in the RTE food (contaminated food) or as resulting from food coming into direct contact with a surface that is contaminated with the pathogen. The FSIS *Listeria* regulation delineates three alternative methods to control *L. monocytogenes* contamination of post-lethality exposed RTE foods.

- Alternative 1: Use of a post-lethality treatment to reduce or eliminate *L. monocytogenes* **and** antimicrobial agent **or** antimicrobial process to suppress or limit the growth of *L. monocytogenes*.
- Alternative 2: Use of a post-lethality treatment **or** an antimicrobial agent or process to reduce, eliminate or prevent the growth of *L. monocytogenes*.
- Alternative 3: **Do not use** a post-lethality treatment or antimicrobial agent or process, instead **relies on** sanitation alone to control *L. monocytogenes* in their post-processing environment (sanitation controls)

A variety of validated post-lethality treatments are noted by FSIS and include steam and hot water pasteurization, radiant heating, high pressure processing, ultraviolet and infrared treatments, and drying to achieve low water activity. The agency expects a post-lethality treatment is designed to achieve at least a 1-log lethality of *L. monocytogenes* before the product exits the production facility.

Antimicrobial processes and agents used to suppress or limit growth of *L. monocytogenes* should be designed to achieve no more than 2-logs of growth over the shelf-life of the food. Antimicrobial agents include modified atmosphere packaging, addition of salt, organic acids and other additives, curing with nitrites, addition of growth inhibitors such as lactates and diacetates, which may be added to the formulation, finished food product or packaging material as appropriate. Common antimicrobial processes include fermentation, drying and freezing that are supported by studies documenting the effectiveness of these processes in limiting or suppressing growth during shelf-life.

No doubt a combination of the above strategies is encouraged to be used through the hurdle concept, including appropriate use of pH, water activity, moisture-protein ratio, and storage temperature to reduce the level of the pathogen during processing and to limit growth during shelf-life.

As stated above a third alternative is to utilize sanitation controls alone where a post-lethality treatment, antimicrobial process or agent are not applicable to control *L. monocytogenes* in the post-processing environment. A sanitation standard operating procedure may be incorporated as a pre-requisite program including the ability to escalate sanitation procedures to counter repeat positive

findings of the pathogen or their indicator microorganisms. Its important to note, regardless of which alternative is applied, maintenance of a robust sanitation program and microbial testing of food contact surfaces for *L. monocytogenes* or an indicator microorganism to verify effectiveness of these activities is expected.

3. Current industry *Listeria* management practices

As prevalence of *L. monocytogenes* in the food supply continues to present complexities, its critical for food manufacturers to rethink their Listeria prevention and control management strategies to drive down Listeriosis incidence across all food categories.

Consider the fact that unlike most other foodborne pathogens, *L. monocytogenes* is essentially an environmental pathogen that finds entry and the potential for harborage in manufacturing milieu through for instance, incoming raw materials, movement of personnel and vehicles, and the uncontrolled use of water. These conditions result not only in niches that support growth of the pathogen but also serve as transfer points to spread contamination across the facility and ultimately to food. Conceptually, measures to mitigate the presence of *L. monocytogenes* in foods can broadly be placed in to four important segments: 1) Formulation: use of low pH, low water activity, antimicrobial agents, etc.; 2) Process: use of heat and other lethality treatments; 3) Facility: use of robust sanitation and environmental monitoring programs; 4) Food: Modified atmospheric packaging and storage temperature. Each of these approaches can be effective yet none alone achieves the level of control needed to address the risks typified by the dynamic nature of contamination and occurrence of *L. monocytogenes* in food production environments. In addition, as Listeria is everywhere, we need everyone involved in food manufacturing to be at the forefront of pathogen management, and this in turn needs increased awareness of Listeria risks through appropriate education and training regimen for personnel.

4. Addressing potential post-lethality contamination in the facility

Among the most frequent causal regions in the facility that engender cross-contamination is the post-lethality environment where food may be exposed prior to packaging. In this discussion, concepts related to environmental monitoring and product sampling will be used to increase the likelihood of identifying potential food contamination during processing and decrease the likelihood of contaminated food from entering commerce.

5. Environmental monitoring and food contact surface testing

Food safety programs to assess growth and harborage of *L. monocytogenes* in a food manufacturing facility have largely revolved around swabbing of surfaces for different microorganisms, particularly indicator microorganisms to determine sanitation efficiency and potential pathogen contamination. As it may be challenging to monitor the entire production environment constantly, care should be taken to select the appropriate monitoring sites that are likely to harbor the pathogen and serve as transfer points for cross contamination. For instance, environmental monitoring of the post-lethality environment is critical in identifying and controlling *L. monocytogenes* in facilities involved with the production of RTE food.

An effective environmental monitoring plan should adopt the ‘seek and destroy’ approach wherein positive indicator findings are deemed as rationale for continuous learning and development of cleaning and sanitation programs. [18] Microbial testing methods that assist with the environmental monitoring program should be directed toward an indicator microorganism such as *Listeria* spp. Seeking out the presence of *Listeria* spp. instead of *L. monocytogenes* is advantageous because they are non-pathogenic and generally found more frequently in the environment than the pathogen. This approach provides a path to identifying growth niches before *L. monocytogenes* can find harborage in the facility.

The challenges of evolving and administering a mature environmental program that can identity harborage of *L. monocytogenes* in a timely manner and take corrective actions rests on good knowledge of the pathogen, the facility and its people and operational footprint. Testing non-food contact surfaces (non-FCS) can provide a good start to a nascent environmental program, and these steps should develop in to both higher frequency activities and cover more relevant regions in the facility where risk is elevated, such as food contact surfaces (FCS). Positive FCS findings reveal both a breakdown in the sanitation system as well as a potential transfer from other areas in the facility. They should be followed

with recleaning, re-sanitizing steps, and retesting procedures to ensure safe production. In some cases, a complete overhaul of these procedures may be warranted including reassessing chemical concentrations, personnel behaviors and tools used for these activities. If positive FCS results are sustained even after these interventions, then root cause analysis steps must be undertaken to provide a more comprehensive assessment of existing risks in the facility.

The criticality of sampling and testing post-lethality regions of the facility and their FCS cannot be understated. Niches or locations in the facility where *Listeria spp*. are found even after cleaning and sanitation have been applied may correspond to transfer points of the pathogen from these areas which lead to persistent product contamination. Indeed *L. monocytogenes* harborage can form anywhere in the facility, but generally, they occur in areas that are not easily identified or accessible for cleaning and thus not controlled. If not identified and addressed in a timely manner, the pathogen slowly migrates from their niches to outer surfaces of the equipment, reaching FCS and contaminating food products.

The presence of any transient *Lm* can be eliminated by following good manufacturing practices (GMPs), effective cleaning and sanitation and a robust environmental program. This approach must include FCS sampling and testing to ensure cross contamination is avoided within the facility and to product. Furthermore, facilities should administer goals that will reduce the number of *Listeria* spp. positives and steps toward continuous improvement of sanitation programs.

6. Risk-based finished product sampling and testing

First and foremost, it should be recognized that depending on the sampling plan and test method, product testing may demonstrate the presence or absence of *L. monocytogenes* in food, but it is not a reliable indicator of the prevalence of the pathogen in a facility. No doubt, food contaminated with *L. monocytogenes* is difficult to assess as it is unlikely to be uniformly distributed throughout the product.

Farber et al. in their recent review of *L. monocytogenes* science recommended that low-risk foods with a regulatory tolerance for the pathogen may incorporate specific end-product verification testing using novel three-class sampling plans as an alternative to existing two-class presence/absence sampling plans [6]. The authors demonstrate some three-class sampling possess higher test performance in detection of the pathogen such that it may serve as a "warning management indicator." The benefit of enumeration (for instance, a quantitative microbiological limit of less than 100 CFU/g) combined with detection (for instance, a qualitative detection limit of 1 out of 4 analytical units of 25g each) include establishing that product contamination is not widespread (only one positive) and not at a very high level (less than 100 CFU/g) of the pathogen in the food.

Of course, any product testing program must follow test and hold procedures to avoid potential food withdrawals or recalls resulting from positive findings among tested samples. Whether testing of products is instituted in a limited way or as a routine measure, utilizing scientifically validated high performance plans can only further generation of more data and inform more accurate risk assessments related to the presence of *L. monocytogenes* in foods.

7. Coupling environmental monitoring and product testing

Historically, product testing was viewed as a futile exercise with many risks for food processor companies, yet many industry supply chain programs incorporate product testing requirements while circumventing the need for carrying out robust environmental monitoring programs. As discussed above, implementing both programs present challenges for food manufacturers and consequently, neither reliable product testing nor adequate environmental monitoring are used to effectively prevent and control the presence of *L. monocytogenes* in the global food supply.

Decades of experience shows the presence of transient *L. monocytogenes* can be eliminated by following good manufacturing practices (GMPs), effective cleaning and sanitation, and robust environmental monitoring programs. The ability to track and eliminate more persistent pathogenic strains and undercut the potential for food contamination requires greater rigor in a facility's food safety verification system. An environmental monitoring program with FCS testing coupled with a scientifically valid product sampling and testing regimen are reliable and needed anchors for the overall food safety system targeting the presence of this ubiquitous pathogen.

8. Conclusion

Even systematic approaches such as good facility hygiene and the 'seek and destroy' process continue to challenge persistence of *L. monocytogenes* in processing facilities, especially in the post-processing environment. Addressing these issues while complying with regulatory zero-tolerance for the presence of the pathogen in food and food contact surfaces is a demanding effort. However, risk-based regulatory approaches can engender effective risk management practices aimed at controlling *L. monocytogenes*. Specifically, a strategy to link food contact surface testing and risk-based testing programs help identify potential entry and harborage of the pathogen, prevent cross contamination, and ultimately minimize the occurrence of the pathogen in food. Both practices can trigger corrective actions to avoid food contamination and restrict the likelihood of allowing potentially contaminated food from entering commerce.

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Quality standardization and certification of traditional food products

Quality standardization and certification of traditional food products

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Abstract. The results presented in this study were obtained during the implementation of the Interreg-IPA CBC project Traditional and Standard Quality - TASQ (HUSRB/1602/41/0146). The main goal of this project was quality standardization and certification of traditional food products in order to expand their market presence within the Serbia and Hungary cross-border region, using innovative processing and marketing techniques. In order to achieve that, the TASQ quality assurance system was developed, and the common certification mark Q was registered by Intellectual Property Office (IPO) in both cooperating countries. Numerous traditional food products were collected and analysed for nutritional and sensory quality as well as for safety. In total, 158 products across nine groups of traditional foodstuffs (meat products, dairy products, honey, vegetable oils, processed fruits and vegetables, juices/beverages, pasta and baker's wares, confectionery, spices and teas) were certified with gold, silver or green Q mark, representing the quality level. A new internet platform (www.tasq.rs) was developed with the purpose to help traditional food producers to promote and sell their products on a wider market. The assigned trademark is clearly indicated for each certified product within the producers' profiles on TASQ internet platform, and represents a guarantee of product quality intended to raise customer confidence.

1. Introduction

Traditional food products (TFP) are an important part of European culture, identity and heritage. Thus, all European countries have cultural traditions linked with specific TFP, which are in most cases elaborated in micro/small processing facilities, consequently resulting in non-standardized quality or even questionable safety [1, 2]. Increasing consumer demand for TFP emphasizes the need for a significant improvement regarding processing, packaging and labelling practices, guaranteeing high standards in food quality and safety, while keeping the original formulations and typical sensory properties.

A certification mark is a distinctive sign that guarantees that a product meets the standards and characteristics pre-established by the proprietor of the mark, who is obliged to monitor and regularly check that the products with the mark manufactured by third parties meet the established requirements [3]. During the TASQ project lifetime, the expert panel developed a quality assurance system (QAS) for sensory, nutritional and processing quality evaluation in order to distinguish between TFP of high quality. Its integral part are the rulebooks for each TFP group and three-level quality rating scheme. The certification mark, Q, was registered in the IPO of the Republic of Serbia and the Republic of Hungary,

and it is used as a guarantee of high sensory and nutritional quality, i.e. the basic quality indicators of TFP. In combination with additional quality indicators, such as organic product, geographical indication, autochthonous/wild variety/species/breed, traditional production and certified HACCP, TFP can be awarded with a gold, silver or green Q certification mark.

Additionally, manufacturers of traditional foodstuffs, for the most part, are not able to reach urban markets due to insufficient and inadequate promotional and sales activities. In order to maintain and expand the market share of TFP, an innovative internet platform (www.tasq.rs) was designed and developed aiming to help traditional food producers to promote and sell their products on a wider market, i.e. to provide a channel for a direct contact between producers in rural areas and consumers in urban ones, who are craving for tasty, local and ethnic foods. The TASQ web-platform allows traditional food producers to set up their own small virtual shops, creating profiles of their products. The quality of certified TFP is clearly distinguished by assigning one of three defined types (colours) of Q certification mark.

2. Materials and Methods

At the beginning of the project implementation period, mapping of traditional food producers, i.e. identification and selection of the initial group was done. In total, 100 manufacturers were involved in project activities in both countries. As a part of the evaluation process, on-site visits were performed and producers were consulted regarding improvement in production processes. Simultaneously, TFP typical for this geographical region were collected and analysed, in order to certify those with the best performing quality characteristics.

Developed QAS consists of basic and additional quality indicators. Basic quality indicators are related to sensory and nutritive quality. Assessment of total sensory quality (TSQ) was done by a six-member sensory panel, evaluating dominant sensory properties (appearance, taste, odour and texture), using 5-point category scales and appropriate coefficients of importance (CI). The sum of corrected score-values (individual scores given to selected sensory characteristics multiplied by the corresponding CI) for all assessed sensory properties represents the TSQ of a product [4]. According to TSQ, products were classified into three categories following the scheme: $TSQ \geq 90\%$ - excellent sensory quality; $80\% \leq TSQ < 90\%$ - very good sensory quality; $70\% \leq TSQ < 80\%$ - good sensory quality.

Determination of the structural/nutritive quality was done according to a previously defined quality aspect for each group and type of TFP. It could be the share of a valuable raw material/component used for processing, the content of a valuable nutrient in the final product or the content of salt, impurities, etc. By quantifying the difference in the content of the above mentioned components with respect to the prescribed minimum/maximum value, the appropriate nutritive quality level was determined.

The additional quality indicators were: organic product; geographical indication; autochthonous/wild variety/species/breed; traditional production and certified HACCP system. These quality indicators were assessed by inspecting the processing facility and production process, interviewing the manufacturer and by reviewing the relevant documentation (certificates, permits, product specifications, etc.). According the combination of basic and additional quality indicators, TFP were awarded with the appropriate type of Q certification mark (gold, silver, green).

Regarding the meat products, the specifications of the most important traditional products (dry-fermented sausages; dry-cured meat products; dry-cured bacon; pork greaves and cooked sausages) were defined within the developed QAS. The specifications contain the legal requirements of the two neighbouring countries, and defined requirements that the product must satisfy in order to achieve a specific level of sensory and nutritive quality. The differentiation between analysed traditional meat products was done according to overall sensory quality and additional quality indicators, while in the case of dry-fermented sausages, the level of nutritive quality was also considered. It was assessed by quantification of meat protein content (Kjeldahl N x 6.25), according to the recommended ISO standard [5]. In this regard, products having at least 25% higher content of meat proteins than required were categorized as excellent quality, while products containing 15% or 10% more meat proteins than required were classified as very good and good quality, respectively.

3. Results and Discussion

Throughout the project lifetime, overall, 158 products across nine selected groups of traditional foodstuffs (80 in Hungary and 78 in Serbia) were awarded with Q certification mark. When it comes to meat products, 28 of them in total were certified, out of which approximately 60% came from Serbia (Table 1).

Table 1. Meat products awarded with Q certification mark

	Total number	Dry-fermented sausages	Dry-cured meat products and bacon	Cooked sausages	Other
Serbia	17	12	2	3	0
Hungary	11	6	2	0	3
Sum	28	18	4	3	3

Regarding the quality level, most of the certified meat products (23) were awarded with gold Q certification mark, indicating an exceptional quality, i.e. excellent sensory and/or nutritional properties, plus added value expressed through some of the additional quality indicators. Three (3) products were awarded with silver Q certification mark, representing top quality product having excellent or very good sensory and/or nutritional properties, plus added value. Two (2) products were granted green Q certification mark. It indicates the prominent quality product that, in addition to very good or good sensory and/or nutritional properties, has the added value expressed through some of the additional quality indicators, or possesses excellent sensory and/or nutritional properties without added value (Fig. 1).

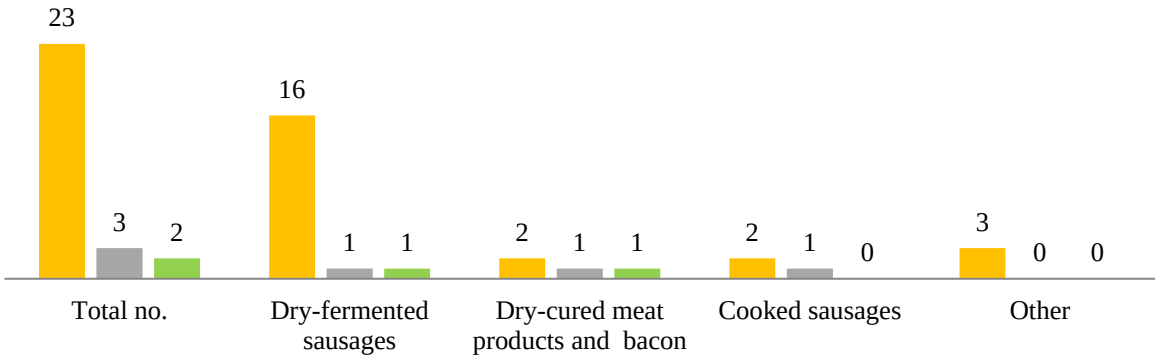

Figure 1. Distribution of different types of Q certification mark (gold, silver, green) among awarded meat products

The majority of certified Serbian traditional meat products (approx. 70%) belong to a group of dry-fermented sausages. *Sremski kulen, Petrovačka kobasica* and *Lemeški kulen,* the most important dry-fermented sausages from Vojvodina (Northern Serbia) protected with designation of origin (PDO) at national level [6, 7], were within this group. Mean values and standard deviations of TSQ and protein content for two samples (different manufacturers) of each of these traditional dry-fermented sausages are given in Table 2. According to TSQ value, four products were rated as excellent regarding sensory quality (≥ 90%), indicating typical sensory properties that are particularly pronounced, without or with some slight modifications and/or errors that do not affect significantly the overall product quality. TSQ values for one sample each of *Petrovačka kobasica* and *Lemeški kulen* indicated products with slight alterations or certain defects of sensory properties, amounting 88.6% and 79.5%, respectively. The highest concentration of protein was found in *Sremski kulen,* being almost the same in both analysed samples (37.3% and 37.4%). Considerably lower content of this valuable nutrient was found in samples of *Petrovačka kobasica* and *Lemeški kulen,* ranging from 30.4% to 32.5%. The obtained results regarding the protein concentration are primarily the consequence of different formulation of raw

sausage mixtures, type of casing and processing conditions. Thus, *Sremski kulen* is made of first category meat, containing very small amount of fat and connective tissue. Despite observed differences in protein concentration, the values of this parameter registered in all examined sausages were much higher, i.e. 25% higher, than minimal requirements (24%) for "domestic kulen" made of coarsely minced first category meat and stuffed into pork appendix or rectum [8].

Table 2. Total sensory quality and protein content of three traditional dry-fermented sausages

	Sample	Total sensory quality (%)	Protein content (%)
Sremski kulen	1	90.8 ± 2.91	37.3 ± 0.06
	2	95.5 ± 2.07	37.4 ± 0.13
Petrovačka kobasica	1	88.6 ± 2.02	30.4 ± 0.16
	2	94.3 ± 2.58	32.5 ± 0.16
Lemeški kulen	1	79.5 ± 2.88	30.4 ± 0.08
	2	97.1 ± 1.97	32.4 ± 0.01

4. Conclusion

Among other food products typical for the Hungary-Serbia cross-border region, a large number of traditional meat products, especially dry-fermented sausages, have peculiar characteristics which arise from the region specific environmental/climatic conditions and use of local raw materials, formulations and manufacturing techniques. In total, 28 meat products were awarded Q certification mark, and 23 of these products were granted gold Q mark, representing exceptional quality product. Serbian traditional dry-fermented sausages with geographical indication certainly possess high quality, confirmed with high value of TSQ and protein concentration. The awarded type (colour) of Q certification mark is clearly indicated for each certified product within the producers' profiles on TASQ internet platform (www.tasq.rs), aiming to raise consumer confidence in products' quality.

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How do we eat meat–the role of structure, mechanics, oral processing, and sensory perception in designing meat analogs

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How do we eat meat – the role of structure, mechanics, oral processing, and sensory perception in designing meat analogs

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Abstract. This study provides an overview of over 50 publications exploring the consumers' motives for choosing meat analogs over real meat, how they perceive them, and what can be learned from meat structure, mechanics, oral processing, and dynamic sensory analysis for meat analog design. Meat analogs' sensory perception is their main lack, while ethics, health, and environmental statements might be used to boost their promotion. Methods for meat structure and mechanics' analysis are well established and translated (to some degree) to meat analog's quality analysis. However, limited information is present concerning meat and meat analogs' oral processing and dynamic perception, which can be seen as a chance for future research and improvement.

1. Introduction

A constant meat consumption rise per capita for the past few decades has been recorded in parallel with the population growth [1,2]. Considering these facts, it can be assumed that larger quantities of meat will be required. At the same time, the resources needed for the production of meat on a larger scale need to be increased, even though some of them are limited.

Consumers enjoy meat because of several reasons. A broad group enjoys meat sensory qualities. Meat nutritional value – the content of essential amino acids, high protein content, and B group vitamins – is another crucial element for meat appreciation.

However, a question that may appear is – will there be enough resources (e.g., land and fuels) to produce meat in a sustainable manner in the future, considering the trends mentioned above of meat consumption and population rise [3]? Also, impacts of meat production on climate change should also be considered [4]. Answering this question is undoubtedly tricky, knowing that many factors could impact the outcome in this case. Nonetheless, being prepared for that and having products capable of replicating the eating experience and nutritional value of meat, for the food producers is a comparative advantage.

In recent years, there is a growing number of products aiming to replicate meat products considering the whole experience of meat consumption. These are known as meat analogs, vegetarian meat, or meat substitutes. Some of these products present on the market are specifically designed using the scientific approach. At present, the products of *Beyond Meat* and *Impossible Foods* companies are especially popular. Both of these apply the technology of using plant proteins (pea protein in the case of *Beyond*

Meat and soy protein in the case of *Impossible Foods*) to produce products aiming to mimic burgers, meatballs, sausages, or ground meat. Otherwise, making meat substitutes resembling the appearance of steaks is still challenging due to meat's complexity concerning its structure and mechanical properties that further reflect on the process of eating (oral processing) and sensory perception [5].

Within this literature review, we strive to answer how consumers perceive meat analogs, what are the benefits and drawbacks of meat analogs compared to conventional meat, and what are the lessons learned from meat structure and mechanics, oral processing, and sensory perception that can be translated to meat analogs' improvement.

2. Consumers' attitudes towards meat and meat analogs

Studies covering consumers' meat appreciation indicated the importance of juiciness, tenderness, fibrousness, color, and meat flavor [6], while it was found that tenderness, juiciness, and flavor play the main role for 13 meat products and meat [7]. Some of these properties were identified as the main drawbacks for meat analogs' acceptance.

Consumer perceptions of three different types of burgers (meat-, plant-, and insect-based) under three testing conditions (blind, expected, and informed) were investigated [8]. The results of this study showed lower liking scores for non-meat origin products. They also reveal the weak points in quality for the meat analogs under scrutiny, mostly related to their texture, i.e., juiciness. However, the influence of testing conditions was also revealed, indicating that consumers will perceive plant-based burgers better if they are not aware of their origin. In another study, 46 consumers were interviewed to get insight into their attitudes towards meat analogs, and sensory testing of six dishes containing meat analog products was conducted [9]. Results followed a similar vein, and lack of uniform taste, compactness, dryness, and softness were found. However, consumers also stated positive sides of these products, referring to the health aspect and some sensory properties (tastiness, crispiness, chicken-like texture, or granular texture). Another study invited over 100 subjects to express their impressions of (four) plant-based chicken and (five) plant-based burgers compared to the real products. Again, insufficient satisfaction was seen, mainly because of the texture attributes, e.g., rubbery, insufficiently juicy, firmness, juiciness, greasiness. It is also worth mentioning that in this research, one plant-based burger surprisingly did not differ from a real burger, promising that it is possible to achieve desired plant-based product quality.

Fulfillment of the expectations related to sensory perception is a must for nearly all foodstuffs. In the case of meat and meat analogs, two groups of consumers with different expectations were identified [10]. The first group drivers for choosing meat analogs are the benefits they provide, discussed below. According to [10], this group is willing to compromise the meat-eating experience while respecting the other mentioned values. Otherwise, the group of meat-eaters that highly respect meat-eating experience is ready to accept meat analogs only if they fully resemble real meat or meat products.

2.1. Benefits of the meat analogs

There are several benefits, discussed in the literature, of meat analogs compared to real meat. These are also important drivers for choosing these products, even though there could be drawbacks in sensory quality at the same time. Among the first are ethical issues. Person- and product-related factors in light of the meat analogs' acceptance were investigated [11]. Ethical issues were important for those who already broadly use this kind of product, and at the same time, this group were satisfied with analogs' sensory profile that does not fully resemble real meat. In contrast, the group that rarely consumes meat analogs, referring to the products' sensory attributes as their weakest point, is not much interested in meat ethics.

Since it was found that high meat consumption could lead to a negative impact on health [12,13], it can be hypothesized that some consumers are interested in replacing these products with healthier alternatives, following the consumers' beliefs that were previously confirmed [14]. An illustration of protein yield from different sources (beef, pork, poultry, fish, clean beef, insects, plants) per area of land, and greenhouse gas production from different protein sources (beef, pork, poultry, plants, insects) has been proposed [15], showing the greatest yields of proteins for insect and plant sources per land area,

while, in contrast, animal sources produced significantly lower protein yields. At the same time, it is noticeable that insect- and plant-based protein production will result in much lower emissions of greenhouse gases, indicating advantages of these concerning environmental protection.

Regardless of the research focus, all the papers referenced here dealing with the consumers' perception of meat analogs suggested that the benefits these products have in the environment, ethics, and health, can be a powerful marketing tool, but still, their sensory perception is the main drawback needs to be improved for the meat analogs to be widely accepted.

3. Structure and mechanics of meat and meat analogs

On a macrostructural scale, meat consists of few different entities (muscle, connective, and adipose tissues), forming a composite, discrete, anisotropic material. The muscle tissue microstructure has been investigated in depth before, and it is well known [16,17]. The connective tissue can be classified based on its morphological role (endomysium, perimysium, epimysium). Collagen and elastin are two connective tissue components affecting its mechanical properties. The collagen is predominant in connective tissue compared to elastin. Opposite to elastin, collagen is rigid – it resists tension, while elastin fibers are stretchable. The negative impact of collagen on meat tenderness has been seen with animal maturation [18] due to changes in the insoluble/soluble collagen ratio. In contrast, elastin's influence on meat tenderness is negligible. Intramuscular fat content, i.e., marbling, is a desired meat property, making certain types of meat (e.g., Wagyu and Kobe beef) especially valuable. A positive impact of marbling on tenderness has been advocated [19]. Considering the fats' melting temperature, their lubricative effect, and temperatures used for meat preparation and serving, it can be further hypothesized that higher marbling will also improve perceived juiciness and impact oral processing.

Several different mechanical tests are used for meat quality evaluation [20,21]. One of the oldest and, nowadays, broadly accepted is the Warner-Bratzler shear force test [22], which describes a standard procedure [23]. This test has been used for muscle fiber strength quantification [24], while its results (maximal shearing force) have been correlated with sensorially perceived texture parameters [25]. Texture profile analysis (TPA) is another popular test used for meat texture analysis and prediction that tends to mimic subsequent two chews, in that way providing several parameters (i.e., hardness, springiness, adhesiveness, cohesiveness, chewiness) [26]. Even though these two tests are widely accepted, the literature review suggests a great diversity of applied testing conditions and sometimes their misuse, e.g., reporting the penetration test as the TPA [27]. Thus, the need for a better understanding of mechanical and sensory definitions and the need for standard testing procedures appear. Besides the Warner-Bratzler and TPA tests, tensile and penetration tests also found application for meat mechanical testing. The tensile test has been used to quantify both fiber strength and adhesion between fibers, depending on the direction of applied deformation [28,29]. The penetrometer test also has been used for adhesion measurements [24]. Other biophysical methods also found application for meat quality assessment [30]. Good examples are applications of near-infrared spectroscopy for predicting the beef sensory hardness and tenderness [31] and proton NMR relaxometry for assessing the state of the water in meat [32], which could be a crucial indicator for predicting sensory juiciness.

Meat analogs are structured using several different technologies. Cultivated meat is produced based on the process of muscle cell replication under controlled conditions. This (pilot) product has disadvantages primarily because of the exclusively high costs of production, but also lack of sensory qualities resembling real meat [5]. Poor sensory quality and low acceptance by consumers are issues of insect-based meat analogs [8]. Otherwise, although slightly more expensive than real meat products, meat analogs produced using textured plant-based proteins (e.g., soy, pea, wheat, peanut, or their blends) are already present on the market and accepted to some degree, although their sensory quality needs improvements. A recent review [33] was on plant-based protein processing technologies for meat analogs production. They recognized two classes of methods used for plant protein texturization, i.e., bottom-down strategies focused on resembling meat fibrousness on a macro scale (extrusion, shear cell technology, and freeze texturization), and bottom-up technologies focused on meat microstructure resemblance (spinning technologies, tissue engineering, and fermentation of filamentous fungi).

However, even though some of these technologies are promising, extrusion is the most frequently used technique to produced meat-like products. For the extrusion process, the vegetable flours or their blends are moistened to a certain degree and undergo a combination of pressure, heat, and mechanical shear inside the extruder. As a consequence, protein polymerization occurs, leading to the formation of layered fibers aligned in parallel with the mass flow direction [33].

The recent publications related to the meat analogs' structure and mechanics examinations witness lessons that have been learned from meat analysis. Table 1 denotes methods used for assessing meat analogs' structural and mechanical properties, as well as the purpose of their application and information obtained.

Table 1. Methods used for assessing meat analogs' structure and mechanics

	Method	Analyzed properties	Application
Mechanics	Compression test	Young's modulus Hardness Chewiness	Examinations of the extrusion parameter [34,35] and binding agent influences [36]
	Tensile test	Young's modulus Tensile strength Anisotropic index	Examinations of the extrusion and feed parameter influences [37][38]
	Shearing test	Maximal shearing force Texture index	Comparison of meat analogs and real meat prepared in sous-vide [39], oyster mushroom protein addition influence on meat analog quality [40]
	Puncture	Penetration force	Effect of biding agents on sausage analog quality [35]
	Texture Profile Analysis (TPA)	Springiness, cohesiveness, gumminess, hardness, chewiness	Examinations of the extrusion parameter influences [41]
Structure	Microscopy (light, scanning electron, laser scanning)	Microstructure and anisotropy	Examinations of extrusion parameter influences and blend ratios in feed [34,37,38]
	X-ray microtomography	Structure porosity (air incorporation)	Blend comparisons [37]
	Nuclear magnetic resonance (NMR)	State of the water	Influences of processing parameters and feed [42,43]
	Fourier infrared spectroscopy	Protein secondary structure	Influences of processing parameters and feed [43]

The literature review of 20 papers published during the past 20 years indicates that meat analog structures and mechanics were recently investigated mainly through the impact of the processing factors (e.g., cooking temperature, screw speed, cooling dynamics, feed composition, the addition of binding agents) on their quality. However, several studies are distinguished based on their approach. Recent research dealing with the examination of different moisture and wheat gluten contents applied an original approach in studying retention of volatile flavor substances, microstructure, moisture distribution, and secondary protein structures of meat analogs [43]. For that purpose, the authors used combined gas and mass chromatography, scanning electron microscopy, low-field NMR, and Fourier transform-infrared (FT-IR) spectroscopy. The results have shown the potential for adjusting blends and processing parameters to achieve desired microstructure properties that further impact volatile retention. This study also opens new questions that could be an entrance for future research, e.g., how do these

structures behave during the oral processing, what are the profiles of sensorially perceived volatiles, or their *in-vivo* release. Answering these should further improve meat analog consumption and perception and reduce the gap between real meat and analogs.

Several factors impact obtaining representative samples for meat mechanical testing, e.g., muscle anatomical position, animal breed, sex, age, pre-, and post-mortem factors. To obtain a better image of meat and meat analogs' mechanical properties, a novel method has been presented [44]. It is based on measuring Young's modulus of the sample using a spherical probe of a small diameter (1 mm, corresponding to the muscle bundle diameter) continuously for the area under scrutiny (300 mm^2), by conducting the measurements in small steps corresponding to the half of the probe's diameter. The results provide images for a broader understanding of meat/meat analog mechanics related to their structure. That can be a powerful tool in analyzing meat and tailoring meat analogs.

An older study proposed a new method for quantifying the fibrous nature of meat analogs using fluorescence polarization spectroscopy [45]. Another introduced image processing for the same purpose, simplifying the data curation and examination execution [46].

4. Oral processing and sensory perception of meat and meat analogs

Food oral processing and sensory perception are in a tied relationship. As mentioned, previous studies covered sensory attributes' importance for meat and meat products' quality appreciation. It was found that tenderness, juiciness, and flavor play a crucial role. However, less is known about dynamic sensory perception. A few reasons are recognized as the cause for that.

Time-intensity (TI), as one of the oldest dynamic sensory methods, has been applied for meat evaluations. However, since it can be considered a time-consuming method, bearing in mind that one or eventually two attributes can be evaluated simultaneously [47], TI may not be a suitable method for deeper understanding of meat and meat analogs' dynamic sensory perception. Temporal Dominance of Sensations (TDS) and Temporal-Check-All-That-Apply (TCATA) are methods that improved on TI by allowing simultaneous selection of several (dominant or present) attributes at a time. Their parallel application with oral processing for meat revealed new information for bolus formation and sensory perception correlation.

TCATA has found its application for commercial ham evaluation [48], showing that the fibrousness is related to the in-mouth sample fragmentation, while the perceived juiciness was linked to the saliva incorporation. TDS application has been used to assess the influence of cooking [49] and Wagyu beef fatting periods [50] on dynamic sensory perception. The changes in perception during the consumption (from hard or firm at the beginning, through fibrous and juicy, to soft at mastication end) were revealed, which witnesses the expediency of applying TDS for a broader understanding of eating experience.

There is a slightly higher number of publications than dynamic sensory studies covering meat oral processing. It was found that the meat cooking method [49], preparation temperature/time combinations [51], and muscle anatomical position and aging [52] impact oral processing, as well as the correlations between it and mechanical parameters (e.g., shear force). Further investigations showed that harder meat would also be differently orally processed, implicating the differences in bolus properties [53], and hypothetically, sensory perception – in line with the previous explanation. Oral processing is even more complex when considering the individual character of mastication [54], and thus, perception.

To the best of our knowledge, there have not been any studies published on meat analogs' oral processing and dynamic sensory perception (and including comparisons with real meat).

5. Conclusion

Following the literature findings, we underline three main conclusions. Firstly, there is room for meat analogs. However, their sensory perception needs to be significantly improved. Secondly, meat structure and mechanics methods of analysis are well established, even though there are some inconsistencies in the literature. They are a good reference point for examining meat analogs and are already implemented to a certain degree for these products. Thirdly, there is a lack of information about meat and meat analogs' oral processing and dynamic sensory perception due to the limited number of publications,

which also can be recognized as an opportunity for future research. Last but not least, recruitment, training, validation and performance analyses of panels involved in both sensory and oral processing studies have to be performed, as these evaluation tools involve human subjects [55]. Finally, we suggest a holistic approach encompassing different classes of methods discussed herewith, with the aim of improving meat analogs' quality.

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Consumer awareness of meat hazards with special reference to sources of meat contamination and microbiological hazards

Consumer awareness of meat hazards with special reference to sources of meat contamination and microbiological hazards

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Abstract. This paper presents the results of consumer opinion testing (n=1000) on hazards in meat (biological, chemical), as well as consumer opinion about causes of meat contamination from farm to retail. Consumer opinion on sources of meat contamination in households is also presented.

1. Introduction

Today's modern food safety system is based on production process hygiene and prevention of contamination with hazards. This system is supported by scientific and regulatory bodies all over the world and legalized in all developed countries. The modern food safety system is based on good manufacturing practices and good hygienic practices (GMP/GHP) and hazard analysis and critical control points (HACCP). The food producer is responsible for food safety and is required to identify hazards and reduce them to an acceptable level or eliminate them in order to make the product (food) safe for the consumer. The value of this modern approach to food safety is reflected in the fact that it encompasses all links of the food chain. It is understood that food safety depends on whether a defined safety system works in all segments of food production. Therefore, the entire food chain should be controlled and checked. The last links in the food chain are food retail, the place where the food becomes available to the consumer (restaurants, for example) and the consumer. However, absolute food safety, no matter how good, functional, controlled and checked the system is, cannot be ensured and guaranteed.

Foodborne diseases are not uncommon and they are, in most cases, caused by biological hazards, primarily bacteria. If these diseases are of an epidemic nature, it is understandable that they attract a lot of media attention and cause consumer concern. Foodborne diseases are not only a danger to human health and life, but also cause huge economic losses (sick leave, medical treatment). The key reasons for the high incidences of foodborne diseases related to households refer to consumer knowledge and information. Today's consumer should have knowledge of and be informed about food hazards, maintenance of workplace and personal hygiene, contamination pathways, food handling (storage

conditions and shelf life), food preparation methods, storage of prepared food and the importance of proper food waste disposal.

The aim of this paper is to examine consumer opinions about meat hazards, as well as knowledge of the main sources of meat contamination.

2. Materials and Methods

For the purposes of this research, a quantitative questionnaire was used to examine consumer attitudes about meat quality and safety. During 2017 and 2018, the survey covered the population from the areas of the city of Banja Luka and Gradiška (urban environments). Data were collected until 1000 validly completed survey forms were filled, which means that surveys that were not completed, or in which answers to questions were incomplete, were not taken into the data processing procedure. Demographic data about participants refer to: age, education and sex. The participants in the survey ranged in age from 20 to over 60, on the basis of which they were classified into five groups (intervals of ten years). In relation to education, the most numerous group in the survey were those with secondary education (53.10%), followed by respondents with tertiary education (38.9%), and the groups with the lowest participation in the survey were postgraduates (3.5%) and those with primary education (4.1%). Respondents were 45.80% females and 54.25% males.

Statistical analysis was performed using Chi square test to determine the significance of differences between means, as a basic statistical method for comparing the frequency of nonparametric landmarks. A level of 0.05 was considered significant. Statistical analysis of the obtained results was done in the statistical package GraphPad Prism software, version 8.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). The results are presented in tables and graphs.

3. Results and Discussion

Consumer opinion about meat hazards is shown in Table 1. According to the respondents in the survey (several answers were possible), consumers believe that the greatest dangers are trichinosis (56.1%), GMO meat (53.3%) and salmonellosis (51.2%). This is quite understandable because these dangers are most often discussed in professional circles and the media [1]. There were significantly fewer responses concerning the two chemical hazards, the presence of hormones (35.3%) and antibiotics (33.9%), since they are less talked about, so consumers are less informed about these hazards. These two chemical hazards were significantly less ($p<0.05$) important for consumers choosing meat than the biological hazards.

Table 1. Significant hazards in meat in the opinion of the respondents (n=1000)

It is especially important for me to know that the meat I buy does not contain:	Answer (%)
Salmonellosis	$51.2^{A,B,C}$
GMO (genetically modified organisms)	$53.3^{D,E}$
Trichinosis	$56.1^{A,F,G}$
Hormones	$35.3^{B,D,F}$
Antibiotics	$33.9^{C,E,G}$

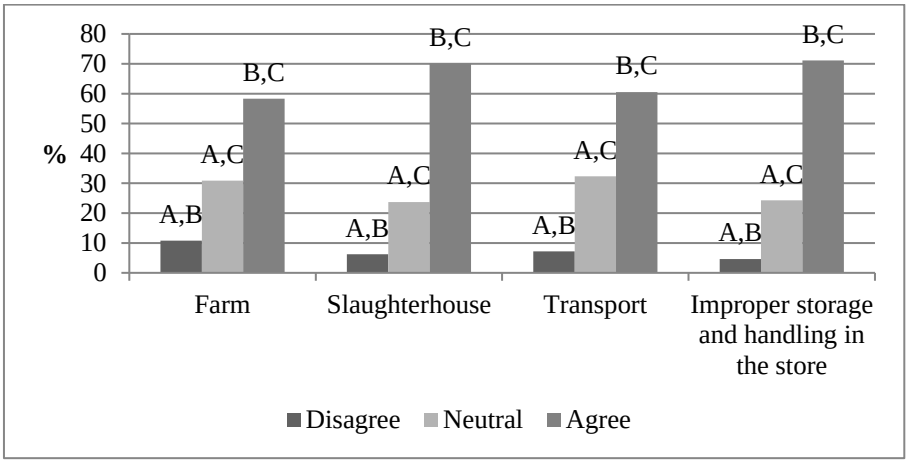
Same letters $^{A, B, C, D, E, F, G}$ - $p < 0.05$; The total is more than 100% due to the possibility of giving more than one answer to the question.

Bernauer [2] in his research noted that the most significant barriers we face today regarding the adoption of GMO food by consumers are low confidence in the security of GMO food supply in important markets, such as the European Union, and relate to long-term health and environmental

consequences. Consumers are informed of the legal ban on the use of hormones and antibiotics in animal nutrition.

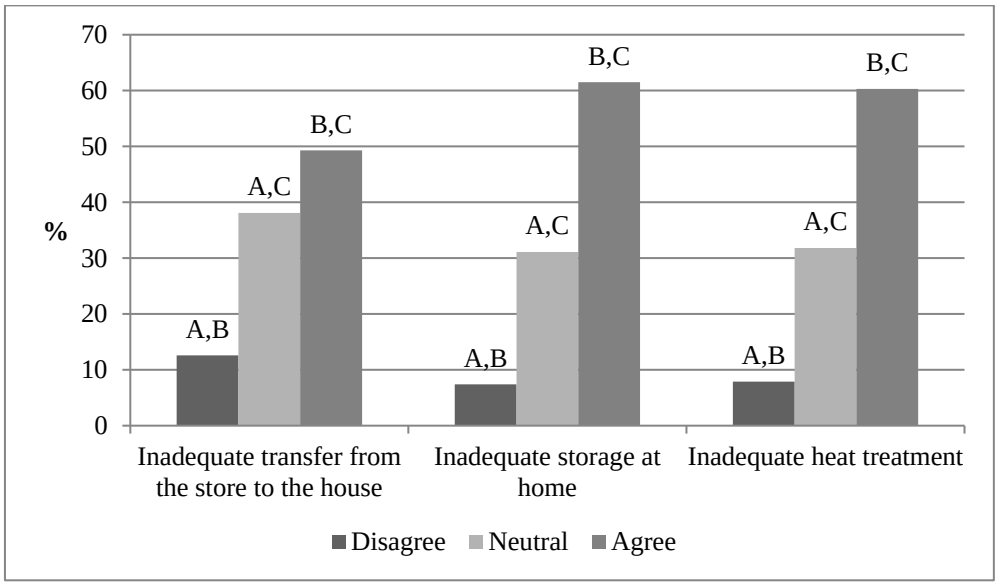
According to consumers, the most significant of the meatborne hazards are (in descending order): trichinosis > GMO > salmonellosis > hormones > antibiotics. The answers indicate that consumers are well informed about the biological and chemical hazards in meat.

According to the results of the survey (Figure 1), consumers think the main sources of meat contamination have the following descending order: inadequate storage and handling at point of sale > slaughterhouse > transport from slaughterhouse to store > farm. The declining number of the main sources of meat contamination in the household, according to consumers is: inadequate storage in the home > inadequate heat treatment > inadequate transfer from the store to the home (Figure 2).



Same letters A, B, C - p <0.05; the degrees of agreement were compared.

Figure 1. The main source of meat contamination from the farm to the consumer



Same letters A, B, C - p <0.05; the degrees of agreement were compared.
Figure 2. The main source of meat contamination in the household

Meat hazards according to the modern approach to food safety are divided into: biological (bacteria, viruses and parasites), chemical (pesticides, heavy metals, antibiotics, hormones and other environmental pollutants) and physical (glass, wood, plastic). Based on the conducted research, it can be concluded that the participants in the survey were aware of the impact that microbiological hazards have on health, that they are concerned about the consequences of consuming meat containing microorganisms, that the presence of microorganisms in meat can be controlled by laws and regulations, that microbiological hazards can be deadly, widespread, can spread rapidly with food, and cannot be easily controlled. Also, survey participants believe that there are not enough regulations related to microbiological hazards in meat, that they are not well regulated by law, that they are not sufficiently implemented and that consumers are not sufficiently informed about this issue [3,4,5,6].

Prevention of bacterial cross-contamination of meat/organs in slaughterhouses should be directed to good hygiene practice. In this regard, the introduction and acceptance of visual inspection of meat (no palpation or incision) itself has no effect on reducing bacterial contamination of the carcass/body under conditions of poor hygiene practices in slaughter in the house. If changes are noticed during the visual inspection of the meat, and in accordance with that, additional controls of the meat are performed by palpation and incision, then the transmission of bacteria can occur [7, 8, 9].

According to Zdolec [10], herd-level measures have a strong effect on the presence of *Campylobacter* spp., *Salmonella* spp., *Yersinia* spp., Shiga toxin-producing *Escherichia coli* (STEC) and two parasitic species *Toxoplasma* spp. and *Trichinella* spp. Serological categorization has a medium effect on the mentioned bacteria and parasites except STEC. Inspection has a limited effect on the presence of *Salmonella* spp., and a strong effect on the presence of *Trichinella* spp. (because examination is mandatory). Inspection is not relevant for the findings of *Campylobacter* spp., *Yersinia*, STEC and *Toxoplasma* spp. Slaughterhouse hygiene has a limited effect on the presence of bacterial species, and no effect on the presence of parasites. Carcass decontamination has a medium effect on the presence of bacteria, and no effect on the presence of parasites. Freezing has a medium effect on the presence of *Campylobacter* spp., a strong effect on the presence of parasites, and no effect on *Salmonella* spp., *Yersinia* spp. and STEC.

Fresh meat has a short shelf-life because its chemical composition favours the growth of many different types of bacteria. Meat contamination is possible at all stages of production and is the main source of bacteria that cause foodborne diseases [9, 11, 12]. The most common causes of foodborne diseases are *Staphylococcus* spp., *Listeria* spp., *Campylobacter* spp. and *E. coli*. Some of them, such as *E. coli* O157:H7, are highly pathogenic and only a few bacteria are enough to cause human disease. The main sources of meat contamination are the animals themselves, slaughter equipment, carcass processing and the workers in the slaughterhouse. The most common cause of carcass contamination is the skin of slaughter animals that is contaminated with faeces. The cause of contamination can also be related to contaminated water and improper application of good hygiene practice. Controlling the level of carcass contamination in the slaughterhouse can contribute to reducing contamination. The level of contamination in the slaughterhouse depends on the training of the workers, the frequency of training, regular health control of the workers, the use of protective equipment, the wearing of jewellery, the employee's health and the prescribed norms of sanitation. At retail market level, the level of bacterial contamination depends on the training of workers, the use of protective clothing, including hair protection, simultaneous handling of meat and money, wearing jewellery, application of detergents and the frequency of washing (daily or not) [12, 13].

According to Pal et al. [14], *Brochothrix thermosphacta*, *Pseudomonas* spp., *Aeromonas hydrophila*, *Bacillus cereus*, *Campylobacter* spp., *Clostridium* spp., *Listeria* spp., *Salmonella* spp., *Staphylococcus* spp., *Yersinia* spp. and *E. coli* are mentioned as meat contaminants. The consumer should be protected from pathogens in the meat and that there should be no health problems because of them. This can be achieved by respecting the principles of GMP, GHP and production process control (HACCP). Some biological hazards are related to particular types of meat, so two parasites (*Trichinella* spp. and *Toxoplasma* spp.) and two types of bacteria (*Salmonella* spp. and *Yersinia* spp.) are associated with pork meat, so this meat should be subject to continuous and systematic controls, according to EFSA

recommendations [15, 16]. *E. coli* and *Listeria* spp. are more commonly associated with beef, and *Campylobacter* spp. is most commonly associated with poultry meat. This, however, does not mean that they are not found in other types of meat [17, 18, 19, 20].

Iroha et al. [21] found in 300 examined samples (beef, poultry meat and horse meat) that 29.3% of the samples were contaminated with *Bacillus cereus*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Shigella* spp. and *Staphylococcus* spp. In the examined samples, the most common of the studied bacteria was *E. coli* (8%).

Williams et al. [22] examined the possibility of bacterial transmission by packaging that is repeatedly used to transport purchased food. There is data that the packaging for the transfer of purchased food is used repeatedly (from two to seven times) in 51% of the examined cases of food supply. A large number of different types of bacteria were identified in the samples of the tested food transfer packaging, and coliform bacteria were found in half of the tested packaging, while *E. coli* was found in 8% of the tested packaging samples. From the results, it can be concluded that packaging plays a significant role in cross-contamination of food. This particularly applies when the same packaging is used to transfer food of plant and animal origin, which, according to a consumer survey conducted, occurs in 25% of cases. It was also determined that packaging is not washed in 97% of cases.

Gerba and Maxwell [23] report that the presence of *Salmonella* spp., *Campylobacter* spp., *E. coli* and *Staphylococcus* spp. in meat is often the result of cross-contamination, or failure to follow procedures with meat based on the principles of GMP/GHP. Meat contamination with these bacteria is beyond the control of households [1]. The most common bacteria in packaging used for food transfer are *E. coli*, *Klebsiella pneumonia* and *Cronobacter sakazakii*. Coliform bacteria are usually indicators of faecal pollution and, in the premises, inadequate sanitation procedures for work surfaces, equipment and hands of workers during meat processing.

Among the viruses that cause foodborne diseases in humans, the Norwalk virus, rotaviruses, astroviruses, hepatitis A and hepatitis E viruses are important. The sources of infection are mainly people who come into contact with food. Foodborne fungi include *Cryptosporidium parvum*, *Cryptococcus neoformans* and zygomycetes. The group of biological dangers also includes prions that are found in some tissues of animals (especially in the nervous system) suffering from transmissible spongiform encephalopathies (such as bovine spongiform encephalopathy (BSE)). The causative agent of BSE is thought to have zoonotic potential [17].

4. Conclusion

According to the test results, consumers most often associate the biological hazards, *Salmonella* spp. and *Trichinella* spp., with GMOs, because these dangers are best known to them from the work of state bodies and the media. Consumers believe that the most common sources of bacterial contamination are the slaughterhouse and improper food handling in retail. In households, consumers believe the greatest danger from bacteria comes from improper storage and inadequate heat treatment.

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Cleaning efficiency of feed production lines after production of feedstuffs with coccidiostats

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Cleaning efficiency of feed production lines after production of feedstuffs with coccidiostats

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Abstract. Intensive broiler production implies regular use of coccidiostats approved as feed additives. However, due to their chemical properties, coccidiostats can stay behind in the production line, and consequently, unavoidable cross-contamination of non-medicated feedstuffs can result in the exposure of non-target animal species and in the potential for coccidiostat residues in foods, such as chicken meat and eggs, derived from these species. In this way, coccidiostats enter the human food chain and can pose a health problem. The aim of this study was to determine the success of line cleaning after the application of salinomycin and maduramicin in feed. We tested the cleaning matrix (wheat groats) in order to demonstrate how many cleaning replicates are needed to safely produce coccidiostat-free feedstuffs. After the application of salinomycin, it is recommended that, for safety reasons, the line be cleaned with at least five batches of wheat groats of 480 kg each. In the case of maduramicin, it is recommended the line be cleaned with a minimum of eight batches, considering the relatively low permissible level of maduramicin as a contaminant in medication-free feed.

1. Introduction

Coccidiosis is a parasitic disease affecting livestock, especially poultry. The disease is caused by protozoan parasites of the genus *Eimeria*. In the case of a mass infection on farms, deaths and large material losses can occur [1]. Today, intensive broiler production implies regular use of coccidiostats approved as feed additives. The use of coccidiostats in the EU is regulated by Commission Regulation 2003/1831/EC [2]. This Regulation classifies feed additives into five categories: technological, sensory, nutritional, zootechnical and coccidiostats or histomonostats. Currently, the eleven following coccidiostats are authorized as feed additives according to 2003/1831/EC [2] – decoquinate, diclazuril, halofuginone, lasalocid, maduramicin, monensin, narazin, nicarbazin, robenidine, salinomycin and semduramicin.

Feed business operators can produce, in one facility, a wide range of animal feeds using one or several production lines. Due to their specific chemical properties, coccidiostats bind to processing equipment, which results in contamination of subsequently produced feed (cross-contamination). This is especially important for production of feed for those species and/or categories of animals for which coccidiostats are not allowed. This unavoidable carry-over or cross-contamination could result in the

exposure of non-target animal species, with potential health risks for animals, as well as the potential for residues in foods, such as meat and eggs, derived from these species. In this way, coccidiostats enter the human food chain and can pose a health problem [3]. Therefore, the testing of coccidiostats in animal feed is included in Serbia's National Monitoring Residue Control Program [4].

In order to avoid cross-contamination and to reduce the level of coccidiostats below the limits prescribed by the valid regulation, feed business operators carry out different cleaning procedures. These procedures include cleaning the lines with corn and /or wheat groats. For the purpose of more efficient cleaning, more rational business and greater product safety, it is necessary to determine which type of cleaning procedure is the most efficient, and in which time period and what amount of cleaning material should be applied in order for the lines to be cleaned.

Maximum levels of unavoidable carry-over of specific coccidiostats is regulated by Commission Directive 2002/32/EC on undesirable substances in animal feed [5]. A carry-over rate of 1% should be considered for feed used during the period before slaughter (withdrawal feed), for other feed to which no coccidiostats are added and for non-target feed for continuous feeding to food producing animals.

In Serbia, the use of eleven coccidiostats as feed additives and unavoidable carry-over in non-target feed is regulated by the Rulebook on the Quality of Feed [6]. Maximum levels of unavoidable carry-over of salinomycin and maduramicin in non-target feed in Serbia are presented in Table 1.

Table 1. Maximum levels of unavoidable carry-over of coccidiostats in non-target feed

Coccidiostats	Maximum levels of unavoidable carry-over, mg /kg feed for laying hens and fattening chickens in the pre-slaughter period in which the use of coccidiostats is prohibited
Salinomycin	0.7
Maduramicin	0.05

The aim of this study was to determine the efficiency of a feed production line cleaning procedure after feed containing salinomycin and maduramicin was produced.

2. Materials and Methods

The experimental study was based on the examination of the line after the production of medicated feed with coccidiostats – salinomycin (50 mg/kg) and maduramicin (5 mg/kg), at different cleaning stages, as well as examination of feed that came first off the production line after the applied cleaning procedure.

- Test samples (12) of wheat groats after salinomycin application - check of cleaning efficiency. Each sample represented one of 12 stages of the cleaning procedure (480 kg wheat groats per stage).
- Test samples (12) of wheat groats after maduramicin application - check of cleaning efficiency. Each sample represented one of 12 stages of the cleaning procedure (480 kg wheat groats per stage).
- Test samples of laying hen feed (6) - verification of the process.

Salinomycin and maduramicin were both purchased from Sigma-Aldrich (St. Louis, USA). Water, methanol, acetonitrile and N,N-dimethylformamide were all HPLC grade and purchased from Sigma-Aldrich (St. Louis, USA). Formic acid LC grade was from Merck (Merck KGaA, Darmstadt, Germany). Individual stock solutions, concentration 1.0 mg/mL, were prepared in methanol and stored at -20 ºC. Working standard solution (mixture of analytes) was prepared in acetonitrile by diluting stock solutions into range that equated to the carry-over levels in feed and stored at 4 ºC.

Portions of ground feed (5 g) were weighed individually into 50 mL polypropylene tubes with caps. Acetonitrile 25 mL was added and the tubes were shaken on a horizontal shaker IKA Yellow line (IKA Werke, Germany) for 60 minutes. The extracted samples were filtered through nylon 0.22 µm syringe filters into HPLC vials. Quantification was carried out using matrix extracted calibrations curves at four levels. Blank feed samples were fortified at four different levels with mixed working standard solution and submitted to the full extraction procedure.

Coccidiostats were analysed with SHIMADZU LCMS 8040 (Shimadzu, Kyoto, Japan). The instrument was controlled by LabSolutions software. The analytical column used for separation was Kinetex 100 x 2.1 mm 2.6µC18 100A with UltraGuard cartridge (Phenomenex, Torens, CA, USA). The oven temperature was set at 45ºC. The chromatographic separation was achieved in gradient mode using water acidified with 0.1% formic acid (mobile phase A) and acetonitrile acidified with 0.1% formic acid (mobile phase B) at a flow rate of 0.3 mL/min. Electrospray ionization (ESI) was used in positive mode, with the following parameters: capillary voltage 3.5 kV, cone voltages 30 V, desolvation temperature 400 ºC. Argon was used as collision gas. The precursor and product ions for analytes are presented in Table 2.

Table 2. MS/MS parameters

Compound	Precursor ion (m/z)	Product ions (m/z)
Salinomycin	773.50	265.10 431.10
Maduramicin	934.8	629.50 647.50

3. Results and Discussion

Salinomycin and maduramicin levels in analysed batches of wheat groats used in 12 cleaning stages after production of medicated feed are shown in Table 3.

Table 3. Levels of salinomycin and maduramicin in wheat groat batches used in 12 cleaning stages

Cleaning wheat groat batch	Salinomycin (mg/kg)	Maduramicin (mg/kg)	Cleaning wheat groat batch	Salinomycin (mg/kg)	Maduramicin (mg/kg)
1	2.872	0.328	7	0.254	0.018
2	1.182	0.190	8	0.250	0.020
3	0.906	0.106	9	0.170	0.013
4	0.524	0.096	10	0.071	0.011
5	0.520	0.078	11	0.066	0.022
6	0.312	0.028	12	0.046	0.018

The level of salinomycin, which had been used to produce medicated broiler feed, decreased from the initial value of 50 mg/kg (in the medicated feed) to 2.872 mg/kg in the first batch of groats used for line cleaning, which represents 5.7% of the initial value. The results indicate cleaning efficiency of almost 95% after the first cleaning batch. However, the absolute value of salinomycin was four times higher than the maximum allowed. Salinomycin at a level below the regulation limit (0.7 mg/kg) was achieved after the 4th cleaning stage. The following cleaning batches effected a gradual decrease of salinomycin to 0.046 mg/kg in the 12th batch. The decrease of salinomycin content in all the analysed batches of cleaning wheat groats is presented in Figure 1. The level of salinomycin decreased rapidly after the first three batches, while from batches 4 to 12, the trend continued but at a significantly slower rate.

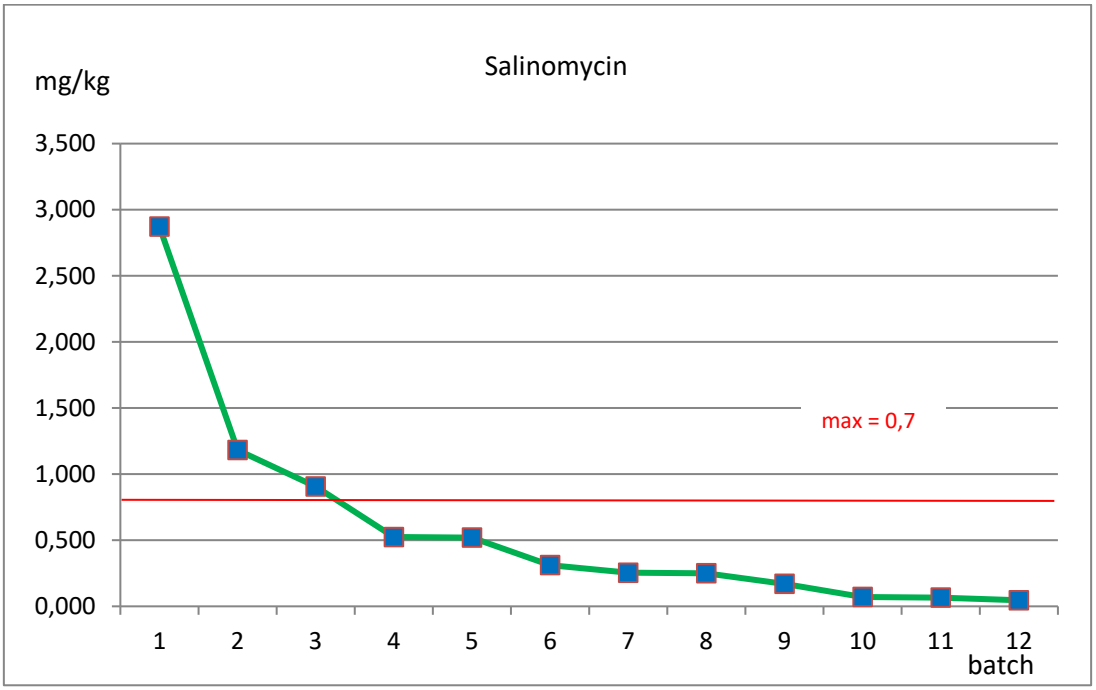

Figure 1. Salinomycin levels in the 12 batches of cleaning wheat groats

The initial level of maduramicin in the medicated broiler feed was 5 mg/kg. After production of medicated feed with maduramicin, levels in cleaning batches of wheat groats decreased gradually from 0.328 mg/kg in the first to 0.018 mg/kg in the last batch (Figure 2). The most rapid decrease in the level of maduramicin in feed was observed in the first three cleaning batches, while the decrease in the following batches was significantly slower. However, a maduramicin level below the maximum allowed limit (0.050 mg/kg) was achieved after the 6th cleaning stage.

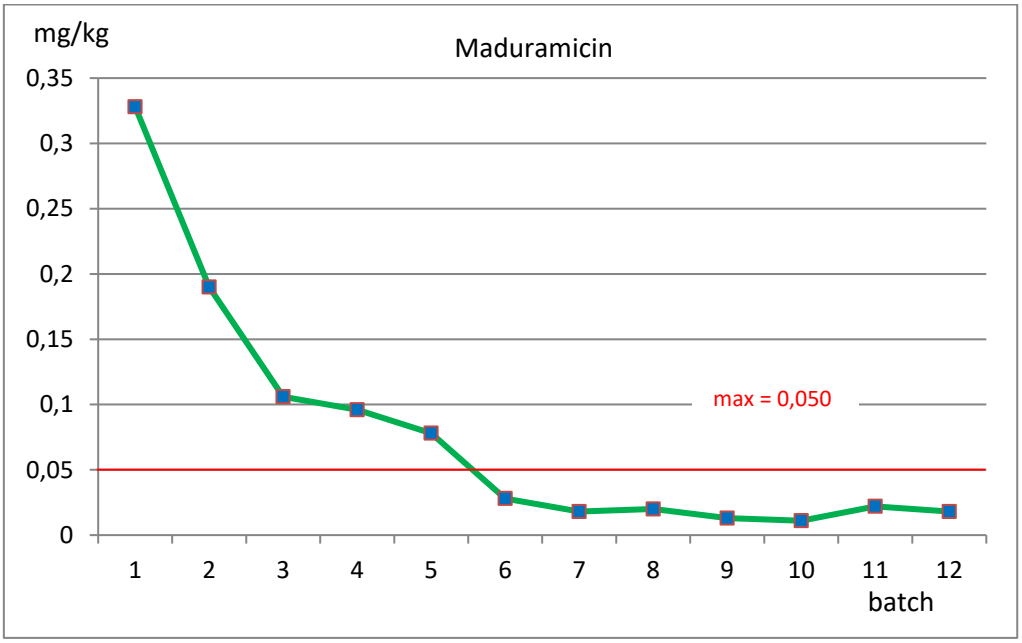

Figure 2. Maduramicin in the 12 batches of cleaning wheat groats

The coccidiostats tested in our study have different chemical structures and properties, and the applied amounts in broiler feed are different – 50 mg/kg for salinomycin and 5 mg/kg for maduramicin. Due to their different chemical structures and the results of this study, these two coccidiostats should be considered separately in terms of cleaning the production lines.

Although the level of salinomycin decreased below the allowed maximum after the 4th cleaning stage, it is recommended that, for safety reasons, the line should be cleaned with at least five batches of wheat groats of 480 kg each. On the other hand, the permissible level of maduramicin is ten times lower than that of salinomycin, which consequently signals the need for more cleaning stages. In the case of maduramicin, it is recommended that the line should be cleaned with a minimum of eight batches of groats.

Dolenc et al. [7] state that the use of feed contaminated with 0.015 mg/kg maduramicin causes residues exceeding 6 μg/kg maduramicin in eggs, while in the case of approximately 0.05 mg/kg in feed, residues exceeding 10 μg/kg were found in eggs [7].

After the cleaning procedure with 12 batches of wheat groats, on the same production line, we produced feedstuff for laying hens without added coccidiostats. On checking the levels of coccidiostats in order to conduct verification of the cleaning procedure, satisfactory levels of salinomycin and maduramicin were measured in this non-medicated feed (Table 4).

Table 4. Salinomycin and maduramicin in laying hen feed

Feedstuff for laying hens (samples taken from one batch)	Salinomycin, mg/kg	Maduramicin, mg/kg
1	0.004	0.006
2	0.005	0.004

3	0.006	0.004
4	0.007	0.003
5	0.015	0.018
6	0.006	0.014

Both coccidiostats were present in the permitted amounts, with a maximum of 0.015 and 0.018 mg/kg for salinomycin and maduramicin, respectively, which indicates a successfully implemented cleaning process in the production line. The coccidiostat levels in non-medicated feed in our study are lower than in a study conducted in Denmark in the period 2004-2007 [8]. The authors stated that of the 111 tested samples of the first batch of non-medical feed, prepared after the production of feed with salinomycin and the cleaning process, 13 samples had noticeable amounts of salinomycin in the range of 0.07-2.95 mg/kg [8].

4. Conclusion

After the production of medicated feed with coccidiostats, it is necessary to clean the production equipment. For salinomycin, the minimum number of cleaning replicates is five while in the case of maduramicin, a minimum of eight batches (480 kg each) of wheat groats are required to ensure the safe, consequent production of non-medicated feed and to avoid the possible harmful effects of coccidiostat carry-over into non-medicated feed.

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Food allergens–food safety hazard

Food allergens – food safety hazard

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Abstract. Food allergy is a life-threatening chronic condition that substantially impairs quality of life. It constitutes a significant public health problem that affects children and adults and is a considerable burden on health, medical systems and emerging economies. Food allergy is defined as an adverse health effect arising from a specific immune-mediated response that occurs reproducibly on oral exposure to a given food and which can be mediated by food-specific IgE antibodies, by cellular mechanisms or by both. Appropriately managing food allergies has become an issue for the food industry because of the rising number of individuals with food allergies. Current initiatives to support consumers at risk include a recognised standard for manufacturers seeking to eliminate an allergen from their production, and the integration of food allergy into training for food business operators.

1. Introduction

Food allergies are a distinctive food safety issue that only affect part of the world's population, with the effects ranging from mild discomfort to, in the worst case, fatality [1]. Food allergies harm only specific parts of the population sensitive to food allergens; this is a significant difference to the other common food safety issues, as microbial or chemical contamination, which impair everyone. Food allergy is defined as an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food [2] or as an adverse reaction to food in which immunologic mechanisms have been demonstrated [3]. According to [4], adverse reactions to food have been classified into different groups on the basis of the pathogenic mechanism. They include immunological reactions, which can be mediated either by IgE antibodies, by cells (non-IgE-mediated) or both (mixed), and non-immunological responses (food intolerance), which are dependent on enzyme deficiencies or pharmacological reactions or, in the majority of cases, arise by unknown mechanisms.

Food allergy is recognised as an important public health issue, requiring collaboration between multiple stakeholders, including the food industry, to be effectively addressed. Management of allergens in the food industry aims to assure the safety of allergic consumers, but has proved particularly challenging because of the unique attributes of food allergy and food allergens. Accurate allergen labelling, minimising unintended allergen presence and circumspect use of meaningful precautionary labelling where required form the cornerstones of effective allergen management. Currently, there are no accessible cures for food allergy. Strict allergen avoidance remains the most efficient allergy management to avoid an allergic reaction, which can be severe and occasionally life-threatening [5].

2. Food allergens

Successful allergen avoidance depends on the identification of the relevant food allergen. In order to enable consumers to immediately identify relevant allergens, the European Union (EU) food information regulation [6], and in Serbia, the Rulebook on declaration, labelling and advertising of food [7] and the Rulebook on health correctness of diet foods [8] require mandatory labelling of 14 allergenic foods or food groups if they are ingredients in the manufacturing of foods. These food groups are: 1. Cereals containing gluten, namely: wheat (such as spelt and khorasan wheat), rye, barley, oats or their hybridised strains, and products thereof, except: (a) wheat based glucose syrups including dextrose, (b) wheat based maltodextrins, (c) glucose syrups based on barley and (d) cereals used for making alcoholic distillates including ethyl alcohol of agricultural origin, 2. Crustaceans and products thereof, 3. Eggs and products thereof, 4. Fish and products thereof, except: (a) fish gelatine used as carrier for vitamin or carotenoid preparations, (b) fish gelatine or Isinglass used as fining agent in beer and wine, 5. Peanuts and products thereof, 6. Soybeans and products thereof, except: (a) fully refined soybean oil and fat, (b) natural mixed tocopherols (E306), natural D-alpha tocopherol, natural D-alpha tocopherol acetate, and natural D-alpha tocopherol succinate from soybean sources (c) vegetable oils derived phytosterols and phytosterol esters from soybean sources (d) plant stanol ester produced from vegetable oil sterols from soybean sources, 7. Milk and products thereof (including lactose), except: (a) whey used for making alcoholic distillates including ethyl alcohol of agricultural origin (b) lactitol, 8. Nuts, namely: almonds (*Amygdalus communis* L.), hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anacardium occidentale*), pecan nuts (*Carya illinoinensis* (Wangenh.) K. Koch), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia vera*), macadamia or Queensland nuts (*Macadamia ternifolia*), and products thereof, except for nuts used for making alcoholic distillates including ethyl alcohol of agricultural origin, 9. Celery and products thereof, 10. Mustard and products thereof, 11. Sesame seeds and products thereof, 12. Sulphur dioxide and sulphites at concentrations of more than 10 mg/kg or 10 mg/litre in terms of the total SO_2 which are to be calculated for products as proposed ready for consumption or as reconstituted according to the instructions of the manufacturers, 13. Lupin and products thereof and 14. Molluscs and products thereof.

Example of international differences and evaluation in case studies is given in the following. Allergen declaration requirements in the United States (US) are according to [9], which requires the declaration of eight allergens and sulphites but currently does not require the declaration of sesame seeds or lupin. These are: cereals containing gluten (i.e. wheat, rye, barley, oats, spelt or their hybridized strains – oats do not contain gluten, but they are commonly produced in the same location as gluten-containing cereals such as wheat, resulting in allergen cross-contact), crustaceans, eggs, fish, milk, peanuts, soybeans and tree nuts (BIG 8). On April 23, 2021, the Food Allergy Safety, Treatment, Education, and Research (FASTER) Act was signed into law, declaring sesame as the ninth major food allergen recognized by the US. This change will become effective on January 1, 2023, so labelling of sesame as an allergen will not be required until that time [10].

Based on systematic and thorough assessments which used all three criteria (prevalence, severity and potency), the Expert Committee of the FAO/WHO [11] recommended that the following should be listed as priority allergens: Cereals containing gluten (i.e., wheat and other *Triticum* species, rye and other *Secale* species, barley and other *Hordeum* species and their hybridized strains), crustacea, eggs, fish, milk, peanuts, sesame, specific tree nuts (almond, cashew, hazelnut, pecan, pistachio and walnut). Due to the lack of data on prevalence, severity and/or potency, or due to regional consumption of some foods, the Committee recommended that some of the allergens, such as buckwheat, celery, lupin, mustard, oats, soybean and tree nuts (Brazil nut, macadamia, pine nuts), should not be listed as global priority allergens, but can be considered for inclusion on priority allergen lists in individual countries. Since current dietary trends include an increased consumption of plant-based foods and diets consisting of alternative protein sources, it was recommended that pulses, insects and other foods such as kiwi fruits be included in a watch list and evaluated for the priority allergen list when data on prevalence, severity and potency become available [12, 13, 14]. Finally, the Expert Committee recommended that foods and ingredients

derived from the list of foods known to cause immune-mediated hypersensitivities should be evaluated on a case-by-case basis for exclusion from declaration on ingredient lists and/or on food packaging.

3. Food Allergen Management

In a global market, it is crucial that there is harmonized understanding of the food allergens and of the measures required to address allergy. In [15], a new Code of Practice on Food Allergen Management for Food Business Operators is presented, which provides guidance on allergen management beginning at primary production and continuing throughout the manufacturing process. Allergen management practices should be part of implemented food safety management systems (FSMS) including prerequisite programme (PRP) activities, good hygiene practices (GHPs), and, where appropriate, HACCP systems, in manufacturing, retail and food service [16,17]. Allergens need to be managed throughout the supply chain and production process. Treatments lethal for pathogenic microorganisms, such as heating, high pressure processing, etc. generally do not destroy allergenic proteins. Processes that degrade proteins, such as enzymatic or acid hydrolysis, should not be relied upon to eliminate or completely destroy allergenic proteins.

The new code is intended to facilitate a proactive approach to managing allergens in food production, rather than a reactive response once a food safety hazard has been identified. It provides guidance on allergen management throughout the production process, including controls to prevent cross-contact where an allergen is inadvertently transferred from a food containing an allergen to a food that does not contain the allergen. Taking a whole supply chain approach, the code spans primary production, manufacturing, and retail and food service, and supplements both of the newly-adopted, revised General Principles of Food. The HACCP plan should include allergens as an independent category of food safety hazard. This involves evaluating the hazards associated with the whole lifecycle of the product, starting with raw materials and assessing every step of the process through to labelling and packaging of the final food for sale. Manufacturers providing partially prepared foods or ingredients from business to business and not to the end consumer must also maintain a thorough allergen management program. The critical points where allergens can be introduced as ingredients or into foods during processing should be identified, and systems need to be established to prevent the unintentional cross contact of allergens to other products.

Allergen risk management starts with investigating the manufacturing process for allergen risks and hygiene, and includes activities to label allergens according to the ongoing work on allergen labelling by the Codex Committee on Food Labelling. The information obtained can be used to develop an Allergen Management Program (AMP). The implementation and use of an effective AMP in conjunction with an allergen risk review approach contributes to food businesses meeting food safety, quality and legal requirements [14].

Food labelling for the presence of allergenic foods/ingredients must identify all foods that intentionally contain the particular food group or ingredients derived from that food. However, voluntary labelling for the possible presence of an allergen (e.g. “May contain”) should be reserved for situations that potentially represent genuine hazards. In recent years, there has been a proliferation of the use of precautionary allergen statements, which range in wording from “May contain” and “Processed in a facility”, to “Made on shared equipment”. This increase has limited consumer food choices. Alarmingly, food-allergic consumers, especially teens, are beginning to ignore precautionary statements, and take risks regarding the food they choose to eat. This can lead to trouble for both the consumer and the industry [18].

4. Allergen analysis

The analysis of a material or surface for the presence and/or amount of an allergen is a valuable tool for a risk-based approach to allergen management. Analytical test results can provide assurance and verification of critical controls within a comprehensive AMP and assist the implementation of a quantitative risk assessment. Understanding the nature of the allergen, its form (i.e. powder, liquid, homogenous or particulate) and its behaviour in the food in which it is used will play a major role in the

choice of methodology applied. Allergen analysis is appropriate for: confirmation of the allergen status of raw materials; validation of appropriate cleaning protocols; verification or ongoing monitoring of cleaning efficacy including flushing and push through volumes; environmental monitoring (which should run in parallel with microbiological and hygiene monitoring); monitoring the effects of critical changes in the process; identifying sources of cross contact; confirming risk assessment assumptions; assessing customer complaints; investigating potential control failures, and; assisting in verification of free-from claims. Analysis should be used for validation and verification purposes as part of a HACCP based food safety program [19].

Recommendations with the aim of protecting the modern buyer/consumer are development, improvement and implementation of allergen protocols and education of employees regarding precisely defined and consistent standards related to the management of safety of allergens, which lead to consistent and sustainable food safety management systems [17].

5. Allergen communications

At the food industry level, the distance between manufacturers and consumers has increased significantly, due to the global food trade, and labels with food allergen information have become even more important to prevent allergic reactions from occurring [20]. It is important to manage food allergies within local contexts. It is critical for countries to understand what allergens are common in a particular population, what foods need to be labelled and how to determine the allowable quantities of food allergens. National food safety competent authorities should: identify or develop a mechanism that regularly monitors the common food allergies in the national context; ensure clarity and readability of food allergen labels and provide education on how to read them, especially to the allergic population; develop or strengthen collaborative mechanisms with the private sector, particularly with food e-commerce platforms and restaurants, to ensure that food allergens are explained to their customers, for example in menus; support research and development of diagnostic tools that facilitate the detection of food allergies in humans and in foods; educate the population on the topic of food allergies through targeted communication campaigns aimed at raising awareness on the topic, and; contribute to global discussions, national case studies and relevant data [21,22,23].

6. Online Shopping

With the increasing rates of online grocery shopping, people with food allergy will rely more heavily on online food label information. This information should be presented in a way that assists consumers with their purchasing choice. Vigilance is required in ensuring online information regarding the ingredient and allergen content is correct, as shoppers are likely to assume that this information reflects the food that will be delivered. It is critical the information online clearly reflects what is on pack. Food manufacturers should have procedures in place that alert retailers and distributers when the allergen status of a food changes so that the shopping websites can be updated. For those who maintain the websites, it is recommended that measures are in place to ensure that the online food label information is up to date [23].

7. Food allergen recall

Food allergen recall is an action taken by a food business to remove unsafe food from distribution, sale and consumption. A consumer-level food recall involves the removal of unsafe or unsuitable food from all points in the production and distribution networks, including any affected food in the possession of consumers. The public must be informed of a consumer-level recall, and this usually involves the use of media such as newspaper advertisements, point of sale notices and publication of information about the recall [23].

8. Conclusion

Food allergies may impact only part of the world's population, but that impact can be lethal. The absence of a comprehensive implementation report for food allergen labelling regulations has resulted in

proliferation of different risk mitigation strategies, leaving consumers uncertain and confused about the safety of food products. It is, therefore, extremely important that food labels contain sufficient information to enable allergic people to avoid the risks of allergic reactions. National strategies, activity by different stakeholders (allergic consumers, health professionals, public authorities and the food industry), identification of the predominance of food allergies, investigation into what foods should be labelled, and definition of appropriate levels of protection from the risks to food-allergic consumers due to the unintended presence of allergen(s) in food, remain pressing priorities.

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Wild thyme *(Thymus serpyllum* L.) supercritical extract as antioxidant in precooked pork chops during chilled storage

Wild thyme *(Thymus serpyllum* L.) supercritical extract as antioxidant in precooked pork chops during chilled storage

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Abstract. The effect of thyme supercritical extract on oxidative stability of precooked and cold-stored (at 4°C for 6 days) pork chops was analysed. Thyme extract was applied with a marinated process (SF1), or was introduced on the surface of the chops after cooking (SF2). Thyme extract in SF1 samples showed a significant protective effect towards oxidation of lipids during the cooking process. Both processes of thyme application showed potential for lipid oxidation inhibition throughout the refrigerate storage period of precooked pork chops, thus protecting colour and sensory characteristics of the samples. Finally, at the end of storage period, the lowest oxidative changes were determined for SF1chops.

1. Introduction

The importance of ready meals in the food markets has been increasing constantly. The increase is especially for chilled ready meals, because most consumers (75%) rather choose this type of product over its frozen counterpart [1]. Consumers choose products which do not require much time, skill or energy for complete home meal preparation. That is why food convenience become one of the main trends in nowadays lifestyles. Convenience foods are produced by high industrial processing degrees [2, 3]. Considering meat products, precooked or ready-to-eat meat products could be the representatives of convenience foods [4]. During industrial processing and storage, meat products are subjected to several changes that could have negative effects to appearance, colour and overall acceptability of the products. In addition, products could undergo formation of off-odours and off-flavours [5]. The main cause of processed meat quality deterioration is lipids oxidation, facilitated by high temperatures during cooking. Lipids oxidation leads to the development of rancid flavour, known as warmed-over flavour (WOF). WOF is especially noticeable in meat which is reheated after being cooked and refrigerate stored. That is why the modern trend of controlling lipid oxidation is increasingly important [6]. Rancidity and WOF development in meat products could be slow down with both synthetic and natural antioxidants. Since there is an increasing preference for natural products, antioxidants of plant origin are gaining an increasing advantage over synthetic ones [5, 7].

For isolation of plants' bioactive components supercritical fluid extraction, as an excellent process, could be used. This extraction could reduce solvent, time and energy consumption. It could gain better extraction yield and prevent degradation of sensitive compounds [8].

Regarding the problem of precooked and chilled stored meat quality deterioration caused by WOF formation, the objective of this paper was to evaluate the effect of wild thyme supercritical extract on oxidative stability and sensory characteristics of cooked and refrigerated pork chops.

2. Materials and Methods

2.1. Supercritical fluid extraction

Supercritical fluid extraction, performed on a laboratory-scale high pressure extraction plant (HPEP, NOVA, Swiss, Effretikon, Switzerland) was used for isolation of thyme extracts and it was described in detail by Pavlić et al. [8]. Extract was obtained at: pressure = 100 bar, temperature = 40°C and CO_2 flow rate = 0.3 kg/h, while separator conditions were 15 bar and 25°C.

2.2. Preparation of marinated pork chops

Three fresh (24 h *post-mortem*) pork loins were purchased from a slaughterhouse. All separable connective tissue, fascia and external fat were removed, and loins were cut perpendicular to the muscle fibres into 2-inch thick chops. Chops were divided to three following treatments: C (meat marinated in water/salt – control), SF1 (meat marinated with thyme supercritical extract – 0.2 µl/g), SF2 (meat marinated in water/salt, cooked, introduced into plastic bags with supercritical extract – 0.2 µl/g, and massaged for 10 min). Chops were cooked in a convection oven (175°C) until 72°C was achieved in the centre of the chop. In order to simulate the common household conditions after cooling chops were transferred to plastic boxes and covered with aluminium foil, to be exposed to air, and stored in refrigerator (4°C) for 6 days. Analyses were performed after cooking (day 0) and during 6 days of storage.

2.3. Colour measurement

Colour measurements were performed on the surface of cooked pork chops using a MINOLTA Chroma Meter (Model CR-400), with aperture of 8 mm in the measuring head and standard additions to measure CR-A33b (Konica Minolta Inc., Osaka, Japan). Colour characteristics were expressed in CIE *L*a*b** system (L*– lightness, a*– redness; b*– yellowness), and total colour changes (ΔE) were calculated using obtained CIE *L*a*b** values [9].

2.4. TBARS determination

Lipid oxidation was estimated as 2-thiobarbituric acid reactive substances test (TBARS), and was expressed as milligrams of malondialdehyde per kilogram of the sample (mg MDA/kg). The TBARS test was performed according to Botsoglou et al. [10].

2.5. Sensory analyses

Nine panellists, with experience in meat products sensory evaluation, performed sensory analysis using a 15-point descriptive scale (where 1 = not noted; 15 = extremely present). Prior to the sensory analyses, chops were reheated for 10 minutes in a convection oven (at 175°C), and were served warm to the panellists. Panellists evaluated odour (cardboard-like, linseed oil-like) and flavour (rancid, vegetable oil-like, marinated oil-like) characteristics of the samples, regarding the descriptors as described by Byrne et al. [11]. Before starting the sensory evaluation, panellists were introduced with referent materials for descriptors.

2.6. Statistical analysis

STATISTICA 12.0 (StatSoft, Inc., Tulsa, OK, USA) was used for statistical analysis. The obtained results were analysed using ANOVA (One-way), and differences among treatment means were compared according to Duncan's multiple range test ($p \leq 0.05$).

3. Results and Discussion

Formation of TBARs was used to determine the extent of lipid oxidation in the samples of precooked pork chops, and the results, expressed in mg malondialdehyde (MDA)/kg of sample, are shown in Fig. 1. For the samples of raw meat TBARs value was 0 mg MDA/kg indicating the absence of lipid oxidation. The TBARs values slightly increased after marinating, what could probably be due to salt addition and meat massaging [12]. Heat is recognised as the one of the main initiators for the lipid oxidation [13] and that is why first increase in TBARs value was determined after cooking. Significantly higher TBARs value increase was determined for control sample, comparing to experimental ones. Marinating meat with thyme extract slow down lipid oxidation caused by high temperatures during cooking, and consequently, SF1 samples had almost no change in TBARs values after cooking, comparing to samples after marinating. During the storage period oxidative rancidity increased in all samples, and significant differences ($p<0.05$) in TBARs values among samples were noted throughout the entire storage period. The highest increase was for control samples, and after 4 days of storage TBARs value for this sample reached 2.08 mg MDA/kg. Thyme extract also showed a significant protective effect towards lipid oxidation during chilled storage, but to the different extents. The lowest TBARs values were determined for marinated chops during the whole storage period. Samples treated with thyme extract after cooking had, during the whole storage period, TBARs values that were higher than in marinated chops, but significantly lower ($p < 0.05$) than in the control ones. Thyme extract's antioxidant activity could be attributed to the monoterpene phenolics, especially carvacrol and thymol, what was in accordance with the results of Šojić et al. [14].

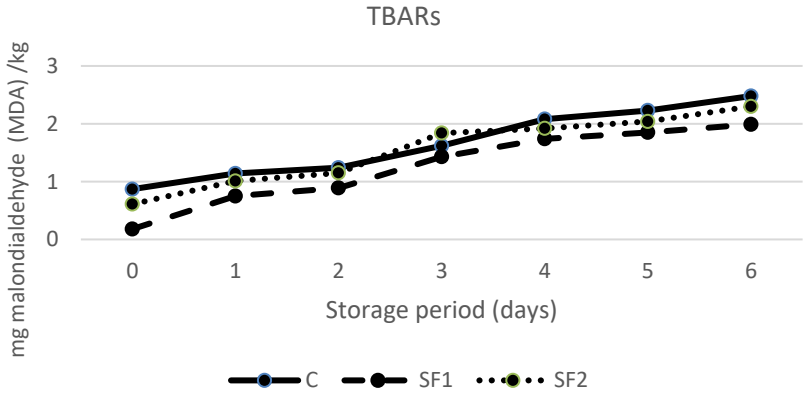

Figure 1. Effect of thyme on TBARS values in precooked pork chops during refrigerate storage

The results of instrumental colour characteristics measurements during 6 days of storage (+4°C) are shown in Table 1. Lipid oxidation influence colour changes. Along with lipid oxidation in a coupled lipid-pigment reaction haem pigments also oxidize, resulting in colour change [15]. Differences in L^* values were noted among samples during the storage period, but no clear pattern was observed. In the experiment for model raw pork batters Hernandez-Hernandez et al. [16] correlated higher TBARs values, as indicators of lipid oxidation, to lower L* values (darker samples). On the other hand, Oliveira et al. [15] did not find any correlation between lipid oxidation and lightness in mortadella. Redness (a* values) of all chops was within the range 0.87-7.25, and majority of the samples were in the range perceived as of grey colour [17]. The redness of the chops decreased during storage, what

was in accordance with results of Fernandez-Lopez et al. [18], who reported that decrease in *a** value for meat products could be correlated with oxidation processes. At the end of storage period, the lowest *a** value was determined for control samples.

In general, the yellowness (*b** values) did not change significantly during 6 days of storage. Changes of colour characteristics during storage could be shown by the value of total colour change, ΔE. When ΔE is higher than 2, modifications in colour are considered to be occurred [19]. For control chops, ΔE was higher than 2 for the whole storage period, while SF1 and SF2 chops reached this value after 6 days.

Table 1. Effect of thyme on colour characteristics of precooked pork chops during refrigerate storage

Day	Sample	L*	a*	b*	ΔE	Day	Sample	L*	a*	b*	ΔE
	C	73.04	3.69^{a}	9.69^{b}			C	75.13	2.11^{b}	9.84^{c}	2.62
0	SF1	72.89	3.58^{a}	10.77^{a}		4	SF1	74.01	2.80^{a}	11.30^{b}	1.47
	SF2	74.28	2.92^{b}	11.00^{a}			SF2	75.48	2.03^{b}	12.18^{a}	1.90
	C	77.26^{a}	7.25^{a}	10.38^{b}	5.56		C	75.22^{ab}	1.30^{b}	10.79	3.42
1	SF1	74.49^{b}	3.07^{b}	11.23^{a}	1.74	5	SF1	76.10^{a}	2.41^{a}	10.90	3.42
	SF2	71.42^{c}	3.20^{b}	10.81a^{b}	2.89		SF2	74.38^{b}	2.42^{a}	11.03	0.51
	C	69.76^{b}	2.09^{c}	9.00^{b}	3.72		C	75.30	0.87^{b}	10.28^{b}	3.67
2	SF1	71.9^{a}	3.07^{a}	10.45^{a}	1.09	6	SF1	74.69	1.97^{a}	11.29^{a}	2.48
	SF2	72.87^{a}	2.62^{b}	10.79^{a}	1.46		SF2	74.13	2.15^{a}	11.37^{a}	0.86
	C	74.13^{b}	1.84^{b}	9.17^{b}	2.21						
3	SF1	73.78^{b}	3.31^{a}	10.21^{a}	1.09						
	SF2	75.77^{a}	3.16^{a}	10.35^{a}	1.64						

^{a,b,c} values in the same column, same day, between different treatments – with different superscript letters – are significantly different ($p < 0.05$)

The results of sensory analyses for precooked and chill stored pork chops are presented in Fig. 2. Differences between freshly cooked and chill stored samples in several odour and flavour attributes were determined. Analysed sensory attributes changed differently during the storage period.

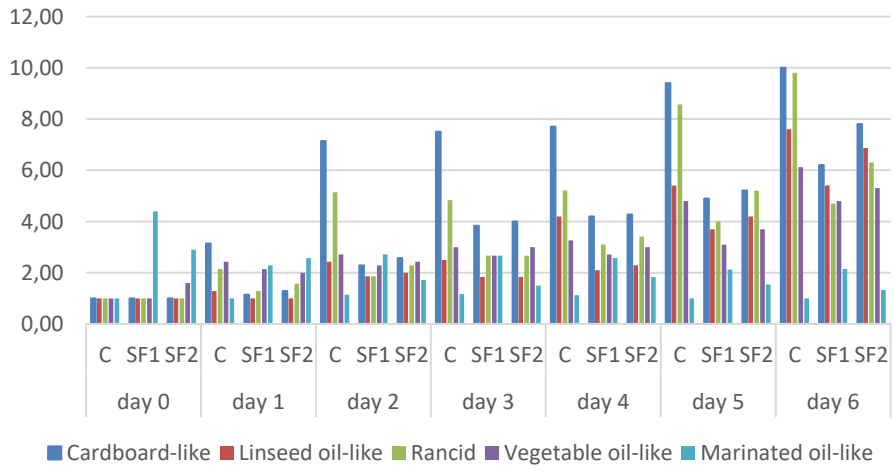

Figure 2. Effect of thyme on sensory characteristics of precooked pork chops during refrigerate storage

The attributes, cardboard like, vegetable and linseed oil odour, and rancid flavour, which are directly associated with WOF development [20], had higher scores during storage period. For control

samples was the highest increase in cardboard odour, followed by SF2 samples. For SF1 samples cardboard odour had the lowest value. Byrne et al. [21] correlated intensity of cardboard flavour with WOF formation, but Campo et al. [22] pointed out that cardboard-like flavour disappeared when the flavour was dominated with rancidity notes.

Rancid flavour increased with storage, and again, the highest values were for control chops. Campo et al. [22] reported positive correlations between rancid flavour and WOF formation with TBARS values. According to Oliveira et al. [15] sensory recognized oxidation, that is WOF formation, for pork and beef products is associated with TBARs values in the range from 0.3 to 1.0 mg MDA/kg. Obtained positive effect of thyme extract on WOF formation was in accordance with the results of Lara et al. [16] which showed efficacy of natural antioxidants, originated from rosemary and lemon balm, in controlling lipid oxidation in processed meat product. In our study, the marinating process resulted in more pronounced thyme aroma, compared with samples treated with thyme extract after cooking.

4. Conclusion

The addition of thyme supercritical extract, as natural antioxidants, in the processing of precooked pork chops, resulted in protective effects against oxidation of lipids during 6 days of storage under refrigeration conditions, consequently preserving colour and sensory characteristics. Usage of extract in the marinating process gain better results, than when applying thyme extract after cooking. Nonetheless, samples with application of extract after cooking were better than the control ones, and the thyme aroma was less pronounced than in extract marinated samples, thus had lower impact in changing the dominant aroma of cooked meat.

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Food contact surfaces and food handler's hygiene in one Serbian retail chain–estimation and trend

Food contact surfaces and food handler's hygiene in one Serbian retail chain – estimation and trend

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Abstract. This research aimed to investigate the efficiency of sanitary procedures (cleaning, washing, disinfection) applied on food contact surfaces and food handlers' hands in one retail chain in Serbia. For that reason, a total of 364 swabs of food contact surfaces and 86 food handlers' hand swabs were investigated for microbiological parameters of process hygiene. The results showed that 15.66% (57 of 365) swabs of food contact surfaces, and 5.81% (5 of 86) swabs from the food handlers' hands, failed to meet the criteria laid down in the self-control plans of the food business operators. Therefore, continuous training of employees on the proper application of sanitation procedures is essential for efficient GHP and HACCP.

1. Introduction

Meat consumption is increasing worldwide due to the rapid growth of the population and urbanization. This has raised concerns and challenges regarding food and meat safety and hygiene because meat and meat preparations are routinely associated with food poisoning outbreaks [1]. Food safety is primarily achieved through a preventive approach, such as implementing a food safety management system based on Hazard Analysis and Critical Control Point (HACCP) principles and good hygiene practice (GHP). Good hygiene practices are prerequisites to implementing a HACCP system and are essential for producing safe food [2]. Furthermore, good hygienic practices at slaughterhouses and during distribution to and storage at retail shops, and during sales are critical points in ensuring the quality and safety of meat and meat preparations to safeguard public health [3]. Also, the implementations of the HACCP system and GHP are mandatory according to EU and Serbian Regulations, and every food business operator must have self-control plans with defined microbiological testing, such as type of examined microorganisms, dynamics, limits, etc.

To prevent contamination of meat, both during its processing and in retail, it is essential to regularly apply cleaning, washing, and disinfection of work surfaces, tools, equipment, and food handlers' hands [4]. Unfortunately, despite an increase in the number of food handlers receiving food hygiene training, many food poisoning outbreaks still occur due to improper food handling practices in the retail industry [5].

Food contact surfaces, such as food containers, utensils, plates, cooking kettle, cutting boards, slicers, knives, steel pallets and spatulas, stainless steel and plastic vessels for food distribution, are a significant concern for foodservice facilities in controlling the spread of food-borne pathogens because cross-contamination via food contact surfaces has been identified as an essential risk factor [6].

Food handlers' hands have been identified as pivotal vectors in the spread of food-borne disease due to poor personal hygiene or cross-contamination, resulting in the hands being contaminated with enteric pathogens [5]. The food handler and contact with contaminated surfaces are potential causes of cross-contamination and, consequently, outbreaks [7]. To prevent infection, people in the food production and food industries should be well trained and motivated to follow good personal hygiene practices, correct handwashing procedures, and follow these procedures while working [8].

Although handwashing may seem trivial to the food staff, failing to do it can have tragic consequences. It is generally acknowledged that a critical vehicle for cross-contamination of food is attributed to food handlers' hands. Accordingly, improved personal hygiene and meticulous hand washing would lead to the primary control of the feces-to-hand-to-mouth spread of potentially pathogenic transient microorganisms [8, 9].

This research aimed to assess microbiological parameters of process hygiene and investigate the efficiency of sanitary procedures applied on food contact surfaces and food handlers' hands in one retail chain in Serbia.

2. Materials and Methods

During four years (from January 2017 to December 2020), an assessment of the process hygiene was carried out in 18 retail shops in Serbia. Overall, a total of 364 swabs of food contact surfaces (FCS) and 86 food handlers' hand (FHH) swabs were investigated for microbiological parameters of process hygiene (Table 1).

Table 1. Number of sampled swabs in each year

Year	Food contact surfaces (FCS) swabs	Food handlers' hands (FHH) swabs
2017	109	/
2018	77	/
2019	101	48
2020	77	38
Total	**364**	**86**

2.1. Swab samples

Swab samples from the food contact surfaces and food handlers' hands were taken after cleaning, washing, and disinfection procedures. Sampling was conducted according to the standard method [10]. On the sampling day, swabs were transported to the laboratory in a cold chain and analyzed within 24h. All samples were analyzed in an accredited laboratory according to SRPS ISO/IEC 17025:2006.

2.2. Microbiological examinations

Swab samples from the food contact surfaces and food handlers' hands were tested for aerobic colony count (ACC) according to SRPS EN ISO 4833-1:2014 [11] and *Enterobacteriaceae* (ENT) in line with SRPS ISO 21528-2:2009 [12]. Results of the microbiological analyses were expressed as a number of bacteria per cm^2 (CFU/cm^2) and number of bacteria per swab (CFU/swab) for swabs taken from the food contact surfaces and food handlers' hands, respectively.

2.3. Evaluation of microbiological results

The estimation of the obtained results of microbiological contamination was carried out following the limit values set by the self-control plans of the food business operators (Table 2).

Table 2. Microbiological criteria in the self-control plans of the food business operators

Microorganisms	Porcelain, glass, smooth metal food contact surfaces	Plastic, wood, stone food contact surfaces	Food handlers' hands (HS)
Aerobic colony count	≤ 10 CFU/cm^2	≤ 30 CFU/cm^2	≤ 2000 CFU/swab
Enterobacteriaceae	≤ 1 CFU/cm^2	≤ 1 CFU/cm^2	≤ 10 CFU/swab

3. Results and discussion

During the four years (from January 2017 to December 2020), in 18 retail shops, 364 swabs from food contact surfaces (cutting boards, slicing machines, knives, refrigerator doors, metalworking surfaces) were examined. During 2017, a total of 109 swabs were examined, followed by 77, 101, and 77 swabs in 2018, 2019, and 2020, respectively (Table 1).

The results showed that 15.66% (57 of 364) swabs of food contact surfaces failed to comply with the criteria laid down in the self-control plans of the food business operators. In 2017, 27.52% of swabs of food contact surfaces were non-compliant with the limits set in control plans, while in 2018, 2019, and 2020, rates of non-compliance were 14.29%, 9.90%, and 7.79% swab, respectively (Table 3). These findings are close to those conducted by Legnani *et al.* [13] in 2004 and Garayoa *et al.* [14] in 2014, while in the research undertaken by Vesković et al. [2], this percentage was significantly higher, up to 41.96%. Reduction of the percentage of non-compliant swabs in 2019 and 2020 compared to 2017 suggests that training of employees on the proper application of sanitation procedures proved to be essential for efficient GHP and HACCP.

The main reason for the non-compliant results of swabs of food contact surfaces in all four years was the high ACC (2017 – 93.33%, 2018 – 90.91%, 2019 – 70.00%, and 2020 – 83.33).

Table 3. Microbiological status of the food contact surfaces

Year	Number of swabs	Non-compliant		Finding	Frequency		Finding	Frequency	
		N	%		n	%		n	%
2017	109	30	27.52	ACC	28	93.33	ACC + ENT	2	6.67
2018	77	11	14.29	ACC	10	90.91	ACC + ENT	1	9.09
2019	101	10	9.90	ACC	7	70.00	ACC + ENT	3	30.00
2020	77	6	7.79	ACC	5	83.33	ACC + ENT	1	16.67

During the two-year period (from January 2019 to December 2020), 86 swabs from food handlers' hands were examined in the same retail shops. In 2019, 48 swabs were tested, while in 2020, 38 swabs (Table 1).

The results of microbiological examinations of swabs from the food handlers' hands showed that 5.81% (5 of 86) swabs were not compliant with the criteria in the self-control plans of the food business operators. However, the results of food handlers' hand swabs showed a similar level of hygiene in both years (non-complaiant in 2019 - 8.33%, and in 2020 - 2.63%) (Table 4). Again, in most findings, the reason for non-compliant results in both years was the high ACC (80.00%). These findings are similar to those reported by Ivanović et al. [15] in 2013, while in a study conducted by Rašeta et al. [4] in 2012, this percentage was significantly higher, 30.0%.

Table 4. Microbiological status of the food handlers' hands

Year	Number of swabs	Non-compliant		Finding	Frequency		Finding	Frequency	
		N	%		n	%		n	%

2019	48	4	8.33	ACC	3	75.00	ACC + ENT	1	25.00
2020	38	1	2.63	ACC	1	100.00	ACC + ENT	/	/

4. Conclusion

According to the results obtained in this study for the presence and enumeration of hygiene indicator microorganisms on food contact surfaces and food handlers' hands, the sanitary conditions in these retail shops were adequate. However, to maintain this level of process hygiene, it is necessary to constantly educate workers regarding the sanitary procedures of work surfaces, tools, equipment, and food handlers' hands. Also, it is obligatory to continue with regular swabs controls of food contact surfaces and food handlers' hands as defined in the self-control plan of the food business operators.

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Resistance of *Salmonella* spp. isolated from poultry meat to antimicrobial drugs

Resistance of *Salmonella* spp. isolated from poultry meat to antimicrobial drugs

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Abstract. Uncontrolled use of antimicrobials in the prophylaxis and treatment of human and animal diseases leads to the appearance of residues in the environment and food. The use of antimicrobials as growth promoters in breeding affirms the importance of their residual finding in foods of animal origin. Bacteria of the genus *Salmonella* are one of the most common causes of food poisoning. All species of this genus are pathogenic to humans and cause various diseases known as salmonellosis. Humans can become infected through the faecal-oral route by consuming contaminated food and water or by direct contact with an animal. *Salmonella* that are resistant to antimicrobial drugs can transfer resistance genes to other microorganisms. In this work, the resistance of 10 *Salmonella* isolates from poultry meat to 8 different antimicrobial substances was examined by the disk diffusion method. All *Salmonella* isolates were sensitive to trimethoprim-sulfamethoxazole and chloramphenicol. All isolates were resistant to amoxicillin and significant percentages were resistant to other antimicrobial drugs. Also, multi-drug resistance of *Salmonella* isolates was found. The best prevention of salmonellosis in humans is constant and comprehensive control of this hazard in food products during production, processing, storage, and sale.

1. Introduction

Salmonellosis is a major health and economic problem. The disease occurs in all animals and intensifies with more intensive farming. In young animals, it usually occurs in the form of septicaemia, while in older animals, it is characterized by acute and chronic enteritis. Despite progress made in *Salmonella* control, this pathogen is still present in food and is the most common cause of foodborne diseases [1],[2].

According to a 2018 report by the European Food Safety Authority (EFSA) and the European Center for Disease Prevention and Control (ECDC), *Salmonella* was the cause of every third food related epidemic reported in European Union countries in year 2018. Salmonellosis is the second most commonly reported gastrointestinal infection in human in the EU (91857 reported cases). In 2018, EU countries reported 5146 food-related epidemics involving 48365 cases. Slovakia, Spain and Poland reported 67% of their total 1581 food-related epidemics were caused by *Salmonella*, and the source of the epidemics was mainly associated with eggs [3]. When it comes to food production all over the world and in Serbia, food safety comes first. Safe food is free of residues, contaminants and pathogenic microorganisms. In food production, primary production itself has a great impact on safety.

For *Salmonella* infections, morbidity in humans is almost 100%, and mortality occurs in environments where general hygiene conditions are poor and where there is no health care [4]. It is estimated that 93.8 million people worldwide are diagnosed with *Salmonella*-induced gastroenteritis each year, with 155000 deaths. *Salmonella* is the most common bacterial cause of foodborne illness in the United States, causing about 44% of confirmed bacterial foodborne infections, and in Europe *Salmonella* infection is immediately behind *Campylobacter* infection [5]. *S*. Enteritidis and *S*. Typhimurium are the serotypes that most commonly cause salmonellosis in humans. Frequent use of antimicrobial drugs results in the occurrence of resistance to various antimicrobial drugs in bacteria, including *Salmonella*. In the 2018 EFSA/EDCA report, antimicrobial resistance was confirmed as one of the main threats to public health [3].

Bacteria can be resistant to one or more different antimicrobial drugs. It is also important to note that antimicrobial drug resistance can be transmitted from resistant to non-resistant bacteria, but also to those that already carry resistance to the same or different antimicrobial drugs, thus creating multiple resistance. As a consequence of *Salmonella* antimicrobial resistance, there is an increase in unsuccessful antimicrobial treatments, an increase in the number of hospitalized cases and increase in mortality [6]. Excessive use of antibiotics in the medical and veterinary industries is one of the main causes for the emergence of multi-resistant foodborne pathogens that are often difficult to treat. In recent years, epidemics caused by multidrug-resistant *Salmonella* have been documented [7].

In cases of invasive salmonellosis, antimicrobial drugs (amoxicillin, chloramphenicol, ceftriaxone, ciprofloxacin, trimethoprim-sulfamethoxazole) are used in therapy [8]. Salmonellosis is known to be a major public health issue, and the aim of this study was to examine the occurrence and prevalence of *Salmonella* in food and their resistance to antimicrobial drugs.

2. Materials and methods

Ten isolates of *Salmonella* from poultry meat were used in this study. The submitted samples were sown on selective-differential media [9]. Suspect colonies grown on solid substrates were sown first on nutrient agar and then on triple sugar iron agar. Biochemical tests (triple sugar, urea agar, lysine decarboxylase broth, indole formation and VP reactions) were used for biochemical identification, thus confirming the physiological characteristics of the genus *Salmonella*. The disk diffusion method according to [10] was used to test the susceptibility of isolates to antimicrobial drugs. The test isolates were first sown on trypticase soy agar and incubated for 24 h at 37°C. *Salmonella* suspensions in saline were prepared from the grown colonies, which corresponded to a density of 0.5 McFarland standard. *Salmonella* suspensions were applied with sterile swabs to Mueller Hinton agar (HiMedia, India), and then commercial antibiotic discs (Liofilchem, Italy) with specific amounts of active substance (amoxicillin 30μg, cefuroxime 30 μg, trimethoprim sulfamethoxazole 25 μg, chloramphenicol 30 μg, tetracycline 30 μg, ciprofloxacin 5 μg, gentamicin 10 μg, nalidixic acid 30 μg) were introduced. After 24 h incubation at 37°C, the results were read by determining the diameter of the inhibition zone and the mean value was calculated. Isolates that were read as intermediate were considered resistant**.**

3. Results and discussion

In the Laboratory for Microbiology and Food, Animal Feed and Water of the Dr Vaso Butozan Public Veterinary Institute of RS, a total of 1240 food samples were examined for the presence of *Salmonella*. Of the examined food samples, 54 (4.34%) did not meet the prescribed microbiological criteria for *Salmonella* [11]. The table (Table 1) shows the results of *Salmonella* testing for the examined food samples.

Table 1. Results of testing foods for the presence of *Salmonella*

Total number of examined samples	Satisfactory (no *Salmonella*)		Non satisfactory (contain *Salmonella*)	
	n	**%**	n	%
1240	1186	95.64	54	4.35

Of the 54 isolates of *Salmonella*, up to 10 isolates were tested for resistance/susceptibility to eight antimicrobial drugs, and the results obtained are shown in Table 2.

Table 2. Antimicrobial resistance of isolated *Salmonella* to antimicrobial drugs

Antimicrobial drug	Number of *Salmonella* examined	*Salmonella*			
		Susceptible	%	Resistant	%
Amoxicillin	10	0	0.00	10	100
Cefuroxime	7	5	71.42	2	28.57
Trimethoprim-sulfamethoxazole	10	10	100.00	0	0,00
Chloramphenicol	9	9	100.00	0	0,00
Tetracycline	10	6	60.00	4	40,00
Ciprofloxacin	10	5	50.00	5	50,00
Gentamicin	10	9	90.00	1	10,00
Nalidixic acid	10	5	50.00	5	50,00

All 10 (100%) *Salmonella* isolates included in our study were resistant to amoxicillin, 50% of isolates were resistant to ciprofloxacin and nalidixic acid, 40% to tetracycline, 28.57% to cefuroxime and 10% to gentamicin. Results of this study are in agreement with the results of other researchers when it comes to *Salmonella* resistance to nalidixic acid, tetracycline and ciprofloxacin to which different *Salmonella* serotypes show resistance worldwide [12, [13],[14], [15].

According to [16], in EU Member States, *Salmonella* isolated from broiler production was resistant to nalidixic acid, ciprofloxacin, tetracycline and sulphonamides. This current study did not show resistance to trimethoprim-sulfamethoxazole (10 isolates tested) or chloramphenicol (9 isolates tested). Some studies of antimicrobial resistance in non-typhoid *Salmonella* have shown a high incidence of resistance in *S.* Typhimurium (77%) to tetracycline, medium resistance (20-30%) to chloramphenicol, sulfamethoxazole-trimethoprim, ampicillin, and nalidixic acid and low resistance (less of 5%) to ciprofloxacin and third generation of cephalosporins [17]. The resistance of 84 *Salmonella* isolates to eight antimicrobial drugs was examined using the disk diffusion method, and it was found that all isolates were resistant to one or more tested antimicrobial drugs. All isolates were sensitive to chloramphenicol. Resistance ranged from 11.9% to sulfamethoxazole-trimethoprim (SXT) to 100.0% resistance to erythromycin [18]. Resistance to amoxicillin, doxycycline, kanamycin, gentamicin and tetracycline was found in 81.81% of *Salmonella* isolates, while 54.54% of isolated *S. enterica* serovars were highly sensitive to ciprofloxacin [19]. In the period between 2007-2011 [20], antibiotic resistance was assessed for 12,582 strains of *Salmonella*, and the results of the study showed increased resistance to ampicillin (12.4 to 18.9%), tetracycline (≈ 15.2 to ≈ 18.9%) and gentamicin (7.0 to ≈ 9.6%). In a study [20] of antimicrobial susceptibility profiles of 1234 strains, resistance to eight antimicrobial drugs was found in 54.5% of isolates.

Multidrug resistance (three or more groups) was observed in 16.4% of strains, with 190 different patterns [20]. Resistance to at least two groups of antibiotics was found in most isolates of *Salmonella enterica*.

Four multidrug-resistant isolates were isolated from animal feed and poultry products [21]. Figure 1 shows results of antimicrobial resistance testing of the *Salmonella* in the current study.

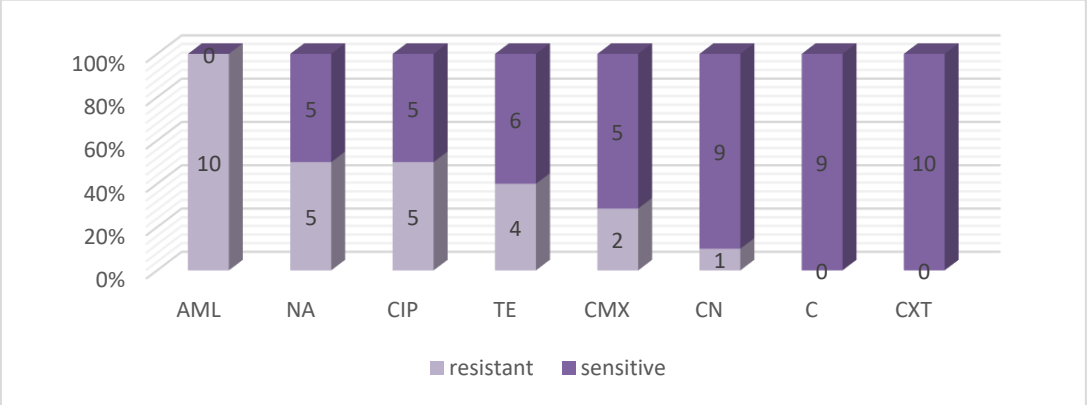

Figure 1. Results of antimicrobial resistance of *Salmonella*

Of the 10 isolates tested, 7 (70%) showed multiple resistance, i.e. resistance to 2 or more examined antimicrobial drugs. Three of the 10 *Salmonella* isolates were resistant to just one antimicrobial drug. Figure 2 shows the number of multidrug-resistant isolates to five, four three and two antimicrobial drugs

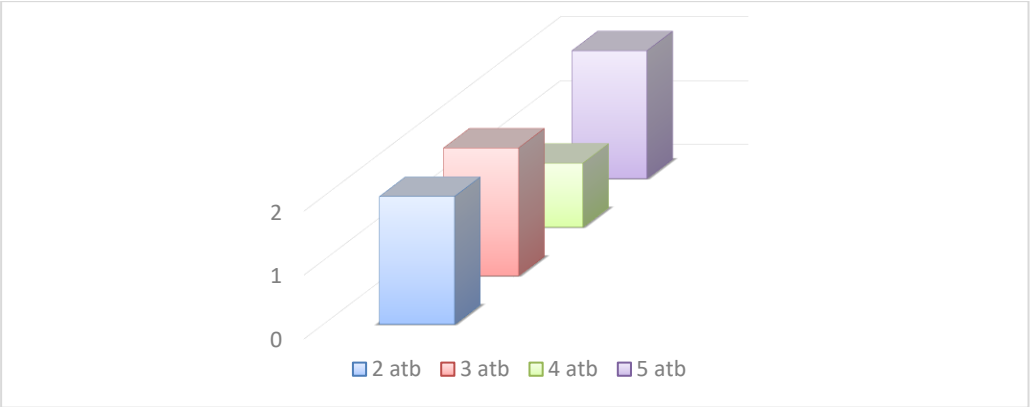

Figure 2. Number of multiresistant *Salmonella* isolates among 10 isolates examined

The results of this study are consistent with other studies on multiple resistance [3], [4], [12]. A particular problem is the occurrence of resistance to key antimicrobial drugs, because it was proven that such plasmids can transfer resistance and create resistance to multiple types of antimicrobial drugs [13]. Available data on antimicrobial drug resistance differ, and the reason could be the distribution and prevalence of certain serotypes in different countries and in different animals. Certain *Salmonella* strains within serotypes could have a specific and characteristic pattern of antimicrobial resistance to a particular antimicrobial drug, which means the drug is ineffective. These results indicate the importance of food chain surveillance and detection of modified patterns in foodborne zoonotic bacteria important to public health.

Inadequate and excessive use of antimicrobial drugs, whether by inappropriate doses, inadequate length of treatment or unnecessary use, results in the occurrence of bacterial resistance to antimicrobial drugs. The problem of resistance itself is mostly associated with the occurrence and spread of the mechanism of resistance in humans, because the appearance of resistance in zoonotic bacteria has begun to affect human therapy. Uncontrolled use of antimicrobial drugs and non-compliance with the

withdrawal time for elimination of drugs in animals used in human nutrition has led to the transmission of resistant bacteria through the food chain to humans and the occurrence of diseases that are difficult to treat. Most drugs used in animal therapy (ampicillin, tetracycline, gentamicin) belong to the some group of antimicrobial drugs used in human medicine, so resistance to one drug from the group could induce resistance to the whole group. The occurrence of resistant strains of *Salmonella* in animals is very significant since poultry, pigs and cattle are known to be the primary reservoirs of *Salmonella*. According to the European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA) and European Medicines Agency, resistance to nalidixic acid, ciprofloxacin, quinolones and cephalosporins have been identified as the most significant public health danger when it comes to *Salmonella* [4]. The results of this study are worrying because they showed that, in our area, there are multi-resistant strains of *Salmonella* that were imported through food or infected poultry.

4. Conclusion

The results of this study clearly show that all (100%) tested isolates of *Salmonella* were resistant to amoxicillin, as one of the most commonly used antimicrobial drugs, and that 70% of isolates were resistant to two or more antimicrobial drugs. Uncontrolled, improper and unprofessional use of antimicrobial drugs results in the occurrence of resistant microorganisms to one or more antimicrobial drugs. In addition to application of preventive and hygienic-sanitary measures at all levels from the field to the table, routine testing of isolated *Salmonella* for antimicrobial drug resistance should be introduced. It is only on the basis of continuous monitoring of bacterial resistance/sensitivity to antimicrobial drugs that adequate therapy for bacterial diseases can be maintained. A national strategy to control consumption and rational use of antimicrobials in animals is needed. The best prevention of salmonellosis in humans is constant and comprehensive microbiological control of food products during production, processing, storage and sale.

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Pork safety – challenges and opportunities

Pork safety – challenges and opportunities

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Abstract. As pork and pork products represent an important part of the diet, the issue of pork safety and quality has become more prominent. Food safety concerns are shaping consumers' attitudes toward safe food. The farm and meat sectors aim at producing healthy animals in a protected environment, which is a key point for food/meat safety. The most common biological hazards in the pork production chain are *Salmonella* spp., *Yersinia enterocolitica*, *Trichinella* spp. and *Toxoplasma gondii*. These hazards are not detectable by conventional meat inspection, and measures rely on prevention or reduction of contamination along the production chain.

1. Introduction

The continuous growth of the world population has led to an increasing trend in food production, which is evident in the meat sector as well. World meat production in 2021 is forecast to expand by 2.2% (pig meat: 4.2%), to 346 million tonnes (pig meat: 114.4 million tonnes), primarily due to meat production in China – especially pig meat, with expansions in other parts of the world as well (e.g. Brazil, United States and the European Union) [1]. Meat production in Serbia reflects the primary production – the most commonly slaughtered animals are pigs and the most common type of produced and consumed meat is pork [2]. During 2019, compared to the previous year, in general, there was an increase in livestock units by 0.8%, while meat production, according to the reports on livestock slaughter in slaughterhouses, decreased by 0.1% [3]. As pork and pork products represent an important part of the diet, especially in some regions in Serbia, in the last decades, the issue of pork safety and quality has become more prominent.

2. Pork, key hazards and safety

Food safety is the foundation of consumer confidence, and therefore, food safety concern is shaping consumers' attitude toward safe food [4]. As from 2009, legislation on the hygiene of foodstuffs, general and specific rules on food hygiene that are mostly in line with the EU "hygiene package" [5-8], become mandatory in Serbia, and food business operators now take full responsibility for the safety of the products they place on the market [9, 10]. There are changes in primary and meat sector systems with the One Health approach, aiming at producing healthy animals in a protected environment and with the emphasis on animal welfare issues [11]. Different farm and husbandry systems (large, medium, small,

backyard) are connected with specific hazards, biological and/or chemical, which are transmitted to the next step in the meat production chain [12], and the next step in the production chain could be the source of contamination as well.

In developed parts of the world, classical zoonoses, such as tuberculosis, trichinellosis and/or cysticercosis, have become much less frequent and less important due to the appropriate preventive measures and control systems focused on them [13]. Nowadays, emerging biological hazards such as *Salmonella* and/or *Yersinia*, carried by the animals without symptoms, are an additional burden on the farm and meat sector, requiring risk-based control measures and mitigation options. In the EU, the primary sector and healthy animals are the key points for food safety, and there are recommended risk-based meat inspections [11]. Mandatory palpation and incision of slaughtered pigs should be avoided to minimise cross-contamination of meat if pigs originate from well-established, integrated farm systems. Depending on the information provided in the food chain information, if there is a need, the official veterinarian decides on additional *post-mortem* inspection. Food chain information should enable a forwards and backwards flow of information on health hazards between different food sectors [11, 14]. In Serbia, reporting procedures for food chain information that contains accurate data from the primary sector could be improved if an easy-to follow guide for farmers was prepared and distributed.

The most common biological hazards connected to pork as a source and vector are *Salmonella* spp., *Yersinia enterocolitica*, *Trichinella* spp., and *Toxoplasma gondii*. These hazards are not detectable by conventional meat inspection, and control measures rely on prevention or reduction of faecal and other contamination, from farm, transport, slaughterhouse environment to carcass and meat during slaughtering operations [15, 16]. The measures are assured by implementing good manufacturing practise, good hygienic practice and hazard analysis and critical control points in all phases of meat production [15, 16]. Meat inspection should be focused to prevent and mitigate those biological hazards, with the primary production sector having a key role in managing these risks [17, 18].

Salmonella is a well-known pathogen and contaminant in the pig/pork production chain. After poultry and eggs, pigs/pork are an important source and vector in human salmonellosis. In the EU, the most commonly reported zoonoses in humans were campylobacteriosis and salmonellosis, respectively [19]. Salmonellosis is the most prevalent intestinal disease in Serbia (in total, 1260 cases in 2019) and was registered almost twice as often as campylobacteriosis [20]. The incidence of salmonellosis in Serbia was 18.14 per 100,000 inhabitants, and there has been a mild decrease in the incidence in the last decade. *Salmonella* can enter the food chain in any stage of production, from the farm, transport, lairage, slaughterhouse, distribution, market, consumer, etc. [18, 21, 22]. For effective control and mitigation, the entire food chain should be involved and have an integrated approach [18]. As reported in one study [23], *Salmonella* was isolated from 23.5% of swab samples (pig carcass) after stunning, and this suggested the possibility that many pigs had become contaminated during transport, in lairage, on the slaughter line etc. In this study, the most frequently isolated serotypes were *S.* Derby, *S.* Infantis and *S.* Typhimurium. Although the occurrence of *Salmonella* on pig carcasses after processing is different from country to country, a significantly lower number of carcasses were positive for *Salmonella* after processing (e.g. scalding, chilling etc.), indicating the importance of using good hygiene and manufacturing practices on the slaughter line [23, 24].

According to an EU report, human yersiniosis is the fourth most common foodborne zoonosis, usually transmitted by raw or undercooked food, mainly pork, and water [19]. Domestic pigs are the most important sources of *Y. enterocolitica*, with a higher prevalence in conventional intensive farming in fattening pigs and which could have a seasonal occurrence [13, 25, 26]. Pathogen prevalence is better controlled in farming systems with high biosecurity levels [13]. During 2019, only 14 cases of human yersiniosis were registered in Serbia, with an incidence rate of 0.2 per 100,000 inhabitants, which was a decrease in the incidence rate compared to the previous year, when it was 0.28; the most prevalent pathogenic serotype is BT 4/O:3 [20, 27]. This serotype is the one most often isolated from pig tonsils on the slaughterhouse [27].

Trichinella infection has been never documented in pigs raised under high containment levels, and the risk of infection is mainly related to the lack of compliance with rules on the treatment of animal

waste [28]. Pigs raised outdoors are at risk of contact with potentially *Trichinella*-infected wildlife [29]. The Balkan region and Serbia have long records of human trichinellosis, and pork and pork products are the main sources of infection. According to official data, human trichinellosis in Serbia has decreased in the last decade [20]. The decrease of human trichinellosis in Serbia evidences the veterinary and public health sectors' efforts in the improvement of animal and human health through the legal framework for detection, surveillance, prevention, control and reporting of zoonoses, and the professional capacity of the competent authorities to implement and enforce standards and regulations for the control of *Trichinella* infections in animals and humans [30]. According to EU legislation, pigs raised under controlled housing conditions do not need to be tested for *Trichinella* any more [15]. In Serbia, there is a variety of farming systems, from controlled housing conditions to backyard farms (including pigs raised outdoors). Well-established and controlled farm systems could achieve *Trichinella*-free status.

T. gondii infection in pigs is an issue mainly in small scale farming and backyard farms [31]. Still, there is a risk to public health even if there is only a very low prevalence of parasites on large farms – a single, slaughter-weight pig produces a lot of portions of meat [32]. In 2019, 73 cases of human toxoplasmosis were reported in Serbia, with an incidence rate of 1.05 per 100,000 inhabitants, and in the last decade, there was a slight upward trend in the incidence of human toxoplasmosis [20]. According to available data, seroprevalence in pigs varies greatly, and depends on country and region [31]. Conventional meat inspection cannot detect *T. gondii*-infected pigs, and additional serological testing can only detect animals that could have been exposed to the parasite. Direct methods for *T. gondii* detection, like molecular tests and bioassays, are expensive and infeasible for daily use.

3. Conclusion

There are various obstacles to improving food safety. Developed countries, with high income and well-informed consumers, have constant pressures from the general public and media in the context of food safety and quality issues as well. In mid-income countries, like Serbia, there are many challenges, but consumer awareness and willingness to pay for safer food is increasing, while consumers' trust in the food they buy is a premise for the food business operators who do invest in food safety to maintain consumer confidence. The food sector is a sector with risks, so food/meat safety must be risk-based to eliminate or reduce food safety risks, with longitudinal and integrated approaches along the food chain, and continuous improvement. The goal is that "food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use" [33].

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The influence of savory on colour, odour and taste of frankfurters

The influence of savory on colour, odour and taste of frankfurters

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Abstract. The aim of this paper was to assess the influence of savory on colour, odour and taste of vacuum-packed frankfurters during 28 days of storage. Powdered, dried savory (0.1%, 0.3% and 0.5%) was added to frankfurters, while control frankfurters were produced without herbs or spices. Assessment of colour, odour and taste acceptability of the frankfurters was performed by a panel of five assessors using a quantitative descriptive test, and the results of the ranking test were analysed statistically. When used at the levels of 0.1% and 0.3% in the sausages, savory did not have a negative influence on frankfurter colour, while quantities of 0.5% adversely affected frankfurter colour. Savory in the smaller amounts of 0.1% and 0.3% stimulated development of a pleasant odour and taste in the frankfurters, while 0.5% savory had an undesirable effect on these sensory attributes.

1. Introduction

Savory (*Satureja hortensis L.*) is a herb and belongs to the family Lamiaceae. Savory has a pleasant, aromatic odour. The taste is aromatic, warm. The smell and taste are similar to pepper and it is used as a substitute [1]. *Satureja hortensis L.* contains from 0.3% to 1.9% essential oil, of which the main ingredients are carvacrol, α- and β-pinen, camfen, γ-terpinen, etc. Savory is also known for its medicinal properties (stomachic, astringent, antiseptic) [2]. According to the literature data and our knowledge of current industry practice, savory is very little used in the Serbian meat industry, especially in pasteurised sausages. One of the reasons this herb is uncommon as seasoning in meat products is its content of the plant pigment chlorophyll, which can have undesirable influences on colour, odour and taste of the meat products [3,4].

The aim of this study was to assess the influence of savory on colour, odour and taste of vacuum-packed frankfurters during 28 days of storage.

2. Materials and methods

2.1. Raw material composition

Frankfurters were produced from beef meat (50%), pork fat (25%) and ice (25%). To 1 kg of stuffing, 18 g of nitrite salts and 3 g of polyphosphate were added. Powdered, dried savory was added to the

experimental frankfurters (0.1%, 0.3% and 0.5%), while the control frankfurters were produced without any herbs or spices. The frankfurter stuffing was filled in artificial cellulose casings. Frankfurters were thermally processed (72ºC in the centre of the product) and then cooled. After cooling, frankfurters were vacuum packed. All packages of frankfurters were stored in the same conditions at 4 ºC and on days 1, 7, 14, 21 and 28 of storage, sensory testing was performed.

2.2. Chemical analysis

The content of essential oils in the dried herb was analysed according to [5].

2.3. Sensory analysis

Sensory evaluations were performed by five trained panellists. Frankfurter colour was analysed using a quantitative descriptive test [6], with grading scale from one to five (1 – unacceptable colour; 2 – very low level of acceptability of colour; 3 – acceptable colour; 4 – good colour; 5 – exceptionally good colour).

Using a quantitative descriptive test [6], with grading scale from one to seven, the frankfurters' sensory properties of odour and taste were analysed (1 – extremely unpleasant odour and taste; 2 – very unpleasant odour and taste; 3 – unpleasant odour and taste; 4 – neutral odour and taste; 5 – pleasant odour and taste; 6 – very pleasant odour and taste; 7 – exceptionally pleasant odour and taste).

2.4. Statistical analysis

Results of the sensory evaluation ranking tests [7] were analysed statistically [8].

3. Results and discussion

The dried savory used in the frankfurters contained 1.6ml/100g essential oil. The minimum quantity of ethereal oils for savory is not prescribed under Serbian regulations [9].

Table 1. Sensory evaluation of the colour of vacuum-packed frankfurters during storage

Day	Percentage of savory in frankfurter	Sum of ranks	Differences in frankfurter colour according to percentage of savory		
			0	**0.1**	**0.3**
1	0	6.5			
	0.1	14.5	8		
	0.3	17.5	11	3	
	0.5	23.5	17**	9	6
7	0	9			
	0.1	10	1		
	0.3	17.5	8.5	7.5	
	0.5	23.5	14.5**	13.5*	6
14	0	6.5			
	0.1	12	5.5		
	0.3	17.5	11	4.5	
	0.5	24	17.5**	12*	6.5
21	0	6.5			
	0.1	14	7.5		
	0.3	16.5	10	2.5	
	0.5	23	16.5**	9	6.5
28	0	7.5			
	0.1	18	10.5		
	0.3	11	3.5	7	
	0.5	23.5	16**	5.5	12.5*

* $p \leq 0.05$ – statistically significant difference; **$p \leq 0.01$) – highly statistically significant difference

During storage, highly statistically significant differences ($p < 0.01$) were observed between the colour of control frankfurters and frankfurters with 0.5% savory.

There was a statistically significant difference ($p < 0.05$) between the colour of frankfurters with 0.1% savory and frankfurters with 0.5% savory on days 7 and 14 of storage. There was a statistically significant difference ($p < 0.05$) between the colour of frankfurters with 0.3% savory and frankfurters with 0.5% savory on day 28 of storage.

No statistically significant differences were detected between the other frankfurters studied.

Table 2. Sensory evaluation of the odour and taste of vacuum-packed frankfurters during storage

Day	Percentage of savory in frankfurters	Sum of ranks	Differences in frankfurter odour and taste according to percentage of savory		
			0	**0.1**	**0.3**
1	0	9			
	0.1	9	0		
	0.3	16	7	7	
	0.5	24	15**	15**	8
7	0	9			
	0.1	12.5	3.5		
	0.3	14.5	5.5	5.5	
	0.5	24	15**	11.5	9.5
14	0	13			
	0.1	12.5	0.5		
	0.3	16	3	3.5	
	0.5	18.5	4.5	6	2.5
21	0	8			
	0.1	17	9		
	0.3	12.5	4.5	5.5	
	0.5	22.5	14.5**	5.5	10
28	0	6.5			
	0.1	23.5	17**		
	0.3	13	6.5	10.5	
	0.5	17	10.5	6.5	4

* $p \leq 0.05$ – statistically significant difference; **$p \leq 0.01$) – highly statistically significant difference

There was a highly statistically significant difference ($p < 0.01$) between the control frankfurters and frankfurters with 0.5% savory on days 1, 7 and 21 of storage. The odour and taste of frankfurters with 0.1% savory and frankfurters with 0.5% savory were statistically significantly different ($p < 0.05$) on day 1 of storage.

The odour and taste of control frankfurters and frankfurters with 0.1% savory were highly statistically significantly different ($p < 0.01$) on day 28 of storage.

No statistically significant differences were detected between other frankfurters on this day.

The results obtained are in accordance with [10], where savory (at different concentrations) was added to dry fermented sausage, as well as with the results of the authors [11]. Savory at levels of 0.1% and 0.3% did not negatively influence the colour of dry fermented sausage, while 0.5% savory had a negative influence. According to [9], savory at the levels of 0.1% and 0.3% stimulated development of a pleasant odour and taste in dry fermented sausage, while 0.5% savory had an undesirable effect.

The results are in accordance with [10], where basil (at different concentrations) was added to frankfurters (basil also belongs to the family Lamiaceae*)*. Basil at 0.1% did not negatively influence the colour of sausages, while larger amounts, 0.3% and 0.5%, did adversely affect the sausages' colour. Basil at levels of 0.1% and 0.3% stimulated development of pleasant odour and taste of sausages, while 0.5% basil had an undesirable effect.

4. Conclusion

Savory at levels of 0.1% and 0.3% did not negatively influence the colour of frankfurters, while 0.5% savory did adversely affect frankfurter colour. Savory at levels of 0.1% and 0.3% stimulated the development of a pleasant odour and taste in the frankfurters, while 0.5% savory had an undesirable effect. In conclusion, frankfurter with 0.1% and 0.3% savory had desirable sensory attributes. The results show that there is a real possibility of using savory in spice mixtures for the production of frankfurters.

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Elimination of phosphate in restructured turkey steaks by the addition of eggshell calcium powder and low methoxyl pectin

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Elimination of phosphate in restructured turkey steaks by the addition of eggshell calcium powder and low methoxyl pectin

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Abstract: This study was carried out to assess the effects of eggshell calcium powder (ESCP) and/or low methoxyl pectin (LMP) as phosphate replacers on the quality parameters of restructured turkey steaks. ESCP, 0.25% or 0.50%, was added to formulation alone or in combination with 0.25% LMP in powder and gel forms. The pH increased with the addition of ESCP. Soluble protein content, water holding capacity, and cooking yield were higher in steaks formulated with ESCP+LMP gel compared to control steaks containing phosphate. Hardness of steaks was decreased by the addition of ESCP and pectin. Pectin in powder form negatively affected the preference of panelists. Oxidation in phosphate-free steaks was more pronounced than in other treatments. The results showed that the binding properties of phosphate could be achieved by using ESCP or ESCP+LMP gel.

1. Introduction

Phosphate salts of sodium or potassium in meat products are used to increase space between meat proteins due to an increase in pH, which results in an increase of the binding ability of proteins to hold more fat and moisture. This, in turn, results in swelling of the muscle fibers and activation of proteins. Swollen and active proteins trap and immobilize water added to the meat system [1]. Hence, the processing yield, tenderness, juiciness, and water-binding capacity of meat products increase [2]. Also, phosphates can bind the Mg^{2+} and Ca^{2+}, then lead to the breakdown of the calcium bridges of actomyosin complex and improve the functionality and solubility of meat proteins. Water holding, gelling, and emulsifying capacities are related quality properties of meat products such as texture, cooking losses, and flavor. These are the properties that could be improved with the changes in meat proteins stimulated by phosphates and the antioxidative effect of phosphates [2,3].

Although phosphate salts have multiple functions in meat products, studies demonstrated a good correlation between the high intake of phosphates and health problems such as reduced calcium absorption and cardiovascular and kidney diseases [4]. All these facts drew the attention of health authorities to re-evaluate the safety of phosphates as food ingredients [5]. However, salts also exist in our daily diet, and a relatively major part of the population has kidney diseases, which means these people cannot tolerate the proposed quantity of phosphates [6]. Taken all together, searching for natural ingredients as phosphate replacer has shined out as a hot research topic to meet the demands of consumers who prefer clean label meat products. Unfortunately, it is a challenge to find a complete phosphate replacer, since the related water and fat binding properties of meat products deteriorate when phosphate is removed from the formulation. Natural phosphate alternatives should be able to compensate

for all the functions of phosphates. For this purpose, the application of blends of citrus flour and sodium carbonate [7], inulin and carbonate [8], and natural calcium powders [9,10,11] as phosphate replacers have been investigated. The use of natural calcium powders was found to successfully replace phosphate salts in meat products in terms of cooking yield and water holding capacity (WHC) [9,10]. Cho et al. [12] stated that the addition of 0.2% oyster shell calcium and 0.3% eggshell calcium to pork products resulted in less desirable textural properties. Accordingly, the combination of natural calcium powders with binding ingredients could be a more effective solution for improving quality of meat products [13]. Eggshell calcium powder (ESCP) is a byproduct obtained from calcination of eggshells through the processing of egg products [14]. Studies regarding the use of ESCP in meat products indicated that calcium powder showed ionic strength raising effects similar to phosphates [10,12].

Another functional ingredient, pectin, is used in formulations due to its gelling and stabilizing capacities. Neighboring pectin chains create a network by the connection of junction zones, and thus, high amounts of water could be entrapped in the network [15]. To the best of our knowledge, no study has been conducted to examine the combined effect of ESCP with pectin in powder form or with pectin gels in phosphate free restructured meat products. In this research, planned in the light of all aforementioned data, the effects of using two different levels of ESCP combined with low methoxyl pectin (LMP) in powder and gelled forms as phosphate replacers in restructured turkey steaks was investigated.

2. Materials and methods

Sodium tripolyphosphate (STPP), ESCP, and LMP were supplied from Pacovis Food Co. (Izmir, Turkey), Essentron Co. (Korea) and Sigma-Aldrich Co. (Istanbul, Turkey) respectively. For preparation of LMP gel, 1 gram of LMP was mixed with 10 ml of boiled distilled water and stirred until all solid matter was dissolved [16]. Control treatment (C) was prepared with 0.5% STPP. Remaining six treatments contained added 0.25% ESCP (E25), 0.50% ESCP (E25), 0.25% ESCP+ 0.25% LMP powder (EP25), 0.50% ESCP+ 0.25% LMP powder (EP50), 0.25% ESCP+0.25% LMP gel (EGP25), 0.50% ESCP+0.25% LMP gel (EGP50) as phosphate replacers. The amount of ingredients and additives were based on total amount of turkey breast meat plus water (Table 1). Breast cuts (2*3*3 cm) mixed with NaCl, STTP, ESCP, or ESCP+LMP by using a metal-tipped mixer operated with a rotation speed of 50 rpm for 5 min mixing - 5 min rest - 5 min mixing. Then, the meat mixtures were shaped in stainless steel ham molds (25x8x10 cm) and cooked at 85°C until core temperature reached 75°C. Lastly, steaks were cooled down and sliced. Sliced steaks were stored at 4°C for 7 days.

Table 1. Formulation of restructured turkey steak

	C	E25	E50	EP25	EP50	EGP25	EGP50
Turkey meat	85	85	85	85	85	85	85
Water	15	15	15	15	15	15	15
Total	100	100	100	100	100	100	100
Additives/Ingredients							
NaCl	2	2	2	2	2	2	2
STPP	0.5	-	-	-	-	-	-
ESCP	-	0.25	0.5	0.25	0.5	0.25	0.5
LMP powder/gel	-	-	-	0.25	0.25	0.25	0.25

Moisture and ash contents of samples were determined according to AOAC [17] procedures. Fat content was analyzed according to Flynn and Bramblet [18]. Protein content of samples was determined using DUMAS method with LECO nitrogen analyzer (FP-528, USA). pH values were measured in triplicate using a pH-meter (WTW pH 3110 set 2, Germany) Color parameters of steaks were measured using a digital colorimeter (Chromameter CR 400, Minolta, Japan) to obtain the color coordinates lightness (L*), redness (a*) and yellowness (b*). Cooking yield was determined by calculating weight differences for samples before and after cooking. Fat and moisture retentions were calculated according to Murphy et al. [19] and El-Magoli et al. [20]. WHC of uncooked turkey steak was determined according to weight differences after heat treatment and centrifugation [21]. Soluble protein content (SPC) analysis was based on the reaction between dye reactive (Coomassie Brilliant Blue G-250) and

extracted proteins. SPC was expressed as mg protein/g [22]. Texture profile analysis (TPA) was performed five times for each treatment using a texture analyzer (TA-XT2, Stable Micro Systems, UK). Turkey steaks were subjected to sensory evaluation for appearance, color, juiciness, texture, flavor, and overall acceptability. Lipid oxidation was followed with Thiobarbituric Acid Reactive Substances (TBARS) analysis [23]. Purge loss was analyzed from the percentage of total weight difference between storage days. The effects of ESCP, LMP and storage on turkey steaks were determined by analysis of variance (ANOVA) and Duncan's Post-Hoc tests in SPSS software.

3. Results and Discussion

The chemical composition of the turkey steaks is set out in Table 1. E25 had the highest moisture content ($P<0.05$) while C, E50, EP25, EGP25, and EGP50 had similar (lower) moisture contents. Higher moisture content was also obtained by Cho et al. [12] in restructured pork where phosphate was replaced with natural calcium powders. Steaks formulated with ESCP and LMP gel had similar protein contents as the C group. Fat, protein, and ash contents of steaks were increased by the cooking. Moisture contents of cooked steaks were similar, except E25. Similar results were previously obtained by Bae et al. [10], who showed the increasing effect of calcium powders on moisture content individually or combined using with binding ingredients. Lower fat contents were obtained in E50 and EP25. All steaks had protein content higher than 20%. Increasing ESCP level increased the ash contents of cooked steaks.

Table 2. Chemical composition of uncooked and cooked turkey steaks

	Uncooked				Cooked			
Sample	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Moisture (%)	Fat (%)	Protein (%)	Ash (%)
C	75.43 ± 0.18^{c}	1.14 ± 0.20^{ab}	20.31 ± 0.18^{a}	3.47 ± 0.00^{b}	73.39 ± 0.41^{a}	1.59 ± 0.26^{ab}	21.34 ± 0.30^{bc}	3.71 ± 0.09^{bc}
E25	76.33 ± 0.26^{a}	1.17 ± 0.12^{ab}	19.78 ± 0.27^{bc}	3.07 ± 0.01^{c}	72.36 ± 0.58^{b}	1.55 ± 0.18^{ab}	22.54 ± 0.21^{a}	3.28 ± 0.05^{d}
E50	75.39 ± 0.12^{c}	1.02 ± 0.07^{b}	20.34 ± 0.18^{a}	3.72 ± 0.18^{a}	73.14 ± 0.16^{a}	1.26 ± 0.06^{b}	20.71 ± 0.22^{d}	4.22 ± 0.11^{a}
EP25	75.40 ± 0.26^{c}	1.08 ± 0.16^{ab}	19.48 ± 0.13^{c}	3.04 ± 0.14^{c}	73.65 ± 0.37^{a}	1.31 ± 0.29^{b}	20.87 ± 0.08^{cd}	3.83 ± 0.16^{b}
EP50	75.84 ± 0.10^{b}	1.29 ± 0.00^{a}	19.74 ± 0.25^{bc}	3.17 ± 0.11^{c}	73.21 ± 0.23^{a}	1.83 ± 0.05^{a}	20.19 ± 0.22^{e}	4.40 ± 0.34^{a}
EGP25	75.47 ± 0.17^{c}	1.18 ± 0.15^{ab}	20.00 ± 0.26^{ab}	3.03 ± 0.01^{c}	73.68 ± 0.71^{a}	1.53 ± 0.02^{ab}	21.37 ± 0.49^{b}	3.26 ± 0.04^{d}
EGP50	75.28 ± 0.18^{c}	1.16 ± 0.05^{ab}	20.16 ± 0.05^{a}	3.11 ± 0.03^{c}	73.71 ± 0.25^{a}	1.56 ± 0.19^{ab}	21.16 ± 0.06^{bcd}	3.44 ± 0.03^{cd}

$^{a-d}$ Means within a row with different letters are significantly different ($P<0.05$).

Cooking yield, WHC, pH, SPC, moisture, and fat retention values of turkey steaks are given in Table 2. The pH of the uncooked steaks decreased with the addition of ESCP and LMP ($P<0.05$), but there was no difference between EP25 and C. However, the pH of steaks was increased by the effect of ESCP and LMP, except for EGP50. Like our results, when ESCP was added to ground pork, pH significantly increased [12]. While the treatment containing 0.50% ESCP and LMP gel had the highest protein solubility, E25 and E50 treatments showed similar protein solubility as was shown by the C group. SPC of restructured chicken meat increased by the addition of 4.5% gelled inulin and 0.2% carbonate as phosphate alternatives [8]. Samples formulated with ESCP+LMP gel had the highest WHC, and the addition of 0.50% ESCP showed a rising effect on WHC ($P<0.05$) due to the pH-increasing effect of ESCP. A high WHC was achieved with the addition of 3% LMP in squid meat [24]. In our study, E50, EP25, and EGP50 had higher cooking yields than other treatments ($P<0.05$). It was observed that the cooking yield was increased by using ESCP alone and/or combined with pectin. Treatments containing pectin gel had the highest moisture retention ($P<0.05$). Gelation is the most unique property of pectin, with gel formed in the presence of Ca^{2+} ions or sugar and acid. Also, pectin traps the liquid by forming a three-dimensional network due to merging or cross-linking of long polymer chains [25]. The highest fat retention values were observed in the E50 and EP25 groups. Bae et al. [10] reported that using ESCP increased pH values and cooking yield in ground pork.

Table 3. pH, soluble protein content, water holding capacity and cooking properties of turkey steaks

Sample	pH (uncooked)	pH (cooked)	SPC (µgprotein/ml)	WHC (%)	Cooking yield (%)	Moisture retention (%)	Fat retention (%)
C	6.00±0.02^{a}	6.36±0.03^{c}	993.55±7.79^{ab}	60.35±0.66^{bc}	82.44±2.34^{c}	61.71±0.34^{c}	86.53±4.48^{cd}
E25	5.91±0.01^{c}	6.43±0.01^{b}	1000.25±10.39^{ab}	59.16±0.34^{c}	84.86±2.23^{bc}	61.97±0.84^{c}	87.45±3.19^{bc}
E50	5.93±0.01^{b}	6.48±0.02^{a}	991.45±10.82^{ab}	61.46±0.40^{b}	88.25±1.15^{a}	63.95±0.14^{a}	92.63±1.73^{a}
EP25	5.99±0.01^{a}	6.51±0.02^{a}	979.58±11.17^{abc}	59.29±0.88^{c}	87.15±1.17^{ab}	61.58±1.02^{c}	95.54±1.82^{a}
EP50	5.90±0.01^{c}	6.42±0.03^{b}	970.78±37.58^{bc}	61.10±0.14^{b}	84.88±0.25^{bc}	61.29±0.20^{c}	85.10±2.09^{cd}
EGP25	5.87±0.01^{d}	6.44±0.03^{b}	951.65±28.89^{c}	64.10±1.73^{a}	84.14±0.05^{bc}	62.30±0.10^{bc}	91.66±0.06^{ab}
EGP50	5.82±0.01^{e}	6.39±0.01^{c}	1009.33±1.64^{a}	65.03±1.44^{a}	86.95±1.87^{ab}	63.59±1.24^{ab}	82.11±2.13^{d}

$^{a-e}$ Means within a row with different letters are significantly different (P<0.05).

Color and textural parameters are shown in Table 4. The addition of ESCP at a level of 0.50% lowered lightness (P<0.05). Increasing the amount of ESCP did not affect a* value; on the other hand, the addition of natural alternatives for phosphate increased a* values (P<0.05). The highest value was found in EP25. These results were likely due to the elevated pH caused by ESCP treatment. At high pH values, a lesser amount of myoglobin is likely to be denatured during cooking, which could contribute to an increase of red color [26]. Using ESCP and/or LMP in powder increased b* values, and the highest b* value was registered EP25. b* values decreased when ESCP level increased in steaks containing LMP (P<0.05). A similar result was also stated by Bae et al. [10] in ground pork meat products. However, this is not consistent with results stated by Lee et al. [27] for sausages formulated with oyster shell powder. Regarding the texture parameters, addition of both pectin forms and ESCP to the formulation decreased the hardness and increased the springiness, except in EGP25 treatment (P<0.05). The addition of mushroom powder as a phosphate alternative in sausage formulation resulted in a soft structure [12]. EGP25 had similar springiness and cohesiveness as C group. The cohesiveness values of turkey steak formulated with ESCP increased compared to C steaks due to binding properties being improved by ESCP. Gumminess of steaks was decreased when LMP was added in powder form (P<0.05). Restructured pork products formulated with potato or rice starch to replace nitrite had decreased hardness, chewiness, and gumminess values [28]. Using ESCP alone improved the chewiness values. As a result, it could be said that even ESCP alone could compensate for textural properties provided by phosphate.

Oxidation was more pronounced by the elimination of phosphate from the formulation (P<0.05). Initially the highest and the lowest TBARS values were recorded in EP25 and C, respectively (Table 5). Steaks with 0.50% ESCP individually and combined with LMP gel had TBARS values close to those of C at the beginning and end of the storage (P<0.05). All cooked steaks showed increased TBARS values when the cold storage increased (P<0.05). Cooking directly influences the extent of oxidation that develops in poultry muscle during storage. This fact shows that the incorporation of additives, except 0.50% ESCP, did not produce any antioxidant behavior during refrigerated storage. TBARS values of cooked chicken ground meat formulated without phosphate reached above 7 µmol/kg [29].

Table 4. Color and textural parameters of turkey steaks

Sample	L*	a*	b*	Hardness (N)	Springiness (mm)	Cohesiveness	Gumminess (N)	Chewiness (Nxmm)
C	70.88±1.05^{ab}	1.69±0.29^{c}	8.99±0.28^{c}	27.14±1.09^{a}	0.20±0.02^{c}	0.19±0.01^{c}	5.44±0.40^{bc}	1.31±0.08^{cd}
E25	71.79±1.49^{a}	3.32±0.41^{ab}	9.58±0.23^{b}	27.28±0.78^{a}	0.26±0.04^{b}	0.24±0.02^{b}	6.19±0.19^{ab}	1.60±0.33^{bc}
E50	69.82±0.58^{b}	3.01±0.26^{b}	9.36±0.21^{bc}	24.48±1.30^{b}	0.31±0.03^{a}	0.28±0.02^{a}	6.96±0.48^{a}	2.19±0.04^{a}
EP25	70.79±0.76^{ab}	3.58±0.42^{a}	10.24±0.3^{a}	17.73±1.17^{d}	0.31±0.02^{a}	0.24±0.01^{b}	4.00±0.65^{d}	1.59±0.34^{bc}
EP50	71.45±1.13^{ab}	3.36±0.36^{ab}	9.47±0.34^{b}	20.81±2.10^{c}	0.28±0.03^{ab}	0.24±0.02^{b}	4.46±0.59^{d}	1.40±0.12^{bcd}
EGP25	71.13±1.03^{ab}	3.13±0.34^{ab}	9.55±0.26^{b}	24.27±0.81^{b}	0.21±0.03^{c}	0.19±0.02^{c}	4.74±0.16^{cd}	1.04±0.08^{d}
EGP50	71.08±0.68^{ab}	2.83±0.36^{b}	8.97±0.14^{c}	20.72±1.08^{c}	0.29± 0.03^{ab}	0.26±0.03^{ab}	5.92±0.94^{b}	1.73±0.38^{b}

$^{a-e}$ Means within a row with different letters are significantly different (P<0.05).

Table 5. TBARS values of turkey steaks

	TBA-RS (mg malonaldehyde/kg)			
Sample	Day 0	Day 3	Day 5	Day 7
C	0.24±0.01^{e,t}	0.61±0.04 ^{f,y}	0.30±0.01^{f,z}	1.57±0.04^{z,x}
E25	1.36±0.01^{c,t}	3.69±0.06^{a,z}	4.98±0.18^{b,y}	5.91±0.04^{b,x}
E50	0.93±0.02^{e,t}	1.87±0.01^{e,z}	2.39±0.02^{e,x}	2.10±0.09^{f,y}
EP25	1.98±0.04^{a,t}	3.51±0.04^{b,z}	6.64±0.05^{a,y}	7.84±0.04^{a,x}
EP50	1.16±0.01^{d,t}	2.76±0.02^{d,z}	4.11±0.04^{d,y}	4.37±0.08^{c,x}
EGP25	1.68±0.01^{b,t}	3.35±0.05^{c,z}	4.46±0.14^{c,x}	4.04±0.05^{d,y}
EGP50	0.95±0.03^{e,t}	1.89±0.02^{e,z}	2.49±0.23^{e,y}	3.85±0.01^{e,x}

$^{a-e}$ Means within a row with different letters are significantly different (P<0.05).
$^{x-t}$ Means within a row with different letters are significantly different (P<0.05).

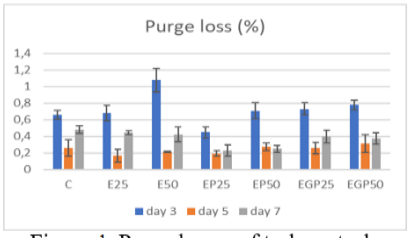

Figure 1. Purge losses of turkey steaks

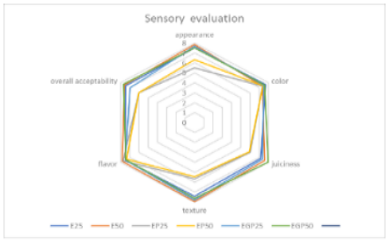

Figure 2. Sensory evaluation of turkey steaks

The highest purge loss was found in E50 (Figure 1), while EP25 had the lowest on day 3 (P<0.05). Purge losses of steaks decreased then increased throughout the whole storage (P<0.05). On day 7, the lowest losses were observed in EP25 and EP50 that were formulated with ESCP+LMP powder (P<0.05). None of the reformulated steaks had higher purge losses than the C group. No statistical differences were found between treatments in terms of color or flavor (Figure 2). The addition of LMP in powder form resulted in a decrement in appearance and juiciness; however, LMP gel increased juiciness scores (P<0.05). EP25 and EP50 steaks had lower texture and appearance scores than other treatments (P<0.05). Tabak et al. [11] showed combined use of eggshell powder with pectin or carrageenan enhanced the technological and sensory qualities of phosphate free chicken patties. Utilization of ESCP alone or in combination with LMP gel did not negatively affect product quality. Consequently, panelists gave lower acceptability scores for EP25 and EP50 steaks (P<0.05).

4. Conclusion

The results of this study showed that using EGSP and pectin gel in combination is a promising natural additive that can be used in phosphate free meat product formulations. Protein solubility and juiciness increased with the use of ESCP in combination with pectin gel. Pectin in powder form resulted in lower sensory scores. However, none of the combinations were able to retard oxidation, and therefore, further studies should focus on the use of natural antioxidants in phosphate free restructured meat products.

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The presence of acrylamide in various type of food products from the Serbian market

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The presence of acrylamide in various type of food products from the Serbian market

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Abstract: Acrylamide forms when some foods are prepared at temperatures usually above 120°C and in low moisture conditions, due to a Maillard reaction between certain amino acids, such as asparagine, and reducing sugars. Acrylamide is carcinogenic to experimental mice and rats, neurotoxic and probably also carcinogenic and genotoxic for humans. The aim of this study was to determine the presence of acrylamide in various groups of food products in which its formation is expected to occur during the production process. In the period December 2017 to March 2021, 529 samples of different types of food products were tested. Samples were collected from the Serbian market. Most of the tested foods, almost half of them (44%), were various types of biscuits. The presence of acrylamide was determined using LC-MS/MS accredited method, with a limit of quantification (LOQ) of 50 µg kg-1and a limit of detection (LOD) of 25 µg kg-1. All samples from the snack product and waffle product groups contained acrylamide. Acrylamide was detected in almost all (98.98%) fine bakery products and biscuits (90.43%). In contrast, only 15.38% of bakery products contained acrylamide. Most of the tested foods contained acrylamide, 83.74% of them.

1. Introduction

Food consumed by human populations is an excellent foundation for various dangers and hazards, whether of biological, chemical or physical origin, with potentially harmful effects on human health. Food can be contaminated at primary and secondary levels. Primary contamination is during all production stages, preparation, processing, treatment and distribution, and secondary contamination occurs due to inadequate and improper storage. Intake of food that contains various hazards can result in diseases in humans [1].

Acrylamide (AA) is a low molecular weight, highly water soluble, organic compound. It forms when some foods are prepared at temperatures usually above 120°C and in low moisture conditions, due to a Maillard reaction between certain amino acids, such as asparagine, and reducing sugars. AA forms in numerous baked or fried carbohydrate-rich foods, including French fries, potato crisps, breads, biscuits and coffee [2, 3, 4].

AA is carcinogenic to experimental mice and rats, causing tumours at multiple organ sites in both species when given in drinking water or by other means [5]. Adverse effects reported in repeated dose

toxicity studies of AA in rats, mice, monkeys, cats and dogs consisted of body weight loss and effects on the nervous system reflected by hind-limb paralysis, reduction in motor performance and/or histopathological changes in peripheral nerves and nervous system structures. In mice, effects reported, in addition to the neurotoxicity, consisted of effects on the testes, including the degeneration of epithelia in spermatids and spermatocytes, the reduction of spermatozoa, and the presence of multinucleate giant cells, as well as forestomach hyperplasia, hematopoietic cell proliferation of the spleen, preputial gland inflammation, lung alveolar epithelium hyperplasia and cataract and for female mice, ovarian cysts [2]. AA is carcinogenic in multiple tissues in both male and female mice and rats. In rats, the major tumours produced by AA are adenomas, fibroadenomas and fibromas of the mammary gland, thyroid gland follicular cell adenomas or carcinomas, and in F344 rats, testes or epididymis tunica vaginalis mesotheliomas. In mice, the major tumours produced by AA are: Harderian gland adenomas, mammary gland adenoacanthomas and adenocarcinomas, lung alveolar and bronchiolar adenomas, benign ovary granulosa cell tumours, skin sarcomas, and stomach and forestomach squamous cell papillomas in females, and Harderian gland adenomas and adenocarcinomas, lung alveolar and bronchiolar adenomas and carcinomas, and stomach squamous papillomas and carcinomas in males [2].

Cancer is leading cause of mortality worldwide. It is assumed that cancer risk is mainly affected by environmental factors including diet habits [6]. AA is neurotoxic and probably also carcinogenic and genotoxic for humans [7, 8, 9, 10, 11]. Since its discovery in food, AA levels were monitored in various countries and the results indicated a public health concern [12]. The food safety control system in Serbia is based on the examination of foodstuffs during import, self-control by domestic producers and monitoring of foodstuffs in circulation by inspection bodies. The levels of reference values for the presence of AA in food (French fries, potato chips, crackers and other products based on potatoes and potato dough, soft bread, breakfast cereals, biscuits and waffles, gingerbread, roasted and instant coffee, coffee substitutes) are defined in Annex 2 of the Rulebook of maximum concentrations of certain contaminants in food [13].

The aim of this study was to determine the presence of AA in various groups of food products in which AA formation is expected to occur during the production process.

2. Materials and Methods

In the period December 2017 to March 2021, 529 samples of different types of food products (biscuits – 230, fine bakery products – 98, snack products – 81, bakery products – 65, waffle products – 29, confectionery – 13, coffee products − 13) were examined. Samples were collected from the Serbian market. The presence of AA was determined using the LC-MS/MS accredited method, after sample extraction and cleanup using the QuEChERS technique [14, 15], with a limit of quantification (LOQ) of 50 μg $kg^{-1}$and a limit of detection (LOD) of 25 μg kg^{-1}. The LC-MS/MS system and software (LabSolutions) used to determine the presence of AA were produced by Shimadzu. All chemicals used were of analytical grade and were used as received without any further purification. The results analysis and graphical presentation of their distribution was performed using Microsoft Office Excel 2016.

3. Results and Discussion

The proportions (numbers and %) of food products in the food groups that contained AA are shown in Table 1. Also, distributions of the results, by groups and in all tested samples, are graphically presented in Figures 1-3.

Most of the tested food samples, almost half of them (44%), were various types of biscuits. On the another side, fewer samples were from the groups of confectionery and coffee products, 2% of all samples for both.

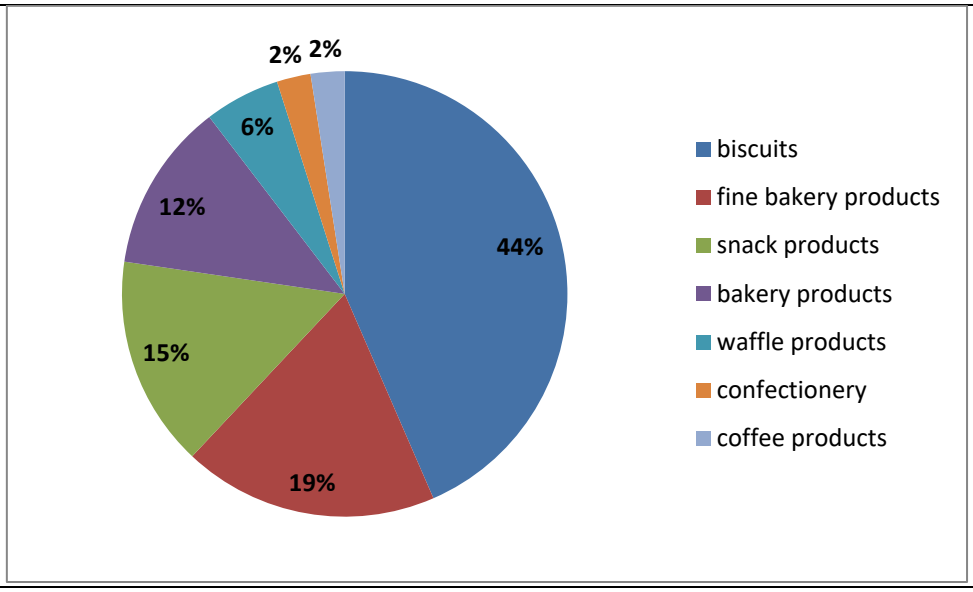

Figure 1. Pie chart showing the percentage distribution of tested food products by food group.

Table 1. Food product samples in defined food groups that were tested for the presence of AA, for the period December 2017 to March 2021

Food product	N	n_d (%)	n_{nd} (%)
Biscuits	230	208 (90.43)	22 (9.57)
Fine bakery products	98	97 (98.98)	1 (1.02)
Snack products	81	81 (100.00)	0 (0.00)
Bakery products	65	10 (15.38)	55 (84.62)
Waffle products	29	29 (100.00)	0 (0.00)
Confectionery	13	10 (76.92)	3 (23.08)
Coffee products	13	8 (61.54)	5 (38.46)
Total	**529**	**443 (83.74)**	**86 (16.26)**

N – total number of tested samples; n_d – number of samples that contained AA; n_{nd} – number of samples where AA was not detected; LOD = 25 µg kg^{-1}

All samples from snack product and waffle product groups contained AA. AA was detected in almost all fine bakery products (98.98%) and biscuits (90.43%). In contrast, only 15.38% of bakery products samples contained AA, which makes this group the only food group within which there were more samples in which AA was not detected (84.62%).

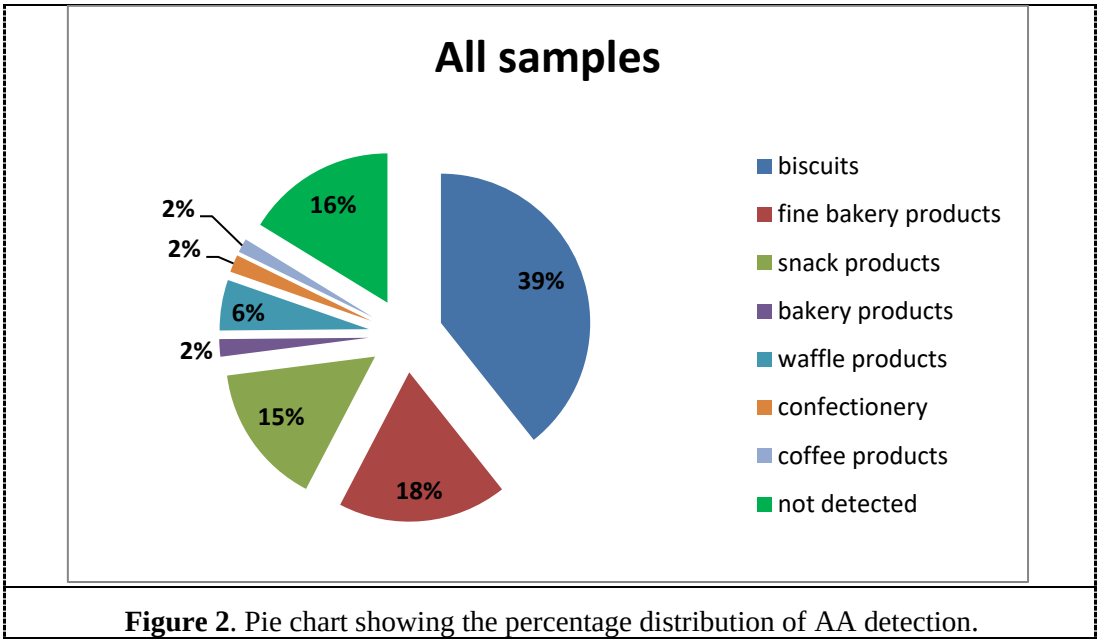

Figure 2. Pie chart showing the percentage distribution of AA detection.

More than a third (Figure 2) of all tested samples were biscuits containing AA. This was expected, as samples of this food product group were the most numerous (Figure 1).

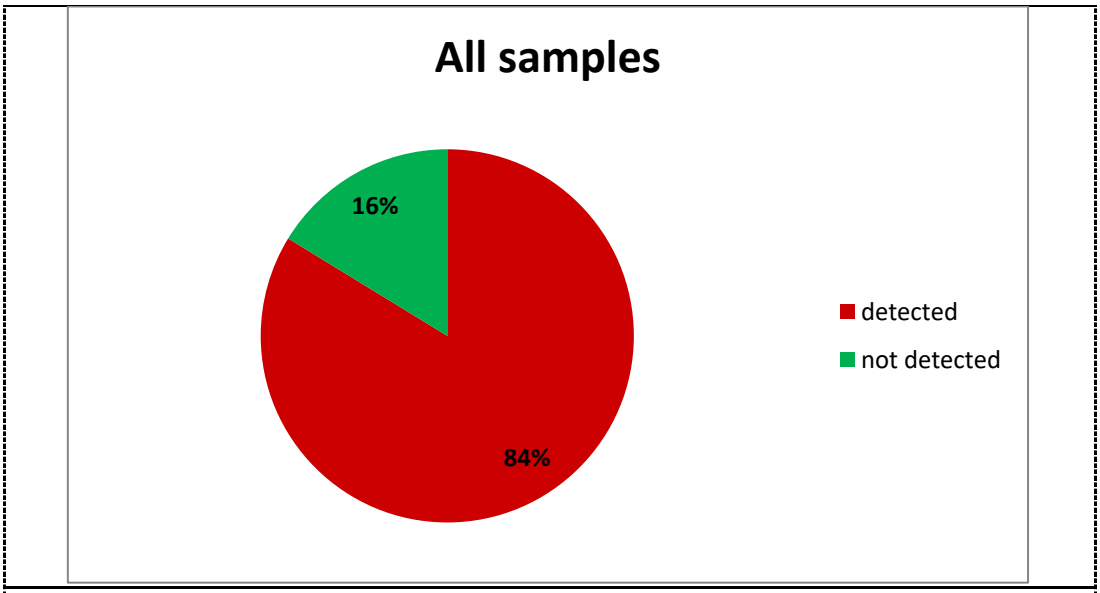

Figure 3. Pie chart showing the percentage of tested samples where the presence of AA was detected and not detected.

4. Conclusion

Most of the tested food product samples contained AA, 83.74% of them. In every sample of snacks and waffle products, AA was detected. Only one sample (1.02%) of fine bakery products did not contain AA. Bakery products were the safest group of samples, regarding AA, with only 15.38% of samples containing this compound. All in all, the results of this study confirm [16] that AA, in most food products that are subject to the formation of AA in terms of their chemical composition and production process,

is unfortunately inevitable. Accordingly, continuous food safety controls are necessary, as well as further research to consider the AA levels in different food groups, and data on the average daily intake of these foods by population groups, in order to integrate these data to assess the health risk of AA intake through food.

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Review of testing for foreign horse and pig DNA in meats in Croatia

Review of testing for foreign horse and pig DNA in meats in Croatia

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Abstract. Several years after the food industry scandal when horsemeat was found in products sold in Europe as beef products in 2013, Croatia began testing food for the presence of foreign protein. For the time being, these tests are not part of routine monitoring, but the result of examining the situation on the market in the city of Zagreb. Namely, in recent years, central Croatia has been trying to establish itself as a tourist destination, and Zagreb hosted hundreds of thousands of tourists from all over the world before the COVID-19 pandemic. The eating habits of the various groups that came to Zagreb were different, and the larger hotel chains recognized the seriousness of the services and sought help to ensure that the food offered was consistent with their declarations and would not conflict with religious requirements. One of these requirements was the testing for foreign proteins such as horse and pork in foods where they were not declared. Although horse and pork are safe for human consumption, they are not part of the eating habits in all countries. The Dr. Andrija Štampar Teaching Institute for Public Health introduced methods for detection of horse and pig DNA in food samples.

1. Introduction

The general principle of food law is to provide consumers with a basis to enable them to be properly informed when choosing the food they consume and to prevent actions that may mislead consumers. The European Union therefore adopted Regulation (EU) No 1169/2011 [1]. The objectives of Regulation (EU) No 1169 are a high level of protection of the health and interests of consumers, with special emphasis on health, economic, environmental, social and ethical circumstances [1].

Europe was rocked by a food industry scandal in 2013 when horse meat was found in products sold as beef products, according to the declaration. Undeclared or misdeclared other meats such as pork were also found in the products. In some products, horsemeat accounted for 100% of total meat. The affair began on January 15, 2013, when it was revealed that horse DNA had been found in frozen burgers sold in Irish and British supermarkets. Shortly after this announcement, the scandal spilled over into many European countries, where products were also found to contain undeclared horse meat in various meat products. Numerous falsely declared products have been withdrawn from supermarket shelves across Europe [2].

Although horse meat is safe for human consumption, it is not part of the eating habits of all European countries. The falsification of declarations and fraud in the food industry usually have financial reasons, as prices for quality meat are high. Horse meat is much cheaper than other types of meat in some countries. There is a similar problem with pork. Indeed, there are large groups of people who do not want to eat pork for religious reasons [3].

Proper labelling of the type of meat contained in food products is important for economic, safety, legal and health reasons. Misdeclared meat is of questionable origin and there is no guarantee that it is safe for consumption. Some people do not consume the meat of certain animal species due to religious customs and laws [4]. In Croatia, food labelling is regulated by the Consumer Information Act (NN 56/13) and Regulation (EU) No 1169/2011 on informing consumers about foods. Nevertheless, the legislation in the Republic of Croatia regarding the examination of the presence of foreign proteins is very open for the time being. Namely, according to Art. 6 of the Regulation on Meat Products NN 62/2018, a meat product that has a prominent animal species in its name must contain at least 75% of the meat derived from that animal species, calculated on the total amount of meat used in the production process [4,5].

This formulation certainly does not satisfy sensitive groups of people regarding eating habits. The question arises as to what amounts of foreign protein such groups would tolerate. Due to such questions, the British Food Standard Agency has developed guidelines for the limits of acceptability of the amount of foreign protein in the product. Currently, a foreign protein level of 0.1-1% is considered acceptable. Back in 2014, the recommendation to the food industry was that this would be technically feasible in terms of good manufacturing practice and acceptable to most consumers. It is considered that a limit of up to 0.1% foreign protein should not be reported but should be monitored regularly. Where control samples show 0.1-1% foreign protein, the reasons for this presence should be investigated and corrective action taken. For products containing 1% or more of a foreign protein, this should be declared or the recall of such products should be encouraged [6].

Testing for the presence of foreign proteins, such as those from horse and pig, can be performed by various methods based on immunological assays, chromatography, and other chemical methods [7]. Most of these methods are limited due to their sensitivity and easy denaturation of proteins by a rise in temperature [8]. Methods based on DNA analysis, such as polymerase chain reaction (PCR), are more robust as well as sensitive and specific. Compared to proteins, DNA is a more stable and resistant molecule. It is resistant to various processes used in the food industry such as food processing at high temperatures and pressures, presence of other chemical compounds, etc. PCR can, therefore, analyse processed foods, as the DNA molecule is not destroyed by food processing such as case for proteins. The PCR method can also be used to successfully identify certain types of meat present in meat mixtures [7,8].

The Dr Andrija Štampar Teaching Institute for Public Health offers services for testing foods for the presence of DNA of horse and pig origin. These DNAs can be successfully detected in various food samples, from fresh meat (e.g. mixed minced meat), to various meat products such as sausage and salami and even in ready-to-eat meals. [9] First, DNA must be isolated from the sample. Then, all necessary amplification reagents are added to the isolated and purified DNA. If foreign DNA is present in the sample, it is amplified, which is noticeable by an increase in fluorescence [10].

2. Materials and Methods

In the last five years from 2016-2020, a total of 43 samples of different types of sausages and salamis were tested for the presence of DNA derived from horses and 51 samples were tested for the presence of DNA derived from pigs. Table 1 shows the number of samples tested.

Table 1. Numbers of meat/meat products examined for horse and pig DNA by year

Year of testing	Horse DNA	Pig DNA
2016	3	-
2017	10	10
2018	10	10
2019	10	21
2020	10	10

2.1 DNA extraction

DNA was extracted according to the protocol using the foodproof Sample Preparation Kit III (Biotecon Diagnostics). The extraction buffer was added to 200 mg of homogenized sample in 2 ml microcentrifuge tubes and vortexed for 30 seconds. Proteinase K (80 µl) was added to the suspension containing sample and extraction buffer. The mixture was incubated at 72 °C for 30 min and the tube was mixed 2-3 times by inverting the tube during incubation. Then centrifugation was done at 12000 x g for 10 min to remove the insoluble material. The supernatant was transferred to a new microcentrifuge tube with 400 µl Binding Buffer and 200 µl isopropanol and mixed gently but thoroughly by pipetting up and down.

The mixture (650 µl) was pipetted into the upper reservoir of a combined filter-collection tube and centrifuged at 5000 x g for 1 min. The collection tube was discarded and filter tube was transferred to a new collection tube. The remaining mixture was added to the same filter-collection tube and centrifuged again at 5000 x g for 1 min. The flow through and collection tube were discarded, and the filter tube was added to a new collection tube. Wash buffer (450 µl) was added to the upper reservoir and centrifuged at 5000 x g for 1 min. The flow-through was discarded and the collection tube was reused for a new step in which 450 µl wash buffer was washed and centrifuged at 5000 x g for 1 min. The flow-through was discarded again and the collection tube reused for a centrifugation of 10 sec at maximum speed to remove residues of wash buffer.

The dried column was transferred to a clean 1.5 mL microcentrifuge tube. Pre-warmed (70 °C) elution buffer (200 µl) was added to the glass fibre fleece and left at room temperature for 5min to ensure the elution buffer was completely absorbed.

Finally, the column was centrifuged at 5000 x g for 1 min to elute the purified DNA, which was used directly or stored at -20 °C for further analysis.

2.2 Polymerase Chain Reaction (PCR) amplification

The extracted and purified DNA was amplified by real-time PCR (PicoReal 24, Thermoscientific). Amplification of DNA was performed with Thermo Scientific PikoReal Software 2.2. The assay is a qualitative duplex real-time PCR, which means that the detection of specific genes and internal control is performed simultaneously using specific primers marked by fluorescent colours. Specific genes for porcine and horse animals and the internal control were detected by FAM (porcine/horse specific gene) and HEX/VIC (internal control) detection channels.

For Porcine detection Lyokit, 25 µL extracted and purified DNA, 25 µL negative control (PCR-grade H_2O), and 25 µL positive control (control template) were added into each PCR tube which already contained lyophilized reagents. The PCR cycler program included pre-incubation in two steps: 4 minutes at 37 °C and 10 minutes at 95 °C; 5 seconds at 95 °C and 60 seconds at 60 °C.

For Horse Species Detection Kit, a reaction mixture was prepared by combining 4µL of primer/probe mixture, 10 µL of real-time PCR mastermix, 6 µL of extracted DNA sample into each PCR tube, so the reaction volume for each tube was 20 µL. Positive and negative controls were used. The program included pre-incubation at 95° for 10 minutes and amplification in two steps: 15 seconds at 95 °C and 40 seconds at 61°C.

3. Results

Figure 1 shows the meat products in which the DNA of horse origin was examined according to year. From 2017 to 2019, tests were carried out on samples of permanently cured meat products declared as 100% pork or beef of domestic or imported origin. In 2017, two meat products were positive for horse DNA. During 2018 and 2019, no horse DNA-positive meat products were found. Samples of domestic and imported origin of brands from individual retail chains and domestic producers were examined. Horse DNA was not found in any of the samples. In 2020, semi-durable meat products such as various salamis, hot dogs, and the like were tested. Only in one sample was DNA of horse origin found in traces.

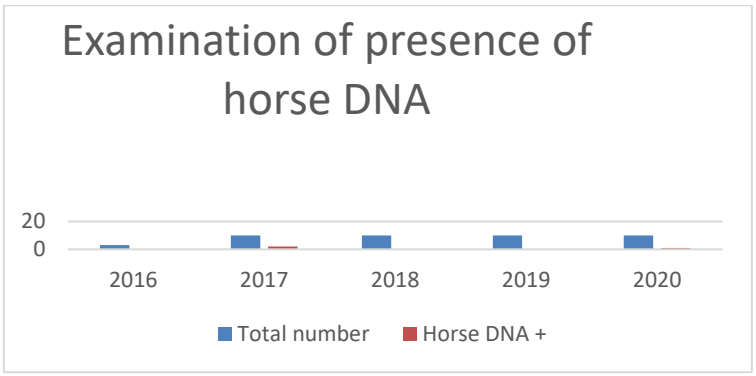

Figure1. Presence of horse DNA in meat products labelled as pork or beef during five years

Figure 2 shows the meat products in which DNA of pig origin was examined according to year. The tested meat products were semi-durable meat products. The figure shows a slow increase in the number of samples positive for pig-derived DNA.

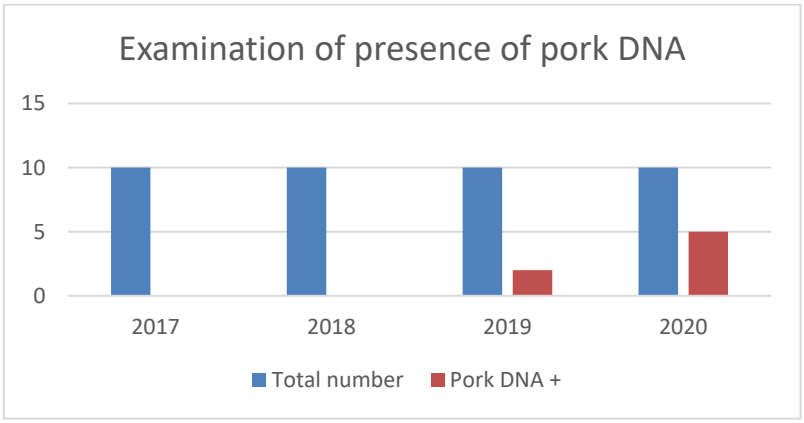

Figure 2. Presence of pig DNA in meat products during four years

Figure 3 shows the results of cleaning control in a company that exports its products to countries where halal is practiced.

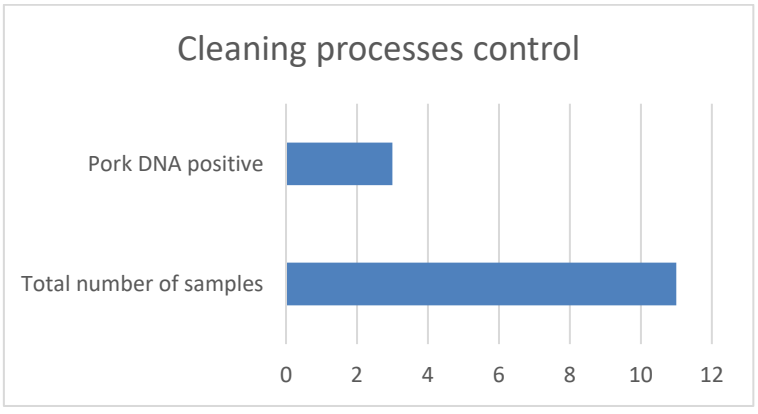

Figure 3. Cleaning processes control

In 2019, some Halal certified companies decided to control their cleaning processes to achieve greater trust of customers in their products. Production lines in smaller companies cannot separate products according to individual types of meat. Therefore, cross-contamination of the products can occur. Sometimes in products declared as 100% non-pig type of meat, traces of unwanted pig DNA were found. Various cleaning and disinfection procedures were tried, until testing showed no traces of unwanted pig DNA. Unfortunately, during 2020, these controls abated due to the pandemic.

4. Conclusion

Foods that are produced or imported before being placed on the market must meet specified standards. For this reason, regular controls are needed to make sure that consumers are consuming exactly the type and quality of meat that is declared. Meat companies have or want to obtain certificates that are mandatory in some countries due to ethical rules, religions or eating habits, and the companies do not rely only on their national regulations or EU regulations, but go a step further and control the presence of foreign proteins in their production.

Acknowledgement

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Adaptation of the two-dimensional electrophoresis method for canned meat

Adaptation of the two-dimensional electrophoresis method for canned meat

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Abstract. Studies of the qualitative indicators of canned meat in accordance with regulatory documents are carried out on average samples of specimens, but when studying by proteomic methods, such sampling does not allow high-quality separation of protein components due to the high fat content in the product. When two-dimensional electrophoresis was carried out on an average sample, fragments of the main muscle and connective tissue proteins of beef were found in small quantities, but the electrophoretogram was not very informative. A significantly better separation was achieved after removing the fat fraction from the product. When studying broth from canned meat, the largest amount of intensely coloured high-molecular-weight protein fractions with a mass of more than 50 kDa was revealed. The electrophoretogram of the meat pieces showed a wide range of proteins across the entire molecular weight range of the polyacrylamide gel, including major muscle proteins. The study of broth together with meat pieces but after fat removal is optimal for the primary screening of the protein component of canned meat.

1. Introduction

Animal foods are among those foods that contain many important nutrients. The food industry uses many technologies to produce products with a variety of shelf life durations. There are already adapted methods for determining the proteomic profile of meat and some meat products. However, processing conditions and methods can change the nutritional value, texture and taste of meat, which makes it difficult to identify proteins in cooked foods [2]. The high temperature processing of the product contributes to the destructive changes in the meat system, which has both positive and negative effects on the nutritional value of products [3]. Understanding the functional mechanisms of protein systems at the molecular level will make it possible to control these processes, possibly also to correlate the risky technological aspects of industrial food production. The range of proteomic technologies can be divided into several main groups, presented in Figure 1. Proteomics techniques complement existing methodologies for quality assurance and food safety authentication. The protein complex of food products is analysed using a variety of high performance separation methods such as one-dimensional and multivariate chromatography, two-dimensional gel electrophoresis, and high-resolution mass spectrometry. The use of these methods makes it possible to control the protein composition of food products and their changes during the production process, while their use is also invaluable in the authentication of toxins, allergens, nutritional value and shelf life or during storage [4].

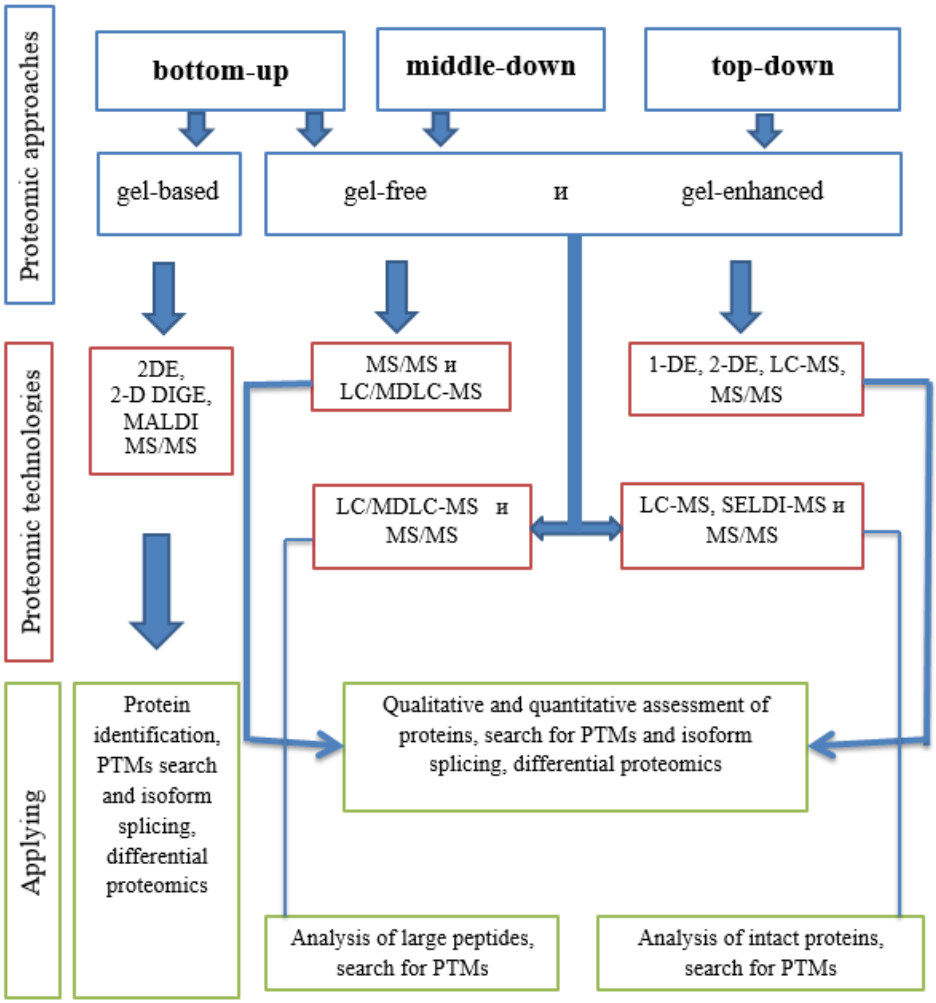

Figure 1. The basic approaches and methods for proteomics. 1DE - one-dimensional electrophoresis; 2DE - two-dimensional electrophoresis; 2-D DIGE - two-dimensional differential electrophoresis; LC - high performance liquid chromatography; MDLC multidimensional LC; MALDI - matrix-activated laser desorption / ionisation; MS - mass spectrometry; MS / MS - tandem MS: PTM - post-translational modifications; SELDI - surface enhanced laser desorption / ionisation.

One of the most famous methods for obtaining proteomic profiles belonging to the “bottom-up” group is two-dimensional electrophoresis of proteins [5]. To separate protein extracts in the first direction, isoelectric focusing in thin columns of polyacrylamide gel (PAGE) is used, with which proteins are separated depending on the value of their isoelectric points. As a method used in the separation of proteins in the second direction, PAGE gradient plate electrophoresis was performed in the presence of sodium dodecyl sulphate as an ionic detergent. Two-dimensional electrophoresis makes it possible to analyse complex protein mixtures and produces electrophoretograms that show more than 1000 fractions [6]. Despite some limitations, mainly related to sensitivity and reproducibility, two-dimensional electrophoresis is used for the systematic study of a wide variety of biological objects [7, 8, 9, 10, 11]. Optimisation of protocols for sample preparation during protein extraction and solubilisation allows these problems to be solved. With that in mind, the purpose of this study was to adapt the method of two-dimensional electrophoresis to determine the protein composition of canned meat.

2. Materials and methods

As an object of research, we took sterilised consumer packages of the canned meat, “Top grade stewed beef”. The ingredients of this canned food were: beef, beef fat, onions, salt and bay leaf.

To produce an average sample, the lids of the cans were cut with a knife around 3/4 of the circumference, and, bending them slightly outward so that the solid parts of the canned food did not pass through the gap, the liquid part was poured into a porcelain cup. The solid part of the canned meat was passed twice through a meat grinder, mixed with the liquid part and ground in parts in a porcelain mortar until a homogeneous mass was achieved, which was then used for further analysis.

The other samples for this study were produced using a modified sample protocol, as described below.

The protein composition of the canned meat system was analysed by the method of two-dimensional electrophoresis according to O'Farrell with isoelectric focusing in an Ampholine pH gradient, as described previously [12].

3. Results and Discussion

Studies of the quality indicators of canned meat in accordance with regulatory documents are carried out on the average sample of the specimen [13]. When studying canned meat pieces using two-dimensional electrophoresis in accordance with generally accepted requirements (Figure 2), fragments of the main muscle and connective tissue proteins of beef were found in small quantities, and the pattern of the location of protein fractions of the studied meat system was preserved. However, the effect of overlapping proteins and a blurred image did not allow for a clear visualisation of the marker zones of the meat system, and therefore, it was decided to change the protocol for sample preparation of canned meat for studies by two-dimensional electrophoresis.

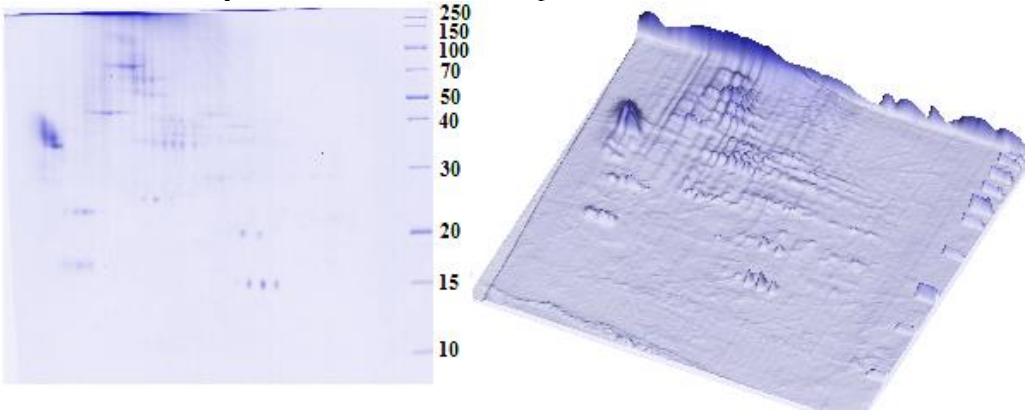

Figure 2. Electrophoretogram of the average sample of canned food “Top grade stewed beef”. The marked ladder is the protein standard with different molecular weights, kDa

In the manufacture of canned meat, rendered fat is used, in which there is practically no protein or raw beef fat, as after sterilisation and melting of fat, small pieces of connective tissue rind can contain up to 0.07% protein, which will not affect the results of electrophoretic studies if the fat component is removed. Therefore, a modified sample preparation protocol for canned meat containing meat pieces was developed before two-dimensional electrophoresis. The modified sample preparation was based on dividing the contents of a consumer package of the canned meat into its component parts to produce three samples and included the following sequence of operations.

Before opening, the can with contents was heated in a water bath for 20 minutes, then opened to 2/3 or 3/4 of the circumference. Then, the can was installed obliquely into a funnel and the liquid part of the canned food was drained into a beaker for 10-15 minutes, while every 5 minutes the can was carefully turned several times. Individual pieces of meat were removed with tweezers or a spoon and were finely chopped prior to electrophoresis. The drained liquid part of the canned food, broth with fat, was cooled in a beaker to 0°C-8°C. The solidified fat was removed from the broth surface. Two further samples were then produced – the liquid broth and a mixture of meat with broth.

The research results are shown in Figure 3. The most informative picture was obtained in the study of pieces of meat (Figure 3A) from the canned food. A large amount of proteins was detected in the entire molecular weight range of the polyacrylamide gel, including the main structural fractions of β-enolase, muscle creatine phosphokinase, phosphoglycerate kinase 1, glyceraldehyde-3-phosphate dehydrogenase, groups of troponins and myosin light chains.

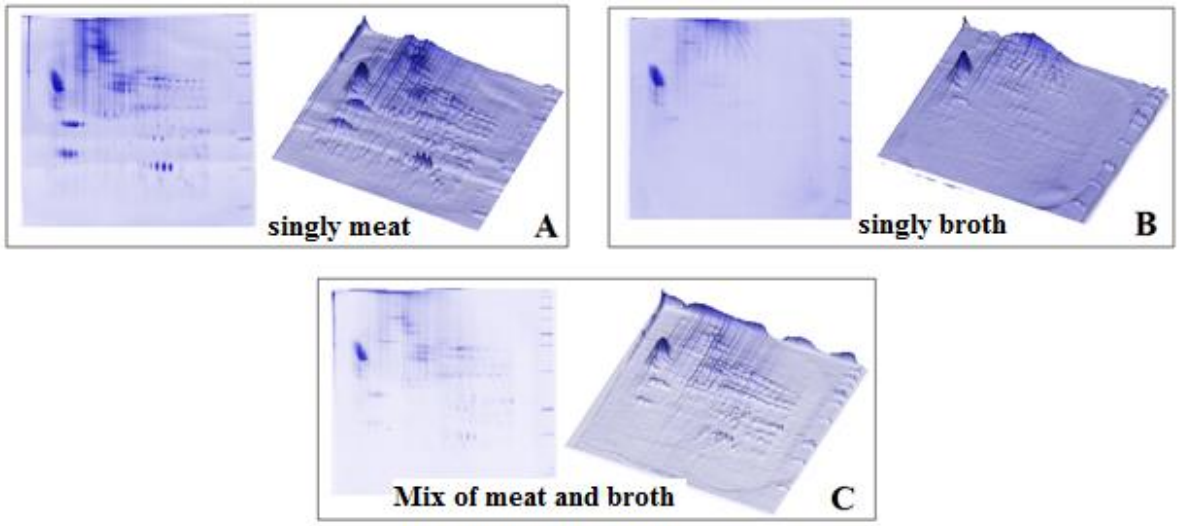

Figure 3. Electrophoretograms of canned food fractions from "Top grade stewed beef "

Electrophoretic study of the broth (Figure 3B) from the canned meat revealed a large number of intensely coloured high-molecular-weight protein fractions with a mass of more than 50 kDa, probably collagen chains from connective tissue, as well as mitochondrial aconitase 2, heat shock proteins, desmin and actin fractions.

For the primary electrophoretic screening of the protein component of canned meat with meat pieces, it was optimal to use the sample option of broth together with meat pieces, with the fat component removed (Figure 3C). This method allowed the main protein components in a wide range of molecular weights to be identified.

4. Conclusion

The conditions for the separation of the protein component in a canned meat product with whole meat pieces were adapted by removing the fatty component of the product. For comparative analysis of high-molecular-weight proteins, it is optimal to study the protein fractions of the broth. To study the variations in tissue proteins, it is reasonable to study the solid component of canned meat pieces. However, to determine the total spectrum of proteins, electrophoretic study of the combined broth and meat pieces is recommended.

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Detection of unlabelled gluten in meat products and gluten-free flour by PCR and ELISA methods

Detection of unlabelled gluten in meat products and gluten-free flour by PCR and ELISA methods

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Abstract. Over the past decade, there has been an increase in the prevalence of gluten intolerance. Since wheat protein is widely used in the food industry, in particular in the meat industry, consumers prone to gluten intolerance should be aware of its presence in food products through the information on the label. Often, however, unintentional contamination of gluten-free products occurs. The aim of this study was to study the prevalence and level of gluten contamination of meat products and gluten-free flour used for the production of Russian-made meat products, which do not contain ingredients containing gluten. To determine gluten, PCR and ELISA methods were used. In four of the nine tested samples, gluten was found at a level exceeding 20 mg/kg.

1. Introduction

Celiac disease is an autoimmune disease caused by the ingestion of gluten proteins from wheat and related cereals (rye, barley and triticale) by genetically susceptible individuals [1]. The prevalence of celiac disease affecting both adults and children in the Western world is estimated at about 1-2% of the population [2]. In the Russian Federation, some data on the incidence of celiac disease diagnosis indicate an estimated prevalence in the range from 1:100 to 1:250 [3]. The overall prevalence of this disease in the world is estimated at 1:184. The disease leads to inflammation, atrophy of the intestinal villi and hyperplasia of the crypts of the small intestine. In addition to intestinal symptoms, celiac disease is associated with a variety of extraintestinal complications, including bone and skin diseases, anaemia, and endocrine and neurological disorders.

Given the widespread use of cereals containing gluten in the food industry (for example, pasta, breakfast cereals, most types of bread, thickeners and stabilizers used in soups, meat products, etc.), excluding wheat and other sources of gluten from the diet presents a challenge for people with celiac disease [5].

The production of gluten-free products is based on the food standards of the international commission, Codex Alimentarius, a joint body of the UN Industrial and Agricultural Organization and the World Health Organization. According to the ALINORM 08/31/26 Codex Alimentarius standard, gluten-free products can be considered gluten-free in which the gluten content does not exceed 20 ppm (20 mg/kg).

Currently, in the Russian Federation, in accordance with the technical regulations of the Customs Union TR CU 027/2012, gluten-free food products must be made from one or more components that do not contain wheat, rye, barley, oats or their crossbred variants and (or) must consist or be made in a

special way (to reduce the level of gluten) from one or more components that are obtained from wheat, rye, barley, oats or their crossbred variants, and in which the level of gluten in the ready-to-eat product is not more than 20 mg/kg.

Several studies have shown the likelihood and levels of contamination of gluten-free products. A 2011 Canadian study found that 82% of oat samples tested (n = 131) were also contaminated with high levels of gluten [6]. Another 2013 study by a group of Canadian scientists found that 32% of gluten-free flour (n = 640) sold at retail outlets in Canada was contaminated with gluten, which can pose health problems for people with celiac disease [7]. A study in Brazil found that 2.8% (n = 180) of traditional Brazilian food samples were inadvertently contaminated with gluten [8]. These studies highlight the risk of gluten-free cross-contamination of gluten-free products.

Unintentional gluten contamination of products that do not have labelling information about the presence of gluten or of gluten-free products is a serious problem. This type of information is important for consumers affected by celiac disease. The study described herein was conducted to determine the prevalence and level of gluten contamination in meat products and gluten-free flour used for the production of Russian-made meat products, which do not contain ingredients containing gluten.

2. Materials and Methods

To determine gluten, six different types of sausages of local specifications and lentil, corn and buckwheat flours were purchased in Moscow retail outlets. The labels of these products did not contain information about the presence of gluten.

2.1. ELISA method

ELISA test to detect wheat protein was performed using the Romer Labs Agra Quant® Gluten test kit, which is a screening test for examination of gluten-free products for the presence of gliadins and prolamins of wheat, barley and rye, and, as indicated by the manufacturer, it demonstrates presence of 4 mg/kg up to 120 mg/kg of gluten. The test is based on the principle of sandwich-like ELISA method, where gliadin was first extracted with 40% ethanol. The acquired extract was diluted with phosphate buffered saline and applied to test wells with conjugated antibodies against gliadin. After incubation and washing off redundant gluten, enzymatically marked antibody was bound to conjugated gliadin if present. A blue colour detected in the last stage was considered to be positive, while light pink colour was negative. Colour was compared to negative and positive controls. The test was performed in compliance with the manufacturer's instructions.

2.1.1. Statistical data processing. For the calculations, the STATISTICA 10 program was used, the results were presented as "Mean ± SD", and the statistical significance was calculated using the nonparametric Kruskal-Wallis test (for three or more independent groups). A probability of 0.05 was chosen as a significant level.

2.2. PCR method

2.2.1. Sample selection. Sausage samples were ground with Retsch GM200 blade homogenizer. Ten samples of 5 ± 1 g were selected from each sausage.

2.2.2. Isolation of DNA. DNA was isolated by the Sorb-GMO-B kit (Syntol CJSC, Russia) according to the instructions.

2.2.3. Conditions for real-time PCR. Primers and probes used in this work were taken from GOST 31719-2012 and MP 4.2.0019-11. The reaction mixture (30 μl) contained 2.5 μl of 10X PCR buffer, 2.5 μl of 2.5 mM $MgCl_2$, 2.0 μl of dNTP, nucleotides at a concentration of 25 mM, 2.5 U of SynTaqpolymerase, primers at a concentration of 300 nM, and 2 μl of DNA. The reagents were manufactured by Syntol CJSC, Russia. Amplification mode: pre-denaturation — 95°C, 420 seconds;

annealing-elongation — 60°C, 40 seconds; denaturation — 95°C, 15 seconds, 45 cycles. Real-time PCR was performed on a ANK-32 thermocycler (Syntol CJSC, Russia). PCR amplification was performed using a tenfold dilution of the original DNA.

2.2.4. Statistical data processing. Statistical analysis of the PCR results was performed using the software supplied with ANK-32 thermocycler.

3. Results and Discussion

It was found that the gluten content exceeded 20 mg/kg in the Braunschweig sausage (manufacturer 2) and the Krakowska sausage (manufacturer 3). The presence of a component potentially containing gluten was not indicated on the labels. The gluten in the sausages could be due to the presence of wheat flour impurities in some ingredients used in the formulation of these sausages, milk powder and dry egg powder. Gluten at levels significantly higher than the acceptable level was found in the studied samples of corn and lentil flour, which can be ingredients of meat products (Table 1).

Table 1. Gluten content in the tested sausages and flour

Samples	Gluten content (mg/kg) detected by PCR	Gluten content (mg/kg) detected by ELISA
Doktorskaya sausage, Manufacturer 1	<20	<20
Russkaya sausage, Manufacturer 1	<20	<20
Doktorskaya sausage, Manufacturer 2	<20	<20
Braunschweig sausage, Manufacturer 2	30.12	23.98
Krakowska sausage, Manufacturer 3	22.34	21.18
Russkaya sausage, Manufacturer 3	<20	<20
Corn flour, Manufacturer 4	80.35	91.21
Lentil flour, Manufacturer 5	150.64	120
Buckwheat flour, Manufacturer 6	<20	<20

All sausages selected for the analysis were produced in accordance with the national standards of the Russian Federation, and they assume gluten-containing ingredients are not present in the products. No gluten warning label was on any of the examined sausages. The same requirement applied to cereal and legume flours.

The most common causes of gluten appearing in meat products are: (1) unreliable suppliers, (2) non-compliance with good manufacturing practices, (3) inadequate analytical method used, (4) improper staff training and (5) inappropriate use of gluten warning labels.

According to the results of the study, the presence of gluten in sausages and flour was confirmed by PCR and ELISA methods. However, there were differences in the gluten levels, from which it can be concluded that further development of the analysis procedure is necessary, especially with respect to cereals and legumes.

4. Conclusions

To reduce production costs, cheaper vegetable proteins are often used in the meat industry. Certain plant sources, such as wheat, are also used as fillers and binders in meat products, but these plants can contain gluten, which causes celiac disease in consumers who are prone to celiac disease. Thus, information on the presence of gluten in such components of composite foods should be available on the label in accordance with the legislation. In addition, gluten can be inadvertently introduced into gluten-free products during transportation, production, storage through cross-contamination. As a result of the conducted research, gluten was detected in two samples of sausages, as well as corn and lentil flour.

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Current view on the assessment of antioxidant and antiradical activities: A mini review

Current view on the assessment of antioxidant and antiradical activities: A mini review

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Abstract. The main problems in assessing the antioxidant properties of plant biologically active compounds are discussed in this review. Antioxidant potential should be considered as a combination of antioxidant and antiradical activities, since antiradical activity is part of the antioxidant activity and does not always coincide with antioxidant activity. The mechanisms of action and the existing experimental and computational methods for their evaluation were reviewed. Methods like FRAP, CUPRAC etc. could be used for assessment of antioxidant activity of plant compounds, but it is necessary to perform studies on cell cultures or laboratory animals in order to determine mechanisms of action on the antioxidant system of a living organism. The current methodological approaches for studying antiradical activity and its mechanisms include experimental methods such as DPPH, ABTS and ORAC, and computational methods based on density functional theory. The main thermodynamic parameters for evaluating antiradical mechanisms (HAT, SET-PT and SPLET) are the bond dissociation enthalpy, ionization potential, proton dissociation enthalpy, proton affinity, and electron transfer enthalpy, among others. The existing approaches for determining the antiradical mechanisms of antioxidants are quite informative, but can still cannot predict or determine by *in vitro* methods the antioxidant mechanism of these compounds in organisms consisting of many complex individual systems.

1. Introduction

The study of substances with antioxidant properties is one of the relevant scientific areas. From 2015 to 2021, 199,681 articles were published in the international database Scopus for the query “antioxidant”. However, the terminology, research methods, and expression of the obtained data are still not uniform, which makes it difficult to interpret and compare the results of the research [1]. Thus, the term “antioxidant” has several definitions, for example, antioxidants are compounds that inhibit oxidation, a chemical reaction that can produce free radicals and chain reactions that may damage the cells of organisms. *Halliwell* and *Gutteridge* proposed a broader definition of antioxidants as “any substance that, when present at low concentration compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate”. Moreover, the term “oxidizable substrate” includes all organic and many inorganic molecules present *in vivo* [2].

On the other hand, the authors used different terms to describe the antioxidant properties, which are characterized by a wide variety and have different frequency of use. The most popular term is

"antioxidant activity", while the terms "antioxidant capacity", "antioxidant content", "antioxidant power", "antioxidant ability" and "antioxidant potential" are used much less often [1]. Despite the prevalence of the term "antioxidant activity", its appropriateness depends on the chosen research methods. The authors often describe the "antioxidant activity" (AOA) of the studied samples, but in fact they studied the "antiradical activity" (ARA).

The use of a specific term to describe the antioxidant potential of research objects depends on the methods and approaches that have been used to study these properties. In addition, for more accurate and comprehensive determination of the antioxidant properties of plant samples, it is necessary to use at least two methods. Thus, the aim of this study was to review the existing approaches and methods for assessing the antioxidant potential of substances of plant origin, taking into account the difference of concepts "antioxidant activity" and "antiradical activity".

2. Antioxidative mechanisms

Antioxidant and antiradical activities characterize the ability of a substance or mixture of substances to act as an antioxidant and reduce the negative effects of oxidative reactions on cells, organs, the body, and even food. However, ARA and AOA describe different principles of activity; therefore, these concepts should be distinguished [3]. ARA characterizes the ability of compounds to react with free radicals (in a single free radical reaction), while AOA describes the ability of substance to inhibit the process of oxidation, which usually involves many different reactions, such as lipid peroxidation [4].

Antioxidant mechanisms are ways of inhibiting oxidative reactions and processes occurring in complex biological systems. These mechanisms include regulating the activity of enzymes by inhibiting the production of oxidases, which promote the generation of reactive oxygen species (ROS), or enhancing antioxidant enzyme activity, in particular, the activity of superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione reductase (GR) and glutathione thiotransferase (GST) [5,6]. The ability of a compound to interact with the free metal ions (iron and copper) with the formation of inactive complexes of these metals also belongs to antioxidant mechanisms [5], does the influence of substances on cell antioxidant responses according to different pathways [5,7]. Despite numerous approaches to the study of antioxidant activity, there is no universal method that allows prediction of the mechanism by which a substance will exhibit antioxidant activity. Therefore, different research methods are carried out depending on the purpose of the work.

Antiradical activity is more clear and encompasses three generally accepted mechanisms of action against free radicals: hydrogen atom transfer (HAT) (1), single-electron transfer followed by proton transfer (SET-PT) (2, 3) and sequential proton loss electron transfer (SPLET) (4-6) [8–10]. SET-PT and SPLET mechanisms are often unified into single electron transfer (SET) [11].

$$ArOH + R^{\cdot} \rightarrow ArO^{\cdot} + RH \quad (1)$$
$$ArOH + R^{\cdot} \rightarrow ArOH^{\cdot +} + R^{-} \quad (2)$$
$$ArOH^{\cdot +} + R^{-} \rightarrow ArO^{\cdot} + RH \quad (3)$$
$$ArOH \rightarrow ArO^{-} + H^{+} \quad (4)$$
$$ArO^{-} + R^{\cdot} \rightarrow ArO^{\cdot} + R^{-} \quad (5)$$
$$R^{-} + H^{+} \rightarrow RH \quad (6)$$

According to these equations, all mechanisms are described for compounds that have an –OH group in the structure. In addition, each mechanism produces a new radical (ArO$^{\bullet}$), which is more stable and less reactive than the original free radical (R$^{\bullet}$) [8]. Antiradical mechanisms are characterized by thermodynamic parameters: bond dissociation enthalpy (BDE) accompanied reaction (1); ionization potential (IP) accompanied reaction (2), proton dissociation enthalpy (PDE) accompanied reaction (3); proton affinity (PA) accompanied reaction (4); and the electron transfer enthalpy (ETE) accompanied reaction (5) [8]. Summarizing, antiradical activity has specific parameters for determination, while antioxidant activity does not have such certain assessment criteria. Thus, it is necessary to clearly

distinguish antiradical activity from antioxidant activity, because these properties do not always coincide [3,4].

3. Methods for evaluating the effect of antioxidants

There are a wide range of methods for comprehensive study the antioxidant properties of compounds or mixtures of substances. However, in the case of dividing the antioxidant potential into antioxidant and anti-radical activity, it is necessary to clearly understand that the choice of method and approach should be carried out relative to the goals set. Biological methods, which include cell cultures, laboratory animals and human studies, are used for studies of antioxidant activity. Biological methods are very expensive and take a lot of time [3], and therefore, such chemical methods as ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC) etc. are more often applied for AOA investigation [12]. The FRAP method is based on the reduction of a ferric (III) 2,4,6-tripyridyl-s-triazine complex by antioxidants to ferric (II) in accordance with the reaction (7) [12].

$$Fe(TPTZ)_2^{3+} + ArOH \rightarrow Fe(TPTZ)_2^{2+} + ArO^{\cdot} + H^+ \quad (7)$$

The CUPRAC method is identical to the FRAP method and based on the reduction of a cupric neocuproine complex (Cu(II)–Nc) by antioxidants to the cuprous form (Cu(I)–Nc) [12]. These methods significantly limit researchers, since they only allow the ability of samples to reduce metals to be evaluated, but they are fast, cheap and easily reproducible, which makes them effective indicators of the antioxidant system condition in oxidative stress and related studies [3].

Test systems, including biological, chemical and biochemical methods of analysis, are more effective in studying the antioxidant activity. Kwanjit Danwilai et al. [13] studied the antioxidant activity of ginger extract by measuring in patients indicators such as the activities of SOD and CAT and levels of glutathione peroxidase, total glutathione (GSH/GSSG), lipid peroxidation products detected as malondialdehyde (MDA) and NO_2^-/NO_3^-. Biswajit Podder et al. [14] assessed antioxidant activity of sea buckthorn extract by measuring the viability of A549 cells using 3-(4,5-dimethylthioazol-2-ly)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase (LDH). We also investigated the antioxidant activity of onion husk extract by measuring the activities of SOD and CAT, the concentration of total glutathione and malondialdehyde, and ferric reducing antioxidant power in blood plasma, brain, and liver in aging laboratory animals [15]. The results of our research clearly demonstrated the effectiveness of a system approach.

Currently, there are a lot of methods for analyzing antiradical activity, but scientists mostly use generally accepted and widely applied methods. Thus, the determination of antiradical activity is most often investigated using the ABTS, DPPH, and oxygen radical absorbent capacity (ORAC) methods, which use stable free radicals and are based on measuring the ability of an antioxidant to suppress these free radicals. All methods with a stable free radical provide information about the activity of trapping radicals, although in many cases this activity does not correspond to the antioxidant activity. It is necessary to carry out research on a real product or in a live system in order to get information about the actual antioxidant activity in relation to lipids or food preservation [4].

According to the ABTS method, pre-formed dark green stable free radical 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) is reduced by a compound [3,16]. The obvious disadvantage of this method is the random nature of the reactions of the free radical, which is a non-physiological free radical [4].

According to the DPPH method, the stable radical 1,1-diphenyl-2-picrylhydrazyl loses its dark purple colour when interacting with antioxidants. The antiradical activity of tested compounds is expressed as a relative or absolute decrease of the concentration of DPPH or as EC50 (concentration of a compound decreasing the absorbance of a DPPH solution by 50 %) [4,9,17]. Despite the prevalence of the method using the $DPPH^{\bullet}$ radical, it has been suggested that it is preferable to use the O-centred stable galvinoxyl (II) radical, which is more closely related to physiologically active oxygen radicals than DPPH, to measure antiradical activity. Gunars Tirzitis et al. [4] mentioned that in some cases, the DPPH test gives

incorrect results, and therefore, recommendations for the correct application of the method were proposed.

The ORAC method is another modern method for determining ARA [18]. This method is based on the inhibition of probe oxidation caused by peroxyl radicals, where peroxyl radicals are formed as a result of the thermal decomposition of azo-compounds, such as 2,2-azobis (2-amidino-propane) dihydrochloride (AAPH) [18,19]. The decomposition of the fluorescent probe is slowed down in the reaction mixture when the antioxidants react with peroxyl radicals [20]. Despite its high sensitivity, the ORAC method is less common than ABTS and DPPH, due to more specific equipment being required. It is worth noting that scientists often use combinations of methods to characterize antiradical activity, since there is no official standardized method [21].

Researchers discussed that, based on the chemical methods ABTS, DPPH and ORAC, it is possible to assume by what mechanism the antioxidant acted. Most researchers claimed that the use of the ORAC method allows determination of the antioxidants acting according the HAT mechanism [18,22,23], while the FRAP method determines antioxidants according to the SET mechanism [22,23]. However, in the case of the ABTS and DPPH methods, there are often conflicting statements about the mechanism to which they correspond. Some scientists believe that the use of the ABTS and DPPH methods allows determination of the antioxidant acting according to the HAT mechanism [3,24] while others argued that the ABTS and DPPH methods, as well as the FRAP analysis, assessing antioxidant acting according to the SET mechanism [18,23], since basic/acid solutions can promote the deprotonating/protonating of susceptible molecules [23,25]. Opposed views arise because the reaction of antioxidant with free radical is influenced by various factors. For example, the reaction with DPPH is significantly affected by the absolute and relative concentrations of DPPH and antioxidants, solvents, hydrogen bond strength, temperature, time, and pH [9,26]. Moreover, the lack of standardization in sample preparation, reaction conditions, analytical protocols, and expression of antioxidant activity makes it difficult to compare results obtained in different laboratories. In addition, the biological activity of compounds depends on the structure of the molecule, the medium and conditions of the reactions, the combination with other compounds, solubility, absorption, etc. Antiradical activity of phenolic antioxidants strongly depends on their structural characteristics [9,27].

4. Computational methods for evaluating the antioxidant potential

Plants are the richest source of natural antioxidants. Plants contain a great variety of such substances in the classes of phenolic compounds and flavonoids, while tannins, carotenoids, anthocyanins, diterpenes, terpenoids, etc. are present in smaller amounts. Scientists can successfully combine analytical and computational methods for more accurate determination of the anti-radical mechanism of action of antioxidants after the isolation, separation and identification of plant compounds by experimental methods. There are already many studies devoted to the investigation of the antioxidant properties of natural compounds, using test systems that include experimental and computational methods of analysis.

Chen et al. [9] assessed relationships between the structure, thermodynamics, and antiradical activity of 20 natural phenolic acids and their derivatives using DPPH scavenging assay, density functional theory (DFT) calculations at the B3LYP/6-311++G(d,p) levels of theory, and quantitative structure-activity relationship (QSAR) modelling. It was observed, that the C=O or C=C in COOH, COOR, C=CCOOH and C=CCOOR groups, and orthodiphenolic functionalities enhanced antiradical activity, while the presence of the single OH in the *ortho* position of the COOH group reduced antiradical activity. In addition, FR scavenging in the gas phase and benzene is most probable by the HAT mechanism, whereas in water and ethanol, the most likely mechanism is by SPLET, according to thermodynamics principles. Similar results were obtained by Milenković et al. [8]. Thermodynamic parameters such as BDE, IP, and PA were calculated for certain dihydroxybenzoic acids in nonpolar (benzene and pentylethanoate) and polar (water) solvents. The researchers found the HAT mechanism for dihydroxybenzoic acids was more probable in benzene, but in water the SPLET mechanism ruled; under the studied conditions, phenolic acids did not exhibit antiradical properties by the SET-PT mechanism.

Spiegel et al. [3] studied influence of the structure on the antioxidant potential of 22 phenolic acids. According to the results of FRAP and DFT, mono hydroxylated compounds and compounds with two hydroxyl groups in *meta* position to each other expressed the lowest antioxidant properties, while compounds with two or more hydroxyl groups in *ortho* or *para* position to each other demonstrated the highest antioxidant properties. *Chen el al*. [28] suggested that all antiradical mechanisms, HAT, SET-PT and SPLET, can occur in the reaction of phenolic compounds with DPPH, while the FRAP method is characterized only by the SPLET mechanism. This hypothesis is based on data from DPPH and FRAP analyses combined with calculations of five thermodynamic parameters for 18 phenolic acids. Thermodynamic parameters were calculated using DFT with the B3LYP/UB3LYP functional and 6–311++G (d, p) basis set. A number of other similar studies evaluated the antioxidant properties of flavonoid compounds [29,30], phenolic compounds [30], natural hydroxycinnamic acids [31], quercetin and its glucosides from propolis [32], chlorogenic acid [33], quercetin and morin [34].

Plant phenolic compounds can scavenge FR using three competitive mechanisms (HAT, SET-PT and SPLET), but the predominance of a particular mechanism depends on the structure of the antioxidant, the reaction conditions, and the solvent. The antioxidant capacity of phenolic compounds is significantly reduced when using a solvent that is prone to the formation of hydrogen bonds with phenolic compounds. For example, alcohols have a double effect on the reaction rate between the phenol and the peroxyl radical. On the one hand, alcohols act as acceptors of hydrogen bonds, and on the other hand, they promote the ionization of phenols to anionic phenoxides, which can react rapidly with peroxyl radicals through electron transfer. The general effect of the solvent on the antioxidant potential of phenolic compounds largely depends on the degree of ionization [11].

Phenolic compounds are better studied; computational methods for studying the antiradical activity were also applied for other plant biologically active compounds. *Vo et al*. [35] investigated nine natural diterpenes by kinetic and thermodynamic calculations. It was revealed that the sequential proton loss electron transfer mechanism is favoured in polar solvents, whereas formal hydrogen transfer is the main pathway for the radical scavenging of these diterpenes in the gas phase and in lipid media. *Vo et al*. [36] also studied natural hydroanthraquinones by kinetic and thermodynamic calculations. Computational methods were used to study the antiradical mechanisms of such plant compounds as products of the lignan family [37], kynurenines [38], coumarin-chalcone hybrids [39], essential oil components [40] etc.

Computational methods for studying the antiradical activity of plant compounds are based on the density functional theory and are used to calculate thermodynamic parameters associated with antiradical mechanisms (HAT, SET-PT and SPLET). BDE, IP, PDE, PA and ETE are the most informative thermodynamic parameters for evaluating the three main antiradical mechanisms. Thus, the results obtained by computational methods alone or in combination with experimental methods allow the thermodynamically advantageous antiradical mechanism for the studied compounds under specific conditions to be determined. However, it is worth noting that the determination of the mechanism of antioxidant action still does not allow prediction of the antioxidant mechanism of these compounds in organisms consisting of many complex individual systems.

5. Conclusions

Antioxidant potential of plant parts and their extracts, biologically active additives, food products, etc. should be considered as a combination of antioxidant and antiradical activities. This approach is not only more informative, but also more accurate. The existing experimental and computational methods and their combinations allow not only evaluation of the antioxidant potential, but also the study and suggestion of the antiradical mechanism of action of the target compounds. However, despite the broad methodological approaches discussed in this review, it is still impossible to determine the antioxidant mechanism of substances *in vitro*. Expensive and long-term experiments on cell cultures and animals are still required for antioxidant mechanism determination.

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Salmonella - foodborne pathogen and antimicrobial resistance

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Abstract. Foodborne diseases encompass a wide spectrum of illnesses and are a growing public health problem worldwide. They are caused by consumption of food or water contaminated by pathogenic (disease-causing) microorganisms such as bacteria, viruses and parasites. The contamination of food can occur at any stage in the process from food production to consumption ("farm to fork") and can result from environmental contamination (water, soil or air). They enter the body through the gastrointestinal tract where the first symptoms often occur like nausea, vomiting, stomach cramps, and diarrhoea. However, symptoms differ among the different types of foodborne diseases and the patient's immune status. Symptoms can sometimes be severe and some foodborne illnesses can even be fatal. Commonly recognized foodborne infections are: campylobacteriosis, *Escherichia coli* O157:H7 infection and haemolytic uremic syndrome (HUS), salmonellosis, cryptosporidiosis, listeriosis, giardiasis. norovirus infection, scombroid fish poisoning, shigellosis, toxoplasmosis, *Vibrio* infection and yersiniosis. One of the top three germs that cause illnesses from food eaten in EU is *Salmonella*.

1. Introduction

Nearly one in three foodborne outbreaks in the EU in 2018 was caused by *Salmonella*. Salmonellosis was the second most commonly reported gastrointestinal infection in humans in the EU (91,857 cases reported), after campylobacteriosis (246,571). *Salmonella* infections are an important public health problem worldwide and antibiotic resistance is one of the biggest public health challenges of our time [1].

1.1. Salmonella species and typing

Salmonellosis is an infectious disease of domestic and wild animals caused by gram-negative bacteria of the genus *Salmonella*. To date, over 2,400 different serotypes of this genus have been isolated from different vertebrate species, of which more than 200 have also been isolated in humans. A small number of serotypes are highly adapted to certain host species, causing severe septicemic forms of the disease. *Salmonellosis* is important from the aspect of food safety of animal origin due to its zoonotic character. In addition, there is also an impact of this infection on pig health and production economics. Within the EU countries, there is legislation that obliges member states to monitor *salmonellosis* in breeding stock

as well as fattening pig farms. *Salmonella* spp. are a group of bacteria which reside in the intestinal tract of humans and warm blooded animals and are capable of causing a disease. *Salmonella* are members of the *Enterobacteriaceae* family, and the genus *Salmonella* contains two species:

1. *Salmonella enterica*
2. *Salmonella bongori*

Salmonella enterica is one of the three most important agent of foodborne illness. This species is sub-classified into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica* of which *S. enterica* subsp. *enterica* is the most important for human health. The genus *Salmonella* can be subdivided into more than 2,400 serotypes. Different salmonella serotypes have different hosts. Some serotypes are pathogenic exclusively to humans such as *S*. Typhi and *S*. Paratyphi, while other serotypes (*S*. Typhimurium and *S*. Newport) infect different species. Non-typhoid salmonellosis in humans is usually caused by several dozen serotypes. Meanwhile, more and more serotypes are being isolated, especially from immunocompromised patients. A key factor that defines a particular salmonella serotype as a successful pathogen is its ability to enter a non-phagocytic host cell through certain molecular mechanisms, and to adapt to a wide range of hosts.

1.2. Transmission of Salmonella

Salmonella can be transmitted to humans in different ways. First, through a direct contact with infected persons or animals occurs, second, by eating food infected with the bacteria i.e. ingestion of contaminated food and third by eating raw or undercooked meat which is the most common way *Salmonella* is spread. Normally, *Salmonella* bacteria cause infections via fecal-oral transmission. Salmonella infection can be prevented by good hand hygiene and food handling procedures.

A significant moment of the bacterium of the genus Salmonella is its ability to survive outside the host. Research shows that in stored samples of feed, grass or dust, spiked with 10^6 to 10^8 *S*. Typhimurium per gram, survival times of one year are not uncommon and the survival up to four years is also observed [7], while in liquid manure, *S*. Typhimurium was re-isolated after 140 days at +10°C [8]. In field experiments, the survival times have not been quite that long, but are still at least weeks to months, depending on temperature and humidity. For example, *Salmonella* can survive in salt solution for several months, and over 80 days in dust. *Salmonella* can be present in rivers, wastewater, sewage and other waters and fertilizers [9].

1.3. Antimicrobial resistance

Antimicrobial drugs, including antibiotics, antifungals, antiparasitics and antivirals are compounds used to prevent and treat infections in humans, animals and plants. Microorganisms that develop antimicrobial resistance are sometimes referred to as superbugs [10]. Antimicrobial resistance occurs when bacteria, viruses, fungi and parasites change over time and no longer respond to antimicrobials, making infections harder to treat and increasing the risk of disease spread, severe illness and death. The largest number of isolated strains of *Salmonella* Enteritidis originates from humans and animals sensitive to most antibiotics, so the occurrence of resistance is very small. The mentioned causative agent is most often isolated from infected people, and it originates from poultry. Because poultry do not cause significant clinical symptoms, the infection is usually not treated with antibiotics, so there is no selective pressure and no development of resistance.

2. Methods

One hundred samples swabs pig carcass were collected in a pig slaughterhouse. *Salmonella* was isolated from the swabs and identified according to the international standard method [11]. First, pre-enrichment was in buffered peptone water (BPW), then selective enrichment was performed in Rappaport Vassiliadis Single Component Enrichment Broth (RVS) and Mueller Kauffman Tetrathionate Broth

(MKTTn). The isolation media used were Brilliant Green Agar (BG) and Xylose Lysine Decarboxylase Agar (XLD) [11].

Antibiotic resistance was determined using the disc diffusion method for each *Salmonella* isolate on Mueller-Hinton agar (OXOID, England). We pour the agar into a sterile glass or plastic petri dish on a flat surface to a uniform depth of 4 mm and left at 37°C overnight to check for sterility. From a pure bacterial culture (not more than 48 hours old except for slow growing organisms), we took individual *Salmonella* isolates with a wire loop and transferred colonies to 5 ml of Trypticase soy broth or 0.9% saline and incubated at 35°C for 4 h, then each isolate was compared with 0.5 McFarland turbidity standards. The cultures were inoculated onto the agar by streaking with the swab containing the inoculum. *Salmonella* isolates were tested for susceptibility to the following nine antibiotics: ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (30 μg), gentamycin (10 μg), nalidixic acid (30 μg), nalidixic acid(30 μg), trimethoprim/sulfamethoxazole, ceftazidime (30 μg), meropenem (10 μg) and tetracycline (30 μg), using the disk diffusion method according to guidelines set by the Clinical Laboratory Standards Institute (CLSI) [12]. Antibiotic impregnated discs were dispensed on the surface of cultures of Muller-Hinton agar and incubated at 35°C for 20 h.

3. Results

All *Salmonella* isolates tested were sensitive to five antimicrobial drugs (ceftazidime, ciprofloxacin, sulfamethoxazole-trimethoprim, meropenem and gentamicin) and were resistant to tetracycline. For all other tested antimicrobial drugs, high sensitivity of the tested *Salmonella* isolates was established. Resistance ranged from 7.41% for ampicillin and chloramphenicol, to 12.96% for nalidixic acid. (Table 1)

Table 1. Results of susceptibility testing of *Salmonella* to antimicrobial drugs obtained by the disk diffusion method

Antimicrobial drugs	Sensitive isolates %	Resistant isolates %
Ampicillin (10 µg)	92.60	7.41
Chloramphenicol (30 µg)	92.60	7.41
Ciprofloxacin (30 µg)	100	0
Gentamycin (10 µg)	100	0
Nalidixic acid (30 µg)	87.04	12.96
Trimethoprim/**S**ulfamethoxazole	100	0
Ceftazidime (30 µg)	100	0
Meropenem (10 µg)	100	0
Tetracycline (30 µg)	0	100

4. Conclusion

To date, various antibiotics have been useful in both human and veterinary medicine. Examples of such antibiotics are gentamicin, ampicillin and amoxicillin. Some antimicrobial drugs, such as enrofloxacin and flumequine, have only been developed for veterinary use. The use of antibiotics as growth promoters in intensive pig and poultry farming has influenced the development of resistance in some bacteria. For these reasons, since January 2006, EC Regulation No. 1831/2003 has banned the use of all antimicrobial drugs as feed additives.

Pathogens that are antibiotic resistant and that are in food could spread through the food chain to humans, in whom they can cause infections. The determined resistance rates of *Salmonella* to the antibiotics studies are in line with the findings of other authors [13]. Of the antimicrobial drugs tested, tetracyclines and ampicillin are particularly commonly used in veterinary clinical practice, while ciprofloxacin is very commonly used in human medicine, and another fluoroquinolone drug not studied, enrofloxacin, has

been developed specifically for veterinary use. This is especially important because it is known that resistance to one antibiotic from a group can sometimes impart resistance to an entire group of antibiotics.

In order to reduce the number of resistant pathogenic microorganisms, farmers, veterinarians, meat processors, doctors and government agencies, all the way to consumers who should be educated, should work together to prevent improper and excessive use of antimicrobial drugs. In primary production, the principles of precondition programs should be applied, which imply the application of GHP and GMP, (good hygiene and good manufacturing practices). This will prevent or reduce the risk of disease in animals, and thus the need for antimicrobial therapy. If therapy is still necessary, it must be applied only in the correct way and under the supervision of a veterinarian. In slaughterhouses and meat processing facilities, the possibility of contamination of meat with the contents of the digestive tract or resistant zoonotic pathogens can be significantly reduced by applying precondition programs and the HACCP concept.

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Pathogens that are antibiotic resistant and that are in food could spread through the food chain to humans, in whom they can cause infections. The determined resistance rates of *Salmonella* to the antibiotics studies are in line with the findings of other authors [13]. Of the antimicrobial drugs tested, tetracyclines and ampicillin are particularly commonly used in veterinary clinical practice, while ciprofloxacin is very commonly used in human medicine, and another fluoroquinolone drug not studied, enrofloxacin, has

been developed specifically for veterinary use. This is especially important because it is known that resistance to one antibiotic from a group can sometimes impart resistance to an entire group of antibiotics.

In order to reduce the number of resistant pathogenic microorganisms, farmers, veterinarians, meat processors, doctors and government agencies, all the way to consumers who should be educated, should work together to prevent improper and excessive use of antimicrobial drugs. In primary production, the principles of precondition programs should be applied, which imply the application of GHP and GMP, (good hygiene and good manufacturing practices). This will prevent or reduce the risk of disease in animals, and thus the need for antimicrobial therapy. If therapy is still necessary, it must be applied only in the correct way and under the supervision of a veterinarian. In slaughterhouses and meat processing facilities, the possibility of contamination of meat with the contents of the digestive tract or resistant zoonotic pathogens can be significantly reduced by applying precondition programs and the HACCP concept.

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Controversy about goods classification: the use of terms and their definitions according to FEACN of the CU

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Controversy about goods classification: the use of terms and their definitions according to FEACN of the CU

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Abstract. The paper considers the problematic terminological questions that arise among declarants and customs officials when classifying goods. Disadvantages of receiving a classification decision are described. The necessity to harmonize the terms “raw fat”, “speck” and “animal rendered fat” in the FEACN of the CU is shown.

1. Introduction

One of the key questions leading to controversy between the customs authorities and participants in foreign economic activity is goods classification according to the Foreign Economic Activity Commodity Nomenclature of the Customs Union (hereinafter referred to as the FEACN of the CU). This trend is quite explicable, as the classification code determines both the rate of customs duties and adherence to prohibitions and restrictions regarding each particular category of foods.

As specified in the provisions of the Customs Code of the Customs Union (hereinafter referred to as the CC of the CU) (paragraph 1, article 20), a declarant and other persons classify goods on their own according to the FEACN of the CU in the customs declaration procedure [1,2]. Control of the correctness of goods classification is carried out by the customs authorities.

Customs statistics data show that more than half of the unreliable declarations are linked with the peculiarities of filling in column 31, as the information about declared goods necessary for assigning to a 10-digit classification code is indicated there (Figure 1). When filling in column 31, declarants face problems with regard to the lack of harmonization in terminology used in normative documents of different levels, ambiguity of several terms/definitions or their absence [3,4].

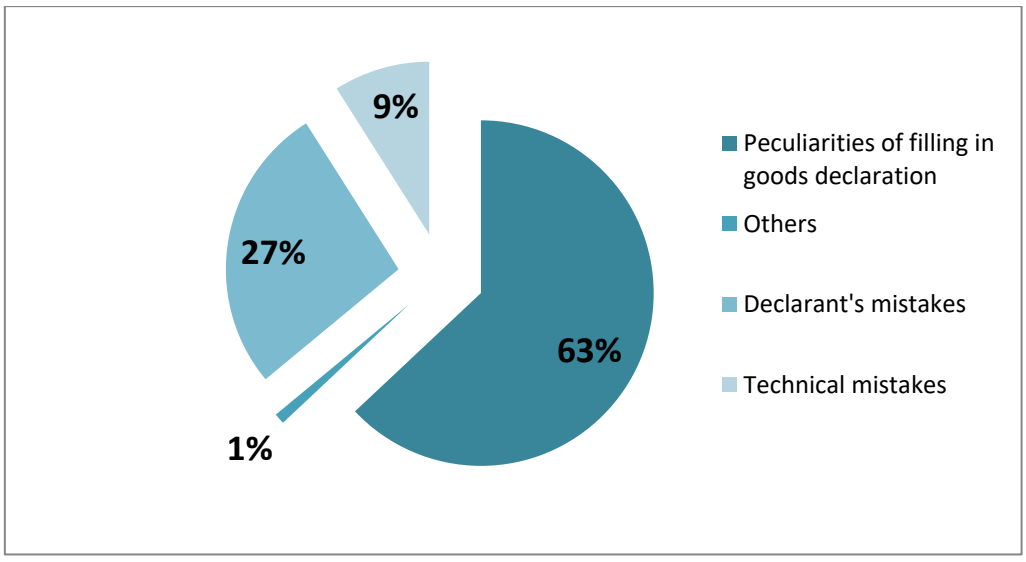

Figure 1. Main mistakes made when filling in goods declarations
*(*Analysis of the declaration array for 12 months of 2020)*

Not infrequently in customs processing of goods, the customs office does not accept a FEACN code chosen by a declarant and insists on using another code that, as a rule, leads to a higher duty rate. In these cases, the practical activities in the sphere of customs procedures indicate that preliminary decisions (also known as customs classification decisions) can help to increase the reliability of declarations. Customs authorities can classify goods upon application before their customs declaration by making preliminary decisions for classification of commodities according to the FEACN of the CU (CC of the CU, paragraph 1, article 21) [1].

Meanwhile, preliminary classification decisions made in the Russian Federation will not be applied in other EAEU countries. Consequently, none of the Union countries is interested in a unified approach to classification of the same goods because, according to CC of the CU (paragraph 4, article 21) [5], preliminary decisions for classification of commodities are obligatory only when declaring goods on the territory of a Union member state, whose customs authority made preliminary decisions.

With that, about two months are necessary to prepare technical documentation required by customs authorities and a minimum of 2-3 months are needed to formalize classification decisions. At the same time, it is unprofitable to organize storage in temporary storage warehouses (TSW) due to the long period of formalization [6]. Moreover, meat product transportation and storage are complicated by the necessity to maintain a temperature regime, which is rather difficult to control in TSW facilities. This situation, to a large extent, complicates both assignment of a certain code to goods by participants in foreign economic activity and identification at customs control and verification of reliability of a claimed code by customs officials. Today, therefore, detection and analysis of existing problems as well as determination of ways to solve these problems are relevant in customs affairs.

The analysis of judicial practice materials regarding disputes over decisions about goods classification for customs purposes, which were caused by the absence of terms in the FEACN of the CU or their varying interpretations, partly revealed some problematic terms, for which judicial practice negative for customs authorities became more frequent. When analysing case materials, the following terms were identified: meat, meat trimmings, fatty tissue, animal fat, beef fat, raw fat and speck. In the FEACN of the CU, there are no clear definitions of terms “fatty tissue” and “raw fat”, while the definitions of “beef fat” and “meat” are totally absent. It can be noted additionally that the 67th Session on amendments to the Harmonized Commodity Description and Coding System of the World Customs Organization held from 12 to 30 April, 2021 considered a suggestion to exclude “fat trimmings of boned meat with chemical leanness of meat less than 20%” from commodity group 02, as notes to this term are not envisaged in group 02.

After fatty tissue separation from a carcass, fat can contain small pieces of muscle tissue, while meat can contain part of the animal’s fatty tissue. These items are classified in FEACN of the CU in different groups that significantly differ from each other by rates of import customs duties. According to the general provisions of group 02 in the FEACN of the CU, “animal fat presented separately” is not included (group 15) (excluding pork fat separated from lean meat…); however, animal fat contained in carcasses or in meat is considered a meat constituent. Therefore, animal fat contained in carcasses or in meat is a meat constituent and is subjected to classification in commodity items of group 02, depending on the processing method. “Rendered pork fat” is classified in group 15 of the FEACN of the CU.

It is profitable for a declarant to classify fatty tissue in group 15 of the FEACN of the CU (where a rate of 5-10% is applied depending on a purpose) and pork fatty tissue in item 0209 (the rate is 15%, but no less than 0.15 euro/kg) [7]. Significantly higher rates of import customs duties were established, and different prohibitions and restrictions are in force, for meat classified in group 02 of the FEACN of the CU.

When analysing court disputes on the above indicated terms, customs authorities cancelled the classification code in all cases and established codes with much higher import customs duties, that is, they suggested classification as meat (Table 1).

Table 1. Problematic terms in classification

On the part of participants in FEA →	code of FEACN of the CU		← On the part of customs authorities
"Frozen beef fat…'	1502 90 900 0; rate of import customs duty – 10%	0202 30 900 8; rate of import customs duty – 50%, but no less than 0.15 euro/kg	"Frozen beef…"
"Frozen beef subcutaneous raw fat…"	1502 90 900 0; rate of import customs duty – 10%	0202 30 900 4; rate of import customs duty – 15%	"Frozen beef …"
"Frozen pork mid-back fat, free of lean meat…"	0209 10 110 0; rate of import customs duty – 15%	0203 29 550 9; rate of import customs duty – 65%	"Fresh, chilled or frozen pork…"

An example of such situation is the judicial case of the Constanta company [8]. The company imported to the Customs Union territory and declared a commodity "pork mid-back fat (speck) separated from lean meat in boxes on pallets, producer – Chili". For this, the commodity code of FEACN of the CU was 0209101100, which corresponded to the rate of import customs duty of 15% and VAT of 10%. However, during customs processing, the customs authority made a decision about classification, according to which the commodity under dispute was assigned to sub-subitem 0203295509 "fresh, chilled or frozen pork", with the rate of import customs duty of 65%.

Dissatisfied with the conclusions of the customs expert, the declarant turned to the V.M. Gorbatov Research Center for Food Systems for commodity identification.

Answering the declarant's question about determination of the commodity name (to what meat raw material type/product type the commodity samples should be assigned), the experts noted in their opinion that according to GOST R 52427-2005 "Meat industry. Food products. Terms and definitions" the term "subcutaneous fat" means raw fat in the form of fatty tissue deposits taken from the external carcass part upon its cutting. Subcutaneous pork fat is called speck.

According to GOST R 54704-2011 "Frozen meat blocks. General specifications", back fat has a mass fraction of muscle tissue no more than 5% and mid-back fat no more than 10%. According to GOST R 55485-2013 "Fat products. Specifications", back fat is a speck taken from the whole length of a pork half-carcass from the last cervical vertebra to the first caudal vertebra over one-third of the rib length. Mid-back fat is taken from two-thirds of the bottom part of a pork carcass. The standard envisages the use of speck with the content of muscle tissue of up to 50% when producing products from speck.

Therefore, all presented samples were pork subcutaneous fat (speck) removed from different parts of carcasses and the customs authority had no grounds to assign this commodity to sub-subitem 0203. There are also many disagreements between customs authorities and declarants due to the absence in the explanatory notes for group 15 of the clear definition of "animal rendered fat"; although, it is implied that this commodity is classified exactly in this group. Instead, the term "lard" is used, which is not enshrined in the Technical Regulation of the Customs Union TR CU 034/2013 "On meat and meat product safety" and other normative documents, and has no description in the FEACN group.

Therefore, reasons for court disputes that arise between the participants in foreign economic activity and customs officers are terminological problems as well as the absence in the FEACN of the CU of identification attributes necessary for correct classification of fatty tissue from slaughtered animals.

At the first stage, the problems of ensuring reliability of the declared code in repeated cases would be solved if preliminary decisions made by customs authorities of a EAEU member state become obligatory for all participant states.

To solve problems at the second stage, it is necessary to harmonize terminology used in the FEACN of the CU and its explanatory notes and in the Russian normative documentation and create clear definitions of such terms as raw fat, fatty tissue, animal fat, meat, meat trimmings, animal rendered fat, speck.

These measures will make identification of the described commodities easier, accelerate the whole process of customs processing and minimize expenses associated with expenditure on judicial trials for all participants in foreign economic activity.

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Stress survival islets contribute to clonal and serotype-specific differences in *L. monocytogenes*

Stress survival islets contribute to clonal and serotype-specific differences in *L. monocytogenes*

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Abstract. *Listeria monocytogenes* is an important opportunistic foodborne pathogen causing listeriosis, an often fatal infection leading to meningitis, sepsis, or infection of the fetus and abortion in susceptible individuals. Diverse ready-to-eat food (RTE) like dairy, meat, fish, vegetables, and complex foods are often linked with listeriosis outbreaks. *L. monocytogenes* is capable of surviving in stressful environmental conditions and grow in refrigerated foods. Regarding stress-related genes, SSI-1 contributes to the survival of cells under suboptimal conditions, such as high salt content and acidic environment. At the same time, SSI-2 is responsible for persistence under alkaline and oxidative stresses.

1. Introduction

Listeria monocytogenes is an extremely diverse species; its population structure is divided into 14 serotypes (including hypervirulent serovar 4) and four phylogenetic lineages (I, II, III, and IV) that have been classified into multiple clonal complexes (CCs) and sequence types (ST) [1,2]. *L. monocytogenes* CCs include: (i) infection-associated isolates, which belong to lineage I and are considerably connected with clinical origins and non-food contact surfaces (including CC1, CC2, CC4, and CC6), (ii) food-associated isolates, which belong to lineage II and are mainly within the production environment and associated with food contact surfaces (including CC9 and CC121), and (iii) intermediate-associated isolates that are isolated from both clinical samples and food [3-5]. Lineages III and IV are usually detected less frequently, showing exceptional biodiversity, and are primarily isolated in animals [6]. *L. monocytogenes* belongs to the *Listeria* genus encompassing 17 species, out of which 11 have been reported in the last 13 years (*L. marthii*, *L. rocourtiae*, *L. weihenstephanensis*, *L. grandensis*, *L. riparia*, *L. booriae*, *L. fleischmannii*, *L. floridensis*, *L. aquatica*, *L. newyorkensis*, and *L. cornellensis*) [7].

Listeriosis is a relatively rare disease but is responsible for high mortality rate in elderly and immunocompromised persons, pregnant women and infants. Infection usually occurs by eating food contaminated with high numbers of *L. monocytogenes* [8]. Diverse ready-to-eat food such as dairy, meat, fish, vegetables, and complex foods are often linked with listeriosis outbreaks [9].

In 2018, a total of 2,459 cases of listeriosis were reported in the European Union, with a hospitalization rate of 97.0% and a fatality rate of 15.6% [10].

2. Stress responses of *L. monocytogenes*

Foodborne transmission of this bacterium is primarily influenced by the ability of *L. monocytogenes* to survive and replicate under a broad range of environmental stress conditions. However, there are differences among different lineages of *L. monocytogenes.* In his study, Hingston et al. [11] concluded that serotypes 1/2a and 1/2b were averagely more cold-resistant compared to serotypes 4b and 1/2c. Subsequent cold growth studies confirmed serotype 1/2a strains as more cold-resistant than serotype 4b strains [12,13]. Furthermore, lineage I strains have been proved to be more salt-tolerant than lineage II strains [14] and serotype 4b strains to be more salt-tolerant than serotype 1/2a and 1/2b [14,15]. Also, lineage I isolates tolerate acid stress conditions significantly better than lineage II isolates [11].

Implementation of cleaning-in-place procedures in food-processing facilities where excesive amounts of oxidizing agents like hydrogen peroxide, chlorine dioxide, peracetic acid, and sodium hypochlorite are used causes alkaline and oxidative stresses for *L. monocytogenes* [16,17]. Manso et al. [18] found that *L. monocytogenes* strains belonging to lineage I (ST5, ST6, ST87, and ST1) are more resistant to oxidative stress than lineage II (ST7, ST9, ST199, and ST321). According to that, this pathogen contains stress-related genes such as stress survival islets SSI-1 and SSI-2.

2.1. Stress survival islets SSI-1 and SSI-2

SSI-1 is an islet comprising five genes that regulate the growth of *L. monocytogenes* under sub-optimal conditions [19]. These include but are not limited to tolerance to acidic, osmotic, gastric, and bile stress. The islet activity enables pathogen survival in food and enhances pathogenicity in the human host [19]. SSI-1 islet encompass following genes: *lmo0444*, *lmo0445*, *pva (lmo0446)*, *gadD1 (lmo0447)* and *gadT1 (lmo0448)* [19].

This islet is a characteristic of both ST-7 (CC7) and ST-8 (CC8) strains linked with persistence in a study of *L. monocytogenes* strains isolated over twenty years ago from food-processing plants. However, this islet can occasionally be found in some sporadic strains isolated from the food establishments [20]. Besides these two CCs, literature data indicate that SSI-1 is also present in *L. monocytogenes* ST3, ST5, ST7, ST9, ST14, ST36, ST199, ST204, ST226, ST296, ST321, ST375, ST379, ST489, ST739, and ST1041 [21,22,23,24]. Zang et al [25] found that SSI-1 was present in both lineages (Ic, Id, IIe, IIg, IIi, IIj, and IIk). The common predecessor of subgroups Ic and Id may have acquired SSI-1. The same acquisition occurred in subgroups IIi, IIj, and IIk. On the contrary, the predecessors of subgroups IIe and IIg obtained SSI-1 independently. Genome sequencing revealed that upstream of SSI-1 islet, there is a respective gene encoding transcriptional regulator protein [25]. Further research studies proved that isolates belonging to serotype 1/2b, the majority of which carried SSI-1 (such as CC3 and CC5), were found to create the strongest biofilms. In contrast, isolates belonging to serotype 4b, most of which did not harbor SSI-1 (such as CC2 and CC6), created the weakest biofilms [26,27]. In the study conducted by Arguedas-Villa et al. [28], the growth of *L. monocytogenes* strains isolated in Switzerland and Canada that harboured SSI-1 was not enhanced in cold environment stress conditions.

SSI-1 offers a wider spectrum of adaption than SSI-2. The SSI-2 islet involves two genes: the transcription factor gene *lin0464* and the PfpI protease gene *lin0465*. On the contrary to SSI-1, the SSI-2 islet confers increased survival during alkaline, and oxidative stress conditions frequently met in food processing environments [17]. SSI-2 is a feature of most *L. monocytogenes* ST121 strains (lineage II), which are the most prevalent clones isolated from food or food processing environments. Additionally, the mutation rate of the SSI-2 islet is extremely low, resulting in almost 100% nucleotide identity shared among various ST121 strains [29,30]. Interestingly, SSI-2 positive *L. monocytogenes* strains are detected in lineage I and III and in *L. innocua* but with slightly shorter islets [17,31]. Although *L. monocytogenes* and *L. innocua* have the highest phylogenetic similarity compared to other members of *Listeria* genus and share the same ecological niches, it has been hypothesized that *L. monocytogenes* obtained SSI-2 islet through the horizontal gene transfer event from *L. innocua* [32].

Most *L. monocytogenes* isolates contain one of the two known stress survival islets, SSI-1 or SSI-2, and/or plasmids carrying genes associated with resistance to stress conditions, heavy metals, or biocides

[3]. These islets and resistance-associated plasmids could be responsible for the survival and development of *L. monocytogenes* under the harsh conditions prevailing in food processing plants [3].

Recent investigations highlight whole-genome sequencing (WGS) to be an affordable, fast, and powerful tool for identifying diverse genetic markers associated with stress (SSI-1 and SSI-2), virulence, antimicrobial resistance, and heavy metal resistance. Also, WGS has been used in a few national studies for *Listeria* outbreak detection and investigations, e.g., in Austria [34,35], Australia [36], the United States [37], Denmark [38], and France [39]. Interestingly, the presence of SSI-1 could provoke a prolonged outbreak associated with the *L. monocytogenes*. RTE salmon products were the likely source of this multi-country outbreak affecting five EU countries: Denmark, Estonia, Finland, France, and Sweden [40].

3. Conclusion

L. monocytogenes is considered to be one of the several important foodborne pathogens transmitted to humans via contaminated food. It can survive under suboptimal conditions (high salt, low pH, and alkaline and oxidative stress) commonly present in food processing environments due to recently identified stress resistance markers SSI-1 and SSI-2. When it comes to the prevalence of SSI's in isolates, there is clear evidence that SSI-1 is equally circulating among infection-associated isolates and food-associated isolates. At the same time, SSI-2 islet is mainly found in food-associated ST121 strains (CC121, lineage II), indicating genetic adaptation and resistance to alkaline and oxidative stresses.

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Spore-forming bacteria in the dairy chain

Spore-forming bacteria in the dairy chain

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Abstract. Spore-forming bacteria form the most diverse and most complex group of bacteria in terms of their elimination from the dairy chain, due to their ability to form highly resistant spores. As ubiquitous microorganisms, spore-formers can enter the product along the milk-processing continuum from different sources, and subsequently cause spoilage in various types of dairy products. The most important classes of spore-forming bacteria relevant to the dairy industry are Bacilli and Clostridia. Bacilli are responsible mainly for the spoilage and decreased shelf-life of fluid milk, while Clostridia cause late gas blowing in cheese. Spore-forming microorganisms contaminate raw milk primarily at the farm level, with potential for recontamination to occur at various points along the dairy production continuum. The most effective measure in reducing spore load at the farm level is adequate pre-milking teat preparation, while at the dairy plant level, bactofugation and microfiltration are applied. Understanding the ecology of spore-formers can improve application of systematic approaches for controlling the spoilage bacteria in dairy processing systems. Also, novel technologies, such as high-pressure processing, ultrasound treatment, irradiation etc., could provide the dairy industry with the powerful tools to eliminate these bacteria from the dairy chain.

1. Introduction

In recent years food loss and food waste are becoming more relevant global issues. In the European Union, around 88 million tons of food are wasted annually, with an estimated associated cost of €143 billion [1]. Together with retail overstocking and discarding products, microbial spoilage is an important cause of dairy food waste [2].

Among spoilage microorganisms important for the dairy industry, spore-forming bacteria are the most diverse and most complex to eliminate from the dairy chain, mainly due to their ability to transform to dormant state – spores [3, 4]. Spores can survive harsh environmental conditions, such as nutrient deficit, osmotic pressure and temperature deviations, owing to their multilayer structure [5]. While the outermost layer protects the spore from enzymatic attacks, inner layers maintain a dehydrated state and provide additional protection against chemicals [4]. Once the environmental conditions are favourable i.e., primarily when specific nutrients (amino acids, sugars, and purine nucleosides), or some non-nutrient factors become available (calcium dipicolinate, alkylamines, high pressure, heat activation) spores can germinate into a vegetative state [6].

There are many ways to subdivide spore-forming bacteria relevant to the dairy industry: based on their taxonomy, metabolic traits, and ability to grow at different temperatures, or in the presence of oxygen [7]. Taxonomically, they are all members of phylum Firmicutes, which consists of five classes: Bacilli, Clostridia, Erysipelotrichia, Negativicutes and Thermolithobacteria [8, 9]. The most important classes of spore-forming bacteria relevant to the dairy industry are Bacilli and Clostridia [10]. The members of *Bacillus* and related genera and *Clostridium* spp. are ubiquitous in nature, can enter the product along the milk-processing continuum from different sources, subsequently grow at refrigeration temperatures, and significantly affect product safety and quality.

2. Spore-forming bacteria relevant for milk and dairy product quality

Pasteurized milk can be contaminated with spoilage microorganisms via two main pathways: recontamination after pasteurization, mainly with Gram-negative bacteria such as *Pseudomonas* [11], and contamination at the farm level with psychrotolerant spore-forming bacteria that survive pasteurization and subsequently cause spoilage during the refrigeration storage [12]. *Bacillus* spp. and *Paenibacillus* spp. are the main spore-forming psychrotolerant spoilage microorganisms found in pasteurized milk [13, 14, 15].

Genus *Bacillus* is the oldest and most diverse genus of bacteria [10]. Species within this genus have a wide range of physiological characteristics, with some strains able to tolerate extreme temperatures, pH and the presence of salt [16]. The expansive physiology of *Bacillus* spp., has allowed its members to colonize almost all natural habitats, such as soil, air, lake sediments and even extreme environments [10]. As *Bacillus* spp. can be isolated from soil (which was until recently considered as their main habitat) and environments contaminated with soil, their presence on dairy farms is inevitable [16]. It is noteworthy that many *Bacillus* spp. can produce thermotolerant lipolytic enzymes that can lead to milk spoilage. These enzymes have optimal temperatures between 60 and 75°C, which are similar to the temperatures used for thermization and pasteurization in the dairy industry [17]. Also, *Bacillus weihenstephanensis*, a member of the *Bacillus cereus* group, can cause the product defect known as sweet curdling [10].

As traditional culture-based methods could not discriminate between these two genera, *Paenibacillus* spp. were formerly assigned to the genus *Bacillus* [3]. Clear distinction can be made regarding growth dynamics of these two genera during pasteurized milk's shelf-life. While *Bacillus* spp. predominate early in the shelf-life of pasteurized milk (1-10 days post-pasteurization), *Paenibacillus* spp. predominate at the end of shelf-life (14-21 days post-pasteurization) [13, 15, 18]. Although *Paenibacillus* spp. are present at very low numbers in raw milk, they can reach more than 10^6 CFU/ml at the end of the shelf-life of pasteurized milk [3]. This suggests that *Paenibacillus* spp. are better able to grow in milk at 6°C whereas most *Bacillus* spp. cannot, with *Bacillus weihenstephanensis* as the most notable exception [19, 20].

Spore-forming bacteria can also survive thermal treatments during extended shelf-life (ESL) milk production processing [21]. Although there are no international standards that define the thermal treatment necessary for ESL milk production, applied thermal treatments are usually in range 125-130°C for 2-6s [22]. During the production process of ESL milk, all vegetative cells are inactivated, but nevertheless, some spores can survive. *Bacillus* spp. can be present as a spore-forming spoilage microorganism in ESL milk, but *Paenibacillus* spp. emerged as a spoilage microorganism particularly attributed to ESL milk [23]. Rysstad and Kolstad [24] recommended that ESL milk should be stored at ≤6 °C, under which conditions, *Paenibacillus* spp. growth is limited. Furthermore, some spores of *Paenibacillus* spp. can survive temperatures as high as 130°C [25].

Ultra-High Temperature (UHT) milk is a "commercially sterile" product, which is intended to be stored at ambient temperatures for prolonged times. However, some high-heat resistant spores can survive UHT treatment. Subsequent spoilage can occur, especially when the product is stored at temperatures above 40°C. Spores isolated from UHT milk usually belong to *Bacillus sporothermodurans* and *Geobacillus stearothermopillus* [23]. Although these bacteria are present in raw milk at very low counts, heat treatment during UHT milk production favours their germination, outgrowth and subsequent vegetative growth [26]. As some strains are proteolytic, they can cause spoilage of the final product [27]. Other *Bacillus* species and *Paenibacillus lactis* can also occasionally cause UHT milk spoilage [28].

Unlike fluid milk products where aerobic spore-formers predominate as sporogenic spoilage microorganisms, spoilage of cheese is usually attributed to anaerobic spore-formers. The main cheese defect attributed to spore-forming bacteria is late gas blowing [10], usually seen in Dutch or Swiss type cheeses such as Gouda, Comté, Emmental and Beufort [29]. Late gas blowing is characterized by slits, cracks and irregular eyes in cheese, due to excessive production of gas [7]. Bacteria causing late gas defect are referred as butyric acid bacteria, due to their ability to ferment lactate to acetate, butyrate and hydrogen gas [30]. They all belong to *Clostridium* spp. and include the species *Clostridium butyricum,*

Clostridium tyrobutiricum and *Clostridium beijerinckii*. Butyric acid bacteria reach raw milk through faecal contamination, when cows are fed with poor quality silage that has undergone aerobic deterioration [7]. As acidification in such silage is not sufficient, spores can germinate and grow [31], and subsequently contaminate raw milk. During the aging of cheese, clostridial spores can germinate and grow, as long as lactate is available as a substrate, and environmental conditions (salt concentration) are not limiting for their growth [32]. Even though it is not considered as a butyric acid bacterium, *Clostridium sporogenes* can also cause late gas defects in cheese, owing to proteolytic activity and subsequent gas formation in the anaerobic cheese environment [29].

Aerobic spore-formers *Bacillus polymyxa* and *Bacillus macerans* were also associated with spoilage of Argentinian cheeses [33]. Spoilage of some cheeses was also associated with the presence of *Bacillus* spp. in raw milk and their survival of heat treatment, or post-pasteurization contamination of the product [10].

3. Sources of milk contamination with spore-formers

The literature data indicate that *Paenibacillus* and *Bacillus* spp. are commonly present in raw milk. These microorganisms contaminate raw milk primarily at the farm level (e.g., during milking, raw milk storage and handling on the farm) with potential for recontamination to occur at various points along the dairy production continuum from farm to final product (during transport or at the processing plant). Cow's teats are considered as a major source of spores in raw milk [34], mainly due to contamination from bedding, feed and dust [35, 36, 37, 38, 39]. The members of the genus *Bacillus* are ubiquitous and have been isolated from dairy farm environments, including silage, pasture, soil, bedding material, faces, water and feed [36]. Moreover, a study by Huck et al. [14] has found evidence for the persistence of selected *Paenibacillus* and *Bacillus* subtypes in processing plants. Thus, a recontamination cycle is re-established.

At the farm level, the most common sources of raw milk contamination with *Paenibacillus* spp. are silage and feed concentrate for dairy cows [40].

The resilience of clostridial spores in the dairy farm environment can be explained by the concept of "the clostridial spore contamination cycle", proposed by Pahlow et al. [31]. Crops used for silage become contaminated with clostridial spores through soil and manure when it is used as fertilizer. If the conditions during the silage fermentation are favourable, spores germinate to vegetative cells, and consequently, microbial loads in silage increase. After being ingested by cows, spores pass through animals' gastrointestinal tract, survive gastrointestinal transit, and are excreted with faces. Through faecal contamination of teats, spores reach raw milk. Also, as manure is usually used as an organic fertilizer, crops are once again being contaminated with spores.

Molecular subtyping methods used for differentiation of subtypes within a bacterial species can serve as sensitive tool for tracking contamination sources of spore-forming bacteria in dairy-related environments [41]. Specifically, sequences of *rpoB* gene, encoding for ß subunit of RNA, are used to differentiate strains of spore-forming bacteria through the entire dairy system. Huck et al [12, 14] used this method to determine the relatedness of spore-forming bacteria in different dairy production chain environments (bulk tank milk, trunks, packed products). Some of the allelic types present in packed product were found at the farm level, indicating that those spore-forming bacteria originate from farm and survive pasteurization. Others were present only in the packed product, meaning that contamination, or recontamination occurred at the processing plant. By applying *rpoB* sequencing, Miller et al. [42] determined that spore-forming isolates originating from raw milk and dairy powder samples are significantly different, and therefore, dairy powder producers should focus on reducing spore-forming bacteria not only at the farm level, but also in the processing plant.

4. Strategies to eliminate spore-formers in the dairy chain

Heat treatment is the most widely used processing technology in the dairy industry in order to reduce bacterial counts in milk. However, as spores can survive thermal treatments, the most effective measures for reduction of the spore count should be applied at the farm level [34].

As spore-formers most likely originate from dirt and faeces attached to teats at the time of milking, the most effective measure in reducing spore load is adequate pre-milking teat preparation [34]. Cleaning teats before milking with a moist washable towel and following with drying with a paper towel can reduce spore counts in milk by up to 96% [36]. Special attention should be paid to cleaning of teats during extremely dry or wet weather, when larger amounts of dirt and soil can contaminate teats [34]. Education of employees to properly clean teats, and establishing cleaning protocols for towels (washing with detergent and chlorine bleach, and fully drying towels), can also result in decrease of spores in raw milk [43]. Milking parlours should be washed regularly, but not while the cows are present on the platform [34].

In order to eliminate spore-formers at the dairy plant level, bactofugation and microfiltration are applied. Bactofugation uses high-speed centrifugation in order to separate milk components of different densities [41]. By using this method, between 90 and 98% of spores can be eliminated from milk. This technology was first applied to remove *Clostridium tyrobutiricum* in order to prevent late gas blowing of cheese. Although it is not a common practice, *Paenibacillus* spp. spores can also be eliminated from raw milk intended for high temperature-short time pasteurization by bactofugation. Microfiltration uses semi-permeable membranes in order to separate bacteria and milk components based on the size of the particles. Microfiltration has higher efficiency in spore removal compared to bactofugation, with 99.1% to 99.99% of spores successfully removed from raw milk [44].

The induction of spore germination before a heat treatment could be an efficient method to eliminate spore-formers from milk and ensure product stability. Spores can be triggered to germinate by various nutritive factors (amino acids, sugars, ribosides and potassium ions), surfactants, or physical treatments (mostly hydrostatic pressure) [45]. Once they germinate into a vegetative state, they are easy to eliminate [46]. Thus, the induction of spore germination, followed by inactivation of spores by thermal, or other, processing technologies can be a strategy for control of these bacteria in milk and dairy products.

5. Conclusion

Spore-forming bacteria are an important group of spoilage microbiota in dairy products. Spores are ubiquitous in nature, and consequently, there are multiple points of entry and multiplication for these microorganisms in dairy systems. With reliable tracking methods, we will be able to understand the ecology of spore-formers and successfully integrate a systematic approach for controlling the spoilage bacteria in dairy processing systems. Also, novel technologies, such as high-pressure processing, ultrasound treatment, irradiation etc., could provide the dairy industry with the powerful tools to eliminate these bacteria from the dairy chain.

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Dressing percentage and meat yield of Hybro G+ provenance broilers

Dressing percentage and meat yield of Hybro G+ provenance broilers

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Abstract. The goal of this paper was to examine the slaughter characteristics of meat from Hybro G+ provenance broilers. Characteristics studied were dressing percentage, breast, leg (thigh and drumstick) and abdominal fat in chilled carcass, and percentage of meat, bones and skin in breast and leg. In this trial, one-day broilers of Hybro G+ provenance were raised under the same zoohygienic and ambient conditions that met the technological requirements for this provenance and were provided with feed and water *ad libitum*. After 42 days' fattening, broilers were slaughtered and the resultant carcasses were air chilled. The average dressing percentage was 73.15%, and in carcasses, the average percentage of breast was 34.33%, leg was 27.91% and abdominal fat was 1.13%. The percentage in breast and leg of meat was 72.61% and 70.38%, of skin was 8.00% and 9.45% and of bones was 19.79% and 19.59%, respectively. The meat:bone ratio was 0.27 for breast and 0.28 for leg.

1. Introduction

In the past decades broiler meat production accounted for 80% of total poultry meat production and underwent the greatest increase of any domestic animal. According to estimates, production and consumption of broiler meat continue to increase due to the birds' good food conversion ratio compared with other animal species, there being no negative cultural and religious aspects (meat is accepted from all nations and religions), the good proportion between meat and fat, attractive meat sensory properties, the animals' quick reproductive cycle and their low price and fast fattening. Domestic broilers are one of the main protein sources for humans and broiler meat has become a major consumer product in many countries [1], whereas white feather broilers with a fast growth rate account for a major portion of global broiler production. Such exponential growth of broiler production is a result of selective breeding, efficient production systems (floor, cage and free-range rearing), improved diet and veterinary care [2].

The main criteria used to evaluate the slaughter value of broilers are dressing percentage, percentages of culinary cuts and muscle content. Carcass composition is also important, as high muscle content, particularly in breast and leg meat, and low fat content are desirable characteristics for consumers. Genotype, feed and environmental conditions are the key factors affecting the carcass and meat quality of broilers. Breast and leg cuts possess great value in the food industry and in households and their external features, nutritional profile and chemical properties play a main role [3].

Dressing percentage depends on *pre mortem* and *post mortem* factors. The main *pre mortem* factors are genetic background, breeding, density, feed, age, sex, transport method and fasting before slaughter [4]. *Post mortem* factors include technological processing of carcasses, chilling method [5] and stunning

method [6]. Genetic background is one of the main factors that influence dressing percentage. That could explain differences between dressing percentage of different provenances.

The goal of this paper was to examine the slaughter characteristics of meat from Hybro G+ provenance broilers; dressing percentage, percentage of breast, leg (thigh and drumstick) and abdominal fat in chilled carcass, and percentage of meat, bones and skin in breast and leg.

2. Materials and methods

In this trial, one-day-old Hybro G+ provenance broilers were used. They were raised under the same zoohygienic and ambient conditions that met the technological normative for this provenance [7]. Feed and water were provided *ad libitum*. The composition of feed mixtures is presented in Table 1.

The proximate composition of feed mixtures was determined according to standard methods [8], and is shown in Table 2. Fattening lasted for 42 days, after which, broilers were slaughtered and carcasses were air chilled to 3°C. After chilling, carcases were butchered into main cuts and samples of leg, breast, and abdominal fat were taken. Meat, skin and bones were separated. Cuts and tissues were measured to an accuracy of 10^{-3}.

Table 1. Composition of feed mixtures

	Pre-starter 1-7 days	**Starter 8-14 days**	**Grower 15-35 days**	**Finisher 36-42 days**
Maize	54.45	50.79	53.84	54.20
Wheat meal	2.00	2.50	1.00	4.00
Soybean meal	25.00	25.00	23.50	23.00
Sunflower meal	5.00	5.00	6.00	5.00
Yeast	3.00	3.00	3.00	3.00
Fish meal	5.00	4.00	3.00	-
Dehydrated alfalfa meal	-	2.00	2.00	2.00
Soybean oil	3.00	5.00	4.50	5.50
Dicalcium phosphate	1.00	1.20	1.30	1.10
Limestone	-	-	0.20	0.40
Salt	0.20	0.20	0.30	0.30
Lysine	0.10	0.06	0.11	0.25
Methionine	0.25	0.25	0.25	0.25
Premix	1.00	1.00	1.00	1.00

Table 2. Proximate composition of feed mixtures

	Pre-starter 1-7. day	**Starter 8-14. day**	**Grower 15-35. day**	**Finisher 36-42. day**
Moisture	11.03	10.72	10.79	20.85
Ash	5.61	5.79	5.96	5.44
Crude protein	22.73	22.23	21.34	19.48
Crude fat	5.93	7.76	7.28	8.16
Crude cellulose	3.94	4.37	4.51	4.37
NNE*	50.76	49.12	50.12	51.69
Calcium	0.95	0.97	0.99	0.81
Phosphorous	0.86	0.85	0.85	0.71
Metabolisable energy, MJ/kg	12.92	13.23	13.12	13.43
Lysine	1.36	1.30	1.26	1.20
Methionine + cysteine	0.97	0.95	0.92	0.84

Tryptophan	0.31	0.31	0.29	0.27

*Non-nitrogen extract

3. Results and discussion

Results of the study are presented in Tables 3 and 4. It is well known there is a high level of direct correlation between weight of broilers before slaughter and dressing percentage [9,10]. The dressing percentage achieved in this study was 73.15%, better than the 68.01% for Hybro provenance broilers reported by Arsenijević et al. [11] and similar to the 71.6-72.1% for broilers fed with different feed mixtures reported by Ristić et al. [12], while Toledo et al. [13] cited better dressing percentages of 73.5-76.1%. Arbor Acres broilers had an average dressing percentage of 69.43% in the investigation by Živkov-Baloš [13], broilers of Hybro provenance achieved 68.1% [11] and Hybro G+ provenance broilers achieved 61.50% [14].

The average percentage of breast in chilled carcass in this study was 34.33% and that of leg in chilled carcass was 27.91%. The percentage of breast in chilled carcass was higher than was obtained by Ivanović [15], Castellini et al. [16] and Toledo et al. [17]. This parameter was under the influence of sex, since females have bigger breast muscles than males. The percentage of leg in chilled carcass was significantly greater than was reported by Ivanović [15]. The percentage of breast in chilled carcass in the current study was higher than the 31.25% reported previously [14], but the percentage of leg in our chilled carcasses was lower than the 32.91% for the Hybro G+ provenance broilers examined in the study by Đorđević [14]. The percentage of breast in chilled carcass in this trial was lower than was cited by Nikolić et al. [18], who found the percentage of breast in carcass ranged from 37.93% to 38.64%. Nonetheless, our results on the percentage of leg in carcass were in accordance with their results (27.60-28.72%) for Cobb broilers [18].

In general, with age, the proportion of bone in broiler carcasses decreases, and one of the main indicators of good carcass conformation is the meat:bone ratio. In another study, this was 1:0.26 for breast and 1:0.37 for leg [19]. In this study, the meat:bone ratio was 1:0.27 for breast and 1:0.28 for leg.

Table 3. Dressing percentage and percentage (%) of breast, leg and abdominal fat in carcass

Dressing percentage		
73.15±1.32		
Breast	**Leg**	**Abdominal fat**
34.33±0.66	27.91±1.24	1.13±0.36

Table 4. Percentage (%) of meat, skin and bones in breast and leg

Meat	**Skin**	**Bones**
	Breast	
72.61±1.70	8.00±0.74	19.79±1.47
	Leg	
70.38±1.63	9.45±0.59	19.59±1.44

4. Conclusion

The average dressing percentage was 73.15%, and in-carcass percentage of breast was 34.33%, of leg was 27.91% and of abdominal fat was 1.13%. The percentage of meat in breast and leg cuts was 72.61% and 70.38%, of skin in breast and leg cuts was 8.00% and 9.45% and of bones in breast and leg cuts was 19.79% and 19.59%, respectively. The meat:bone ratio was 1:0.27 for breast and 1:0.28 for leg.

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Preservation of meat products with natural antioxidants from rosemary

Preservation of meat products with natural antioxidants from rosemary

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Abstract. Oxidative reactions can reduce the quality of meat products. Synthetic antioxidants can delay the formation of oxidation products but their use in muscle foods has been reconsidered among modern consumers willing to purchase clean label products. Rosemary is a relevant source of antioxidants that can be explored as natural additive in muscle foods. This review aims to provide an overview of the protective effect of rosemary active against the oxidative decay in meat products. The use of rosemary essential oil or extract can slow the progression of oxidative reactions and preserve redness, reduce the accumulation of primary and secondary lipid oxidation and protein oxidation products, and slow the increase of perceived rancidity in sensory analysis. These effects were reported during the storage of patties, burgers, meatballs, sausages, and nuggets. In this sense, rosemary extracts and essential oil can be explored as natural antioxidant in meat products.

1. Introduction

The occurrence of oxidative unbalance and formation of free radicals is a major route to cause loss of quality in meat products [1]. Oxidative reactions are initiated by expose of meat products to atmospheric oxygen, UV radiation, endogenous enzymes, free radicals, and transition metals [1,2]. The absence of preventive actions and the consequent progression of oxidative reactions in susceptible components (such as myoglobin and lipids) leads to changes in quality and acceptance by consumers (color deterioration and off-odor formation) during processing and storage of meat products [1,2].

In order to delay the loss of quality derived from oxidative reactions, antioxidants have been applied in meat products [3]. Although these compounds are widely used in meat products, the concerns about the potential effects in health among consumers have been stimulating a transition towards the production of clean label products [4]. Among the several options of natural antioxidants, rosemary (*Rosmarinus officinalis* L.) stands out due to the presence of antioxidant compounds such as carnosic acid; carnosol; camphor; 1,8-cineole; and α-pinene and pleasant sensory attributes [5]. Due to the

importance of searching for natural additives for the meat industry, this review aims to discuss recent advances in the application of rosemary extracts in the protection of meat products against oxidative reactions.

2. Incorporation of rosemary active compounds in meat products

The presence of numerous antioxidant compounds with high antioxidant activity supports the evaluation of rosemary essential oil and extracts in meat products such as patties, burgers, and meatballs (Table 1). These meat products are convenient and tasty but are affected by oxidation [6–11]. In this sense, Al-Hojazeen and Al-Rawashdeh [12] evaluated two concentrations of rosemary extract in the preservation of raw and cooked chicken patties. Patties produced with either 300 or 350 ppm had lower levels of lipid and protein oxidation than control samples produced without antioxidants during 7 days at 4 °C. The instrumental and sensorially preserved redness were also better preserved in samples produced with these levels of rosemary extracts. Additionally, the intensity of rancidity was lower in these samples in comparison than control.

Table 1. Effect of rosemary antioxidants in patties, burgers, and meat balls

Meat product	Rosemary antioxidant	Storage conditions	Effect in oxidative stability	Ref
Raw and cooked chicken patties	Extract (300 and 350 ppm)	4 °C for 7 days	Reduced lipid and protein oxidation, redness loss (raw) and rancidity odor (cooked); increased sensory score of color (cooked)	[12]
Raw beef patties	Extract (0.1%; 3, 6 and 10% of carnosic acid in the extract)	4 °C for 90 days	Reduced peroxide and TBARS[a] values and loss of redness	[13]
Raw pork patties	Extract (0.1%)	4 °C for 15 days	Reduced loss of redness, metmyoglobin formation, conjugated dienes, and peroxide and TBARS values	[14]
Raw chicken patties	Extract (1%)	4 °C for 5 days	Reduced lipid oxidation and redness loss	[15]
HPP-treated raw pork patties	Active packaging with extract (4.5% of carnosic acid in film)	5 °C for 25 days	Reduced radical formation and lipid oxidation in superficial and inner section	[16]
Raw pork burger	Extract (0.1, 0.2, and 0.3%)	4 °C for 10 days	Reduced lipid and protein oxidation and redness loss (0.2%)	[17]
Raw chicken burgers	Extract (0.02%)	4°C for 21 days	Reduced lipid oxidation	[18]
Raw chicken burgers	Extract (18 and 480 mg/kg)	-18 °C for 4 months	Reduced lipid oxidation (480 mg/kg); no effect in sensory attributes	[19]
Raw mixed meat burger	Extracts (0.015, 0.03, and 0.06)	-12°C for 120 days	Low lipid oxidation and loss of redness; high acceptance among panelists	[20]
Raw beef burgers	Extract (28 mL/kg)	-18°C for 60 days	Reduced metmyoglobin formation, lipid oxidation, and redness; no effect in sensory properties	[21]
Deep-fried pork meatballs	Extract (0.02%)	-18 °C for 180 days	Reduced peroxide and TBARS values	[22]

[a] TBARS: Thiobarbituric acid reactive substances

A similar experiment with raw beef patties with rosemary extract (extracts with 3, 6 and 10% of carnosic acid) and paprika oleoresin indicated lower levels of peroxides and thiobarbituric acid reactive substances (TBARS) than samples produced without antioxidants during 90 days at 4 °C [13]. The preservation of red color in patties was also observed during storage. Interestingly, the authors compared the capacity of butilhidroxianisol (BHA) and rosemary extract to preserve the color of paprika oleoresin. The highest concentration of extract preserved the intense and characteristic red color of paprika oleoresin whereas a complete discoloration was observed in samples containing BHA after 15 days.

The combination of rosemary antioxidants with packaging systems has been providing interesting results to improve the oxidative stability of patties. This strategy was explored by Hwang et al. [14] with rosemary extract in vacuum packaged raw pork patties. The formation of metmyoglobin was reduced and a slight but significant preservation of redness was also reported by the authors. In terms of lipid oxidation, the formation of primary oxidation products (conjugated dienes and peroxides) was delayed and less intense than observed in control samples. The formation of secondary oxidation products (measured with TBARS assay) was also limited by rosemary extract in these patties. A related experiment was carried out to explore the effect of modified atmosphere packaging (63.2% O_2 and 30.6% CO_2) and rosemary extract [15]. Both lipid oxidation and loss of redness were delayed in raw chicken patties produced with rosemary during 5 days of refrigerated storage. Interestingly, this study also explored the use of plasma treatment to reduce the microbial load. Again, rosemary antioxidants reduce the intense oxidation induced by plasma treatment in patties' myoglobin and lipids.

Another relevant experiment obtained results that support the use of rosemary to preserve patties treated with non-thermal processing technologies was carried out by Bolumar et al. [16]. According to these authors, treating raw pork patties with high-pressure processing can induce the formation of free radicals and oxidation in myoglobin and lipids. The addition of rosemary extract in an active packaging reduced the formation radicals and also delayed the loss of redness and formation of lipid oxidation products during 25 days at 5 °C.

The effect of rosemary antioxidants was also reported in burgers (patties with other food additives and seasonings). For instance, Yin et al. [17] explored the effect of rosemary extract level in the preservation of raw pork burger. Using 0.2% of extract generated the lowest levels of lipid and protein oxidation during storage. Moreover, this treatment also reduced the loss of redness. A related study in raw chicken burgers stored at 4°C for 21 days indicated lower levels of lipid oxidation in comparison to control samples (without antioxidant) [18]. Similarly, Pires et al. [19] reported significant differences in the preservation of frozen stored chicken burgers (−18 °C for 4 months) with two levels of rosemary extract. The samples produced with higher level (480 mg/kg) displayed lower levels of lipid oxidation whereas no significant effects were reported in sensory analysis.

A related experiment reported a similar outcome in a burger produced with beef, chicken, and turkey meat [20]. In this case, all levels of rosemary extract reduced oxidative degradation of color and lipids during frozen storage (-12°C for 120 days). Moreover, sensory analysis also revealed that increasing the level of rosemary extract caused the sensory perception of "freshness" in this burger, which increased the acceptance from panelists. Gahruie et al. [21] reported a similar protective effect of rosemary extract during frozen storage of raw beef burgers. However, the authors indicated a significant reduction in redness.

The quality decays derived from oxidative reactions in meatballs can be delayed by rosemary antioxidants. The recent study carried out by Hęś & Gramza-Michałowska [22] revealed that lipid oxidation in deep-fried pork meatballs stored for 180 days at -18 °C was reduced in relation to control samples (without antioxidant). In the same line of thought, Can et al. [23] produced an active film with rosemary extract to preserve raw chicken meatballs. According to the authors, both films (0.3 and 0.5%) were effective to reduce lipid oxidation and preserve sensory attributes and extend the shelf life by 3 days in relation to control samples (without antioxidants).

Sausages are meat products obtained from comminuted meat and animal fat with seasoning and additives (nitrate and/or nitrite) that is processed to acquire defined characteristics [24]. The wide production and consumption of this category of meat products are attributed to the vast options of

ingredients and processing conditions as well as the individual value attributed from consumers to these meat products [24]. Since these products are obtained in conditions that favor oxidative reactions (such as meat comminution, thermal processing and long periods exposed to atmospheric oxygen), the use of ingredients with antioxidant effects is a common practice. Consequently, oxidation can occur and lead to the formation volatile products and sensory-active compounds that compose the expected odor and flavor of these meat products [25–28]. In this case, nitrite (added as sodium salt or produced during fermentation stage) has a central role to prevent quality decay from oxidative reactions [29]. Replacing nitrate/nitrite is current major challenge in the meat industry due to the absence of an adequate replacer with similar or higher capacity to inhibit oxidative reactions and also assist in the physicochemical and microbial stability and safety [29,30].

Alternatively, rosemary essential oil and extracts have been proposed as natural antioxidant to improve the oxidative stability of sausages (Table 2). For instance, Abbasi et al. [31] observed that rosemary essential oil reduced the formation of lipid oxidation products in a cooked cured chicken sausage in relation to the control with nitrite salt. These authors also observed that redness was reduced but there was no effect on sensory attributes between rosemary and control sausage. In a similar experiment, Zhou et al. [32] observed a concentration-dependent effect of rosemary extract on the quality of a cooked pork sausage. Increasing rosemary concentration (especially in the range 0.3-0.5%) reduced the formation of oxidation products and increased redness in relation to control with nitrite. Additionally, minor changes in other physicochemical attributes were also reported by these authors.

Table 2. Effect of rosemary antioxidants in sausages and nuggets

Meat product	Rosemary antioxidant	Storage conditions	Effect on oxidative stability	Ref
Cooked cured chicken sausage	Essential oil (5%)	Final product	Lower redness and lipid oxidation; no effect in sensory attributes	[31]
Cooked and smoked pork sausage	Extract (0.1, 0.2, 0.3, 0.4, and 0.5%)	Final product	Reduced oxidative state; increased redness (0.3-0.5%)	[32]
Cooked and smoked pork sausage	Extract (0.1, 0.2, 0.3, 0.4, and 0.5%)	Stored at 4 °C for 20 days	Increased redness, reduced lipid oxidation and VBN[a] (0.5%)	[33]
Cooked cured beef sausage	Extract (250 mg/kg)	Stored at 4 °C for 25 days	Lower peroxide value and TBARS[b] values	[34]
Fermented pork sausages	Extract (0.48%; 100 g/kg rosemary extract in the oil phase)	Stored at 7 and 20 °C for 49 days	Reduced POV[c], TBARS and hexanal content, redness loss, and rancidity (sensory analysis)	[35]
Chicken nuggets	Extract (150 ppm of carnosic acid and carnosol)	Stored at -18 °C for 9 months	Reduced lipid oxidation; no effect in color, and rancidity odor and taste	[36]
Caiman nuggets	Extract (0.05%)	Stored at -18 °C for 120 days	Preserved redness, reduce lipid oxidation; no effect in sensory attributes	[37]

[a] VBN: Volatile basic nitrogen
[b] TBARS: Thiobarbituric acid reactive substances
[c] POV: Peroxide value.

In a later experiment from the same research group, the protective effect of rosemary extract was evaluated during the refrigerated storage of a cooked and smoked pork sausage [33]. Again, rosemary extract exerted a concentration-dependent effect to inhibit the formation of lipid oxidation products and the formation of volatile basic nitrogen (VBN) during 20 days at 4 °C, principally in 0.5% treatment. Redness increased with rosemary concentration and was stable throughout the storage period. Likewise, rosemary extract reduced the peroxide and TBARS values in a cooked cured beef sausage during 25 days of storage at 4 °C. The experiment, carried out by Erdmann et al. [35], also highlighted the antioxidant effect in fermented sausages. In this case, the authors studied the influence of an emulsion system containing rosemary extract to delay oxidation during storage in *Chouriço*, a traditional Portuguese sausage produced with pork meat. According to the authors, the sausages produced with rosemary extract displayed lower lipid oxidation levels (peroxide and TBARS values and hexanal content), loss of redness and perceived rancidity in sensory analysis in relation to control. It is also relevant to mention that rosemary extract can exert antioxidant activity regardless of nitrite presence and no pro-oxidant effects were reported in any of the aforementioned studies.

Another relevant meat product susceptible to oxidative reactions is nuggets. These meat products are obtained from minced meat and covered with layers known as predust (usually composed of wheat flour), batter (can be prepared with cold water with corn starch and flour), and breading (breadcrumbs). Then, shaped nuggets are pre-fried (> 165 °C for a short period of time) and frozen [38,39]. Differently to other meat products discussed above, nuggets are deep-fried (usually in unsaturated vegetable oils), which causes the absorption of the frying oil and so can favor oxidative reactions during frozen storage [40]. The use of rosemary extract can improve the oxidative stability of nuggets. This statement is supported by the study carried out by Teruel et al. [36]. The natural extract in three forms (liquid ethanol, liquid acetone, and powder acetone) reduced lipid oxidation during 9 months of frozen storage in comparison to control without antioxidants. However, no differences were reported for color, rancid odor and taste between rosemary and control nuggets. In the same line of thought, Paiva et al. [37] evaluated the effect of rosemary extract in caiman nuggets. Lipid oxidation was reduced and a better preservation of redness during frozen storage was reported by these authors. No significant effect in sensory attributes was reported among treatments.

3. Conclusion

Delaying the progression of oxidative reactions is an essential action to preserve the quality in meat products. Antioxidants naturally found in rosemary can be of great value to obtain meat products with enhanced oxidative stability and increased shelf life. The antioxidant effect of rosemary is observed in the use of both essential oils and extracts regardless of meat product (patties, burgers, meatballs, sausages, or nuggets), presence of other additives and ingredients and preservation technologies (packaging systems). The protection against oxidative reactions induced by innovative and non-thermal technologies (plasma and high-pressure processing) is also observed. Therefore, rosemary antioxidants can be of great value in the production of these muscle foods. Future studies with rosemary antioxidants could explore their application in production of healthier and functional meat products.

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The nutritional and health value of beef lipids - fatty acid composition in grass-fed and grain-fed beef

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The nutritional and health value of beef lipids – fatty acid composition in grass-fed and grain-fed beef

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Abstract. Interest in meat fatty acid (FA) composition stems mainly from the need to find ways to produce healthier meat with a suitable ratio of polyunsaturated (PUFA) to saturated fatty acids (SFA) and a favourable balance between n-6 and n-3 PUFA. Studies of grass feeding versus grain feeding cattle have been conducted in different regions throughout the world and suggest that grass-based diets can significantly improve the fatty acid (FA) composition of beef. Compared with grain diets, grass diets contribute to a lower total fat content and more favourable SFA composition i.e. higher proportion of low density lipoprotein (LDL) and cholesterol-neutral stearic acid (C18:0). Feeding fresh grass compared to concentrates results in higher levels of n-3 PUFA in muscle lipids and a more favourable n-6/ n-3 PUFA ratio that is within the nutritional recommendations for the human diet.

1.Introduction

Consumers are increasingly concerned with the quantity and nutritional value of fat present in the foods they consume. Beef is considered as a significant source of protein, vitamins and minerals, but consumers recognize it as red meat with a high content of saturated fatty acids (SFA) [1]. The greatest importance for human health have SFA with less than 18-carbon atom chain length, because they contribute the most to the increase of total serum and LDL cholesterol and therefore increase the risk of cardiovascular disease [2]. The recommendation of the World Health Organization [3] is that SFA should be represented around 10% of total fat in the diet. At the same time, the recommended PUFA to SFA ratio (P:S) should be increased to above 0.4**.** More recently, nutritionists have focussed on the type of PUFA and the balance in the diet between n-3 PUFA formed from α-linolenic acid (ALA,18:3n-3) and n-6 PUFA formed from linoleic acid (LA,18:2n-6). According to current nutritional recommendations the n-6/n-3 ratio within the PUFA, should not exceed 4.0 [4]. A high n-6/ n-3 PUFA ratio is typical of Western and, increasingly, global diets and is associated with an increased risk of cardiovascular disease, obesity, type 2 diabetes and breast and prostate cancer, especially in people with genetic predispositions [5].

Due to the above, the last two decades, many studies have been conducted in order to finde ways to produce healthier beef, with enhancing the amount of PUFA. Different strategies were developed to improving quality of beef fat, but diet has the largest potential to alter the fatty acid composition of bovine muscle. The aim of this article is to summarise the results of several studies which compared fatty acid (FA) composition in grass-fed beef and grain-fed beef.

2.Fat content and fatty acid composition of beef

Changes in food consumption patterns and recommendations to limit intake SFA have led to production leaner beef. Reduced fat content in beef carcass provided application of different production factors such as genetic, animal diet, weight, sex and butchery techniques [6]. Intramuscular

fat (IMF) level is associated with sensory traits: juiciness, aroma and texture (tenderness and mouthfeel), wich are key factors considered when consumers purchase beef.
Lean beef has a low IMF content, typically 2-5% and in many countries this is accepted as being "low" in fat. IMF consists on average, of 45-48% SFA, 35-45% monounsaturated fatty acid (MUFA) and up to 5% PUFA of total FAs. The predominant SFA are C14:0 (myristic acid), C16:0 (palmitic acid) and C18:0 (stearic acid). Linoleic (LA,18:2n-6) and α-linolenic acids (ALA,18:3n-3) are the main PUFA, while oleic acid (18:1n-9) is the most prominent MUFA [7]. Beef also contains small amounts of the long chain n-3 long chain FAs, eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic (DPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). The P:S ratio for beef is typically low at around 0.1 except for very lean animals (<1% IMF) where the P:S ratio is much higher, ~0.5–0.7. The n-6:n-3 ratio for beef is beneficially low (usually <3), reflecting the significant amounts of desirable n-3 PUFA, particularly ALA , but also EPA, DPA and DHA [8] (Table 1). Beef lipids are the richest naturaly source of conjugated linoleic acid (CLA) and *trans*-fatty acids (TFA) wich are recognized to have biological activites that include inhibition of carcinogenesis and atherosclerosis. Most dominant TFA in beef is vaccenic acid (C18:1*t*) with health promoting biological properties, as disting to industrial TFA [9].

Table 1. Typical fatty acid composition of beef *longissimus* muscle

Fatty acid	Percentage of fatty acid
C12:0	0.08
C14:0	2.66
C16:0	25.0
C16:1*cis*	4.54
C18:0	13.4
C18:1*trans*	2.75
C18:1cis-9	36.9
C18:1cis-11	2.33
C18:2n-6	2.42
C18:3n-3	0.70
C20:3n-6	0.21
C20:4n-6	0.63
C20:5n-3	0.28
C22:4n-6	0.04
C22:5n-3	0.45
C22:6n-3	0.05
P/S ratio	0.11
n-6/n-3 ratio	2.11

Source: Enser, M. et al. 1996. Meat S cience, 42, 443– 456 [29]

Beef fat quality can be influenced by many factors, including breed or genotype, age or live weight, anatomical location, gender and nutrition [10]. It is generally acknowledged that genetic factors have a smaller influence than dietary factors on the FA composition of beef. Breed differences in the FA composition of beef are generally small, but do reflect differences in underlying gene expression or activities of enzymes involved in FA synthesis [11]. The fatty acid composition in ruminant tissues is very complex as the result of biohydrogenation in the rumen. The action of enzymes present in microorganisms in the rumen producing stearic acid and wide range of intermediate products such as *trans*- and *cis*- FAs, MUFA and FAs with conjugated double bonds. Most of dietary PUFAs, which mainly consist of C18:2n-6 and C18:3n-3, extensively hydrogenated by rumen microbiota to SFA, particularly to C18:0, and small part of them escape ruminal biohydrogenation. Despite the high levels of ruminal biohydrogenation of dietary PUFA, manipulating of diet is the major route for increasing the content of beneficial FAs in beef [12].

3. Fatty acid composition in grass-fed and grain-fed beef

Research spanning three decades suggests that feeding fresh grass compared to concentrate can significantly alter the FA composition and improve the overall antioxidant content of beef. Feeding n-3 PUFA rich forage leads to leaner carcass, reduces deposition of intramuscular fat and improve fatty acid profile beef lipids, i.e increases the proportion of PUFA to SFA. Fresh grass is usually the cheapest form of cattle feed, but at the same time increased time to market and the need to be able to source pasture or conserved forage [13]. The quantity and composition of PUFA in beef is very much dependent on the supply of PUFA in the diet, and associated dietary and animal factors (e.g., feeding behaviour and rumen conditions), which influence the degree of biohydrogenation [14]. In general, pathways used for biohydrogenation of LA and ALA, the major FA in typical cattle diets, are influenced by the forage to concentrate ratio [15]. Forages like grass and clover contain a high proportion (50-75%) of their total fatty acids as ALA [16], which is the building block of the n-3 series of essential fatty acids, and elongation and desaturation of ALA result in the synthesis of EPA and DHA. Feeding fresh grass compared to concentrates results in higher concentrations of n-3 PUFA in muscle lipids, both in the triacylglycerol and phospholipid fractions [17-21].

Table 2 summarises the SFA content reported by several studies that compared the lipid profiles of cattle fed either a grain or grass diets [17-21]. The findings of these studies shows generally inconsistent differences in total SFA content between grass-fed and grain-fed cattle (Table 2). Despite percentage increase in SFA from grass-fed beef, it does not impact to an increased intake of total SFA, due to the lower total fat content of beef from grass-fed cattle. In fact, the data suggest that because of its lower total fat content, grass-fed beef contains up to 1.4 g less total saturated fat per 100 g than does grain-fed beef. The various SFAs differ in their effects on the blood lipoprotein profile. The medium-chain SFA lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acid are nutritionally undesirable because they increasing plasma cholesterol level, while stearic acid (C18:0) has no effect on serum cholesterol [22]. Grass-fed beef tend toward a higher proportion of cholesterol neutral stearic acid (C18:0) and therefore many health organizations proposed classification of SFA with carbon chain lengths from C12 to C16 as “cholesterol-raising fatty acids”, excluding stearic acid [23].

Table 2. Literature comparison of mean saturated fatty acid composition between grass-fed and grain-fed cattle

Breed, diet	C14:0 Myristic	C16:0 Palmitic	C18:0 Stearic	Total SFA	Total lipid	Reference
			%FAs within IMF			
Simmental bulls						
Grass	1.82	22.56*	17.64*	43.91	1.51*	Nuernberg et al. [17]

Grain	1.96	24.26*	16.08*	44.49	2.62*	Nuernberg et al. [17]
			g/100g lipid			
Mixed cattle						
Grass	2.84*	26.90	17.00*	48.80*	2.80*	Leheska et al. [18]
Grain	3.45*	26.30	13.20*	45.10*	4.40*	
			% of total FAs			
Angus X-breed						
Grass	2.19	23.10	13.10*	38.40*	2.86*	Garcia et al. [19]
Grain	2.44	22.10	10.80*	35.30*	3.85*	
			g/100g lipid			
Crossbred steers						
Grass	1.24*	18.42*	17.54*	38.76	9.76*	Alfaia et al. [20]
Grain	1.84*	20.79*	14.96*	39.29	13.03*	
			% FAs within IMF			
Hereford steers						
Grass	1.64*	21.61*	17.74*	49.08	1.68*	Realini et al. [21]
Grain	2.17*	24.26*	15.77*	47.62	3.10*	

Source: Daley et al. Nutrition Journal 2010 [24], 9:10; *significant difference (at least <0.05) between feeding regimens

The feeding system had a major impact on PUFA composition of beef (Table 3). Results of the large number of studies are consistent and show that grass-based diets resulted in significantly higher levels of n-3 PUFAs in the beef lipids, without affecting n-6 PUFAs content. Higher percentage of n-3 PUFAs resulting in a more favourable n-6:n-3 PUFAs ratio, which is one the most important indices to evaluate the nutritional value of fat [24]. Green pastures are a good source of ALA, in contrast to concentrate diets, which explains the higher percentage of ALA in animals fed with grass. Certain of researchers suggest that is for the higher content of PUFA in beef lipids from pasture-fed cattle responscehigher amount protection of FAs in fresh grass from ruminal biohydrogenation, compare to grain-fed beef [25]. On the other hand, this increased content PUFA may explain presence of secondary plant metabolites during ruminal digestion, which inhibit microbial biohydrogenation [26]. Feeding n-3 PUFAs rich diets like grass results in beneficial responses in the content of n-3 long chain FAs, EPA and DHA in beef. Results of health promoting biological properties EPA and DPA including development and maintenance of neural and visual tissues throughout life and important role in reducing the risk of cardiovascular disease and cancer [27].

Table 3. Literature comparison of mean polyunsaturated fatty acid composition between grass-fed and grain-fed cattle

Breed, diet	C18:2n-6 Linoleic	C18:3n-3 Linolenic	C20:5n-3 EPA	C22:6n-3 DHA	Total PUFA	n-6/n-3 ratio	Reference
			% FAs within IMF				
Simmental bulls							
Grass	6.56	2.22*	0.94*	0.17*	14.29*	2.04*	Nuernberg et al. [17]
Grain	5.22	0.46*	0.08*	0.05*	9.07*	8.34*	
			g/100g lipid				
Mixed cattle							
Grass	2.01	0.71*	0.31	na	3.41	2.78*	Leheska et al. [18]
Grain	2.38	0.13*	0.19	na	2.77	13.6*	
			% of total FAs				
Angus X-breed							
Grass	3.41	1.30*	0.52*	0.43*	7.95	1.72*	Garcia et al. [19]
Grain	3.93	0.74*	0.12*	0.14*	9.31	10.38*	
			g/100g lipid				
Crossbred bulls							
Grass	12.55	5.53*	2.13*	0.20*	28.99*	1.77*	Alfaia et al. [20]
Grain	11.95	0.48*	0.47*	0.11*	19.06*	8.99*	
			% FAs within IMF				
Hereford steers							
Grass	3.29*	1.34*	0.69*	0.09	9.96*	1.44*	Realini et al. [21]
Grain	2.84*	0.35*	0.30*	0.09	6.02*	3.00*	

Source: Daley et al. Nutrition Journal 2010 [24], 9:10; *significant difference (at least <0.05) between feeding regimens
na – value was not reported in original study

Due to the importance of n-3 long chain FAs for human health, intake recommendations around the world are for a minimum of 250 mg EPA+DHA/day. Some studies did report higher EPA and DHA levels in grass feed beef, but still, they were less than the required 40 or 80 mg EPA+DHA per 100g food product. It appears that lean grass feed beef can make a modest contribution to n-3 long chain FA intake goals while contributing a limited amount of total fat to the diet [28]. Grass-based diets result in more favourable n-6/n-3 ratios in meat, within the nutritional recommendations for the human diet.

Summarised data shows n-6/n-3 ratios are 1.72 to 2.78 and at 3.00 to 13.6 for grass and grain-fed beef, respectively.

4.Conclusion

It is evident that nutritional value is an important dimension of beef quality and this is reflected by efforts to improve the FA composition of beef. Feeding grass results in important beneficial responses in meat: the content of n-3 PUFA, low n-6/n-3 ratio and; more favourable SFA composition with higher proportion of LDL cholesterol-neutral stearic acid. These changes in FA composition of beef are associated with higher nutritional quality and beneficial effects on human health.

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From designing diets for animals to designing food of animal origin – overview

From designing diets for animals to designing food of animal origin – overview

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Abstract. In recent times, food is not only observed from the point of view of the required intake for growth, development and regeneration of the body, but also has a leading role in the quality of human life. Therefore, the diet focuses on optimizing the daily intake of both nutrients and non-nutritive ingredients of food, all in order to preserve health and, above all, reduce the risk of chronic non-communicable diseases. Functional food can be considered food that has been scientifically proven to have a positive effect on certain body functions (in addition to the usual nutritional value) that contribute to human health and reduce the risk of disease. At the same time, it is important that the food has a standard form and that the positive effect on health is manifested by consuming the usual amount of food. The functionality of food is achieved by the presence in it of bioactive components (one or more) which have been scientifically proven to have positive effects on human health in the quantities in which they are present in food. The nutritional value of foods of animal origin depends on many factors, but certainly animal diet has the greatest impact. In human nutrition the so-called designed products of animal origin (meat, milk, eggs) are used, which are due to the specific animal diets enriched with n-3 fatty acids, vitamins, carotenoids or trace elements. Today, there are nutritional strategies by which we can access functional foods for the purpose of health promotion.

1. Functional food

The beginning of the 21st century still presents food science with new challenges. Food is no longer viewed only from the aspect of the need for adequate intake, primarily for the purpose of proper growth, development and regeneration of the body. Food today has one of the leading roles in the quality of human life. For that reason, the previous so-called balanced diets (a term used in human medicine) have grown into the level of optimally balanced diets (also a term used in human medicine), which are focused on optimizing the daily intake of both nutrients and non-nutritive food components in order to promote health and reduce the risk of occurrence of chronic non-communicable diseases [1].

The term “functional food” was first used in Japan in the mid-1980s, and referred to food that, in addition to being nutritious, also contains ingredients useful for supporting certain bodily functions. Nutrition science is no longer just about ensuring proper nutrition and avoiding malnutrition and nutrient deficiencies, but also about discovering biologically active substances in food that have the ability to improve health and reduce the risk of disease [2,3]. In 1988, the Japanese government set up a project to investigate potential positive food functions in order to reduce treatment costs. The category of foods with potential positive health effects, which is the result of these studies, is known as “foods for specific health needs” (foods for specific health use - FOSHU food). This category of food appeared in 1991 and represents food that is expected to exhibit a certain, beneficial health effect, as a result of the presence of specific components.

Functional food is not easy to cover with a single definition, since this food is primarily a concept, not a well-defined group of food products. There are several working definitions of functional foods. In 1998, the European Union, in coordination with the International Life Science Institute Europe (ILSI Europe), adopted the following definition "a food can be considered functional if it has been satisfactorily shown to have a beneficial effect on one or more functions of the organism, outside the usual nutritional effects and in a way that is important for general health or to reduce the risk of disease" [2,4,5].

Functional foods, due to their specific and modified composition in relation to classic foods of the same type, have positive effects on human health and are most often used to preserve optimal gastrointestinal functions, raise levels of antioxidant defenses, reduce risk factors involved in the etiology of cardiovascular disease and cancer. The listed effects of functional food often show due to the presence of one or more bioactive components in their composition, which scientific research has determined that, in the quantities in which they are present in food, have positive effects on certain physiological or biochemical processes in the body. The biologically active compound can be a macronutrient (resistant starch or n-3 fatty acid), a micronutrient (vitamin or mineral), or a non-essential food ingredient that has a certain energy value (oligosaccharides, conjugated linoleic acid, plant sterol).

A functional ingredient can also be a phytochemical (isoflavones, phytoestrogens) or a living microorganism (probiotics). After consuming a functional food, a biologically active compound is released in the digestive tract, which acts at the site of release (dietary fiber, probiotic) or is resorbed and distributed to target tissues, where it will have a beneficial effect [4,5,7]. In recent years, there has been increasing talk about the application of nanotechnologies in the production of functional food, and its advantages, challenges and possible disadvantages [8].

The development and production of functional food requires a multidisciplinary approach and is a long and complex process [9]. The most common functional ingredients used in the enrichment of foods of animal origin are: selenium, omega-3 fatty acids and vitamin E. These ingredients are attractive primarily because for many years in the development of functional foods they have been used to modify a number of products to preserve heart health and to reduce overweight, since these are the biggest problems of the modern way of life [10].

Recently, there is an increasing trend of excluding pharmaceutical preparations from animal feed, so nutritional solutions are becoming an increasingly important alternative in existing production systems. All this is the reason why the concept of functional food should be built on solid scientific foundations, and at the same time recognized and accepted by both farmers and consumers of food of animal origin (meaning all of us) [4,6,7].

2. Animal nutrition

The science of nutrition is a relatively young science, and its importance lies in the fact that all living beings, plants and animals, including humans, intake food/feed regularly, that is, they bring energy and nutrients into the body, which is the material basis of life. Energy and nutrients ingested by food/feed ensure the development of basic biochemical processes and functions of individual tissues and organs, and thus the organism as a whole. Domesticated animals that intake feed every day do so to meet their needs for life support, growth, reproduction and work, as well as for the production of meat, milk and eggs. Appropriate animal nutrition, i.e. the optimal combination of nutrients, can increase production, but also improve the

quality of food obtained for human consumption [11]. The fact that animal nutrition can affect the quality of food of animal origin is very important because the modern way of life of people indicates the need for quality food and prevention of chronic non-communicable diseases. With a proper diet, we can significantly control the oxidative status of the organism, and thus the overall health. The official expert recommendation is that you should take four important antioxidants every day and for life: vitamin C, beta-carotene (a precursor of vitamin A), vitamin E and selenium. For all of them, there is evidence that they have anticancer properties, and that they are important for the prevention of chronic non-communicable diseases [12,13].

3. Selenium

One of the ways to produce functional food, i.e. the "third generation of food with a positive aspect of health" is to enrich food with selenium (meat, eggs, milk), which has been confirmed by numerous studies [4,10,14,15,26]. Selenium is an essential element for humans and animals and as such is necessary in a certain amount in animal feed. Its most important function is that, together with vitamin E, it forms a multicomponent system of protection of biological membranes from oxidative degeneration. Selenium is the active ingredient of the enzyme glutathione peroxidase (GSH-Px) in which about 40% of the total selenium in the body is present. The reaction of free radicals, especially present in tissues with intensive oxygen circulation, causes peroxidation of phospholipids and damage to the structure of membranes, and thus the function of cell membranes. Selenium, together with vitamin E, has a protective role when it comes to heavy metals and some drugs and chemicals. In addition, it has other important functions in the body because it is an integral part of many selenoproteins. The creation of geo-botanical maps of selenium has been carried out in several regions of the world and indicates the existence of numerous selenium-deficient areas. Given this fact and all known functions of selenium in the body, there is a need for the production of selenium-enriched products (meat, eggs). In animal diets, selenium has been present as an essential element for decades in inorganic form in the amount of 0.15-0.30 ppm. Numerous researchers around the world have confirmed that the amount of selenium deposited in tissues depends on the levels and forms of selenium (organic, inorganic) used in the diet, i.e. that using increased amounts of organic selenium (0.5 ppm and more) allows its content in tissues to increase [7,14,15].

Studies [7,16,17] show the results of the influence of different amounts and sources of selenium from animal feed on its content in tissues and organs of poultry, i.e. the possibility of increasing the amount of organic selenium in the blood to achieve higher GSH-Px activity in the blood, but also a larger amount of selenium in muscles and organs, which is important from the aspect of consuming food of animal origin.
One of the experiments [7,14] was organized on a total of 150 one-day-old broilers of Hubbard provenance divided into three groups and the animals were fed complete mixtures for fattening broilers, with standard raw materials and chemical composition. The groups differed only in the amount of added organic form of selenium (selenized yeast) in the feed, namely 0.3, 0.6 and 0.9 ppm. All three groups of broilers also received an increased amount of vitamin E (100 IU) through feed. At the end of the experiment, in addition to better production results (body weight, body weight gain, conversion in the group with 0.6 ppm of added organic selenium) but without statistical significance ($p>0.05$), a significant result of this experiment was the higher ($p <0.01$) content of deposited selenium (in the group with 0.9 ppm vs. 0.3 pp), namely 0.61 mg/kg (breast meat), 0.54 mg/kg (drumstick with thigh), 0.96 mg/kg (liver) and 0.48 mg/kg (heart). The content of vitamin E was significantly higher ($p <0.01$) in the liver (4.49 mg/kg) with supplementation with 0.6 and 0.9 ppm

selenium vs. 0.3 ppm. In addition, in the experimental groups with increased content of selenium and vitamin E, there was significantly ($p < 0.01$) less fat in meat, and a more favorable fatty acid composition (atherogenic index) [14].

From the obtained results, it can be concluded that by increasing the content of organic selenium in feed mixtures, the content of selenium in meat and organs also significantly increased ($p < 0.01$). Since it is known that the soil of Serbia is poor in selenium, and that the body absorbs it best when it is ingested through meat, milk and eggs, it is certainly necessary to include it in the diets of animals that excrete it in their products [18,19]. Since pork and chicken meat are consumed in large quantities, it is considered to be one of the best ways to get selenium, although there is increasing talk about milk and eggs enriched with this microelement. In countries with developed biotechnologies, products of animal origin have been used for many years, by which this important microelement is introduced into the body in the most natural way.

4. Improving the fatty acid composition of meat

Today, numerous studies in the world refer to the impact of fats on human health and the appearance of chronic mass non-communicable diseases. Fats are of multiple importance to human health, being energy source, carriers of fat-soluble vitamins, participants in important physiological processes in the body and are indispensable in many biological functions, including growth and development of the body. However, few studies can consistently show a link between the intake of fats or certain fatty acids and health, so that in scientific circles, there is no complete consensus on the relationship between fat intake and health, which can be seen from the differences in the recommendations on the intake of fats and fatty acids [20]. The importance of the use of n-3 and n-6 fatty acids and their ratio is especially emphasized (Tables 1 and 2).

Table 1. Ratio of n-6 PUFA to n-3 PUFA in human nutrition

Period-area	**n-6/n-3**
Paleolithic	0.79
Greece prior to 1960	1.00 - 2.00
Current Japan	4.00
Current India, rural	5 - 6.1
Current UK and Northern Europe	15.00
Current US	16.74
Current India, urban	38-50

Source [21]

The human body does not have the ability to synthesize essential fatty acids, namely linoleic acid (LA) and alpha-linolenic acid (ALA). Deficiency of these fatty acids can be the cause of human diseases, so they must be taken with food. By extending the chain, LA and ALA can be converted into components that play the role of hormones and participate in protection against inflammation. As such, essential fatty acids are involved in a number of physiological processes, such as blood clotting, wound healing and protection against inflammation.

Also, the importance of conjugated linoleic acid (CLA) for human health is increasingly being discussed. CLA is a mixture of positional and geometric isomers of C18: 2n-6 linoleic acid (LA). A number of CLA isomers have been isolated, but all physiological effects known so far are related to the two isomers cis-9, trans-11 (c9t11CLA) and trans-10, cis-12 (t10c12CLA) isomer. Most of the studies related to the health

effects of CLA have examined the effects on the occurrence of cancer, cardiovascular disease, diabetes, obesity, and on the immune system and lipid metabolism.

Table 2. Fatty acid content in platelet phospholipids and mortality rate from cardiovascular diseases

Parameter	Europe and the US	Japan	Greenlandic Inuit
C20:4, n-6 (%)	26	21	8.3
C20:5, n-3 (%)	0.5	1.6	8.0
n-6/n-3 ratio	50	12	1
CVD mortality (%)	45	12	7

Source [22]; CVD - cardiovascular diseases

In accordance with the previous facts, but also the constant demands of some consumers for food of high quality, food production systems of animal origin try to produce food with as many positive effects on human health as possible. Unsaturated fatty acids are currently a hot topic in the food industry, and therefore, increasing their amount in the food is attracting ever more attention from both the public and the food industry [23].

To make the content and ratio of fatty acids in pig meat more favorable, it can be influenced by the optimal choice of nutrients for pig nutrition. The aim of the study in two experiments [24, 25] on pigs was to examine the effect of commercial flax preparation, i.e. commercial CLA preparation, added to pig feed on the fatty acid composition of pig meat. Since flaxseed has a desirable fatty acid composition, many producers are interested in improving the fatty acid composition of adipose tissue and pig meat by including it in the final fattening diet of pigs [24,25]. The basis of these and similar studies is the fact that the fatty acid composition of human foods reflects the fatty acid composition of tissues - that is, foods of animal origin that we get from animals in intensive breeding.

The aim of one study was to examine the influence of different fat sources (sunflower, flax and soy) in the diet of fattening pigs on the fatty acid composition and meat quality [25]. The study was performed on 30 pig crossbreeds (Yorkshire × Landrace), with an initial weight of 60 kg. The pigs were divided into three groups and the experiment lasted 46 days to a mean pig weight of about 100 kg. They were fed a standard mixture for pigs in the last stage of fattening (finisher), and the groups differed only in that the first experimental group (EI) received sunflower grain in the meal as a source of fat and fatty acids, while the second experimental group (E-II) had a commercial preparation of flax (Vitalan ®, Vitalac, France) in the recommended amount of 2.5% in the mixture, and the third experimental group (E-III) received full-fat soybean meal in the diet. The composition of fatty acids in feed and meat, i.e. the content of n-3 and n-6 fatty acids, as well as their mutual relationship (n-6/n-3) were examined. Similar studies, i.e. examination of the influence of nutrients (fats, flax or their mixtures) on the fatty acid composition of meat have been performed on poultry [27].

The aim of a second study was to investigate the effects of adding conjugated linoleic acid (CLA) preparations to the diet of fattening pigs, on the fatty acid composition in the meat of these pigs. Sixty

crossbreeds of pigs (Yorkshire × Landrace), with an initial body weight of 60 kg were used in this experiment. Pigs were divided into two groups (control and experimental), with 30 pigs in each and were fed with a standard mixture (NRC, 1998), for fattening pigs from 60 to 110 kg (fattening period of 60 days). The meal differed between the groups only in that the experimental pigs received feed with added commercial preparation of conjugated linoleic acid 60% CLA (Lutalin®, BASF, Germany), at the recommended amount of 2.0%. The mixtures were balanced and fully met the needs of the animals at this stage of production.

The meat of pigs fed with these supplements had a significantly more favorable ratio of n-6/n-3 fatty acids, compared to the meat of pigs fed without the addition of flax, i.e. CLA [25,28]. Similar results were obtained when examining the effect of adding CLA to a poultry diet [29].

The results of testing the content of specific saturated, monounsaturated and polyunsaturated fatty acids in the muscle tissue of pigs show that the content of saturated fatty acids was higher, and monounsaturated and polyunsaturated fatty acids were lower in the experimental group of pigs. The differences in the content of n-3 and n-6 fatty acids of the experimental and control groups of pigs were not statistically significant, but the ratio of n-6/n-3 was more favorable in the experimental group of pigs. The presence of both isomers of this preparation has been proven in the meat of pigs fed with the addition of CLA. The presence of these isomers has not been demonstrated in the meat of pigs fed without the addition of CLA [28].

In these studies, two isomers of CLA were used, namely c9t11CLA and t10c12CLA as a feed additive for pigs. Both of these acids were also found in pig meat at the end of fattening, with the content of c9t11CLA being almost twice as high as the content of t10c12CLA. It is noticeable here that the same degree of uptake and incorporation of both isomers of conjugated linoleic acid observed in feed is not the same. The c9t11 isomer is much more suitable for incorporation into intramuscular fat depots [28].

5. Conclusion

Animal meat can be enriched with functional ingredients through diet i.e. meal design. The relationship between organic selenium ingested with feed, and selenium content in animal tissues and products is not linear. By ingesting the organic form of selenium in feed, the selenium content in fresh tissues reaches the level of 0.3 to 0.4 mg/kg. Organs, liver and heart, contain higher concentrations of selenium, liver up to four times in relation to skeletal muscles. The addition of flax, i.e. CLA, to pig feed improves the value of pig meat from both the nutritional and health aspects. The above facts unequivocally indicate that functional foods, primarily meat enriched with the mentioned bioactive additives, will increasingly be an indispensable part of the human daily diet. Food production exceeds the need to survive and satisfy hunger. The development and creation of functional food is a scientific temptation that must be ahead of the economic temptation, and which must be founded on scientifically based facts.

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The intake of phosphorus through meat products: a health risk assessment

The intake of phosphorus through meat products: a health risk assessment

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Abstract. The aim of this study was to assess the dietary exposure of phosphorus in the Serbian adult population by combining individual consumption data with available data for analysed meat products. During a three years period of investigation (2018 to 2020), a total of 682 samples consisting of 425 cooked sausages and 257 smoked meat products were collected from different local retail markets across the Serbia to examine phosphorus concentrations. The mean phosphorus concentration, expressed as P_2O_5, varied from 4.68±0.88 g/kg to 6.05±1.30 g/kg in finely minced cooked sausages and smoked meat products, respectively. The average estimated daily dietary intake (exposure) (EDI) of phosphorus ranged from 1.115 mg/kg BW (body weight)/day (finely minced cooked sausages) to 1.441 mg/kg BW/day (smoked meat products). Phosphorus dietary intake (exposure) averaged 3.08% of the acceptable daily intake (ADI: 40 mg/kg BW/day). According to our results, the average phosphorus exposure in the Serbian adult population from consumption of these meat products is far below the European ADI.

1. Introduction

Phosphorus, an essential nutrient, performs vital functions in skeletal and non-skeletal tissues and is pivotal for energy production. Phosphorus is involved in many physiological processes, such as in the cell's energy cycle, in signalling pathways in the form of cyclic monophosphates, in cellular regulation through phosphorylation, and in the mineralization of bones and teeth [1]. About 85% of the body's phosphorus is in bones and teeth, while 14 % is in soft tissues, including muscle, liver, heart and kidney, and only 1 % is present in extracellular fluids [2]. The major dietary contributors to phosphorus intake are foods of animal origin, i.e. milk and dairy products, followed by meat, poultry and fish, grain products and legumes [3]. Inorganic phosphates are derived mainly from food additives. Phosphorus additives (E 338-341; E 343; E 450-452) are increasingly being used in processed and fast foods, especially in the meat industry, cheeses, baked products and beverages for several technological purposes. They increase water holding capacity (WHC), preserve moisture or colour, emulsify ingredients and enhance flavour, as well as stabilize foods. It has been estimated that 50% of daily phosphorous intake in the Western world is from food additives [3,4]. Phosphorus in food additives is rapidly and almost completely absorbed, whereas the natural constituent of protein-bound phosphorus is more slowly and less efficiently absorbed (60%) [5]. An association between high serum phosphate levels and cardiovascular morbidity and mortality in patients with chronic kidney disease and bone

health complications has long been known [6]. Therefore, high phosphorus intakes from additives should be taken into account as a potential public health concern. These additives were evaluated by the Scientific Committee for Food [7], which derived a group acceptable daily intake (ADI) for phosphates expressed as phosphorus of 40 mg/kg body weight (BW) per day and concluded that this ADI is protective for the human population. Also, European Directives on food additives [8] require that Member States monitor intakes to ensure that consumers do not have an excessive intake of each given food additive, which could lead to a health hazard. The Serbian standard maximum limit for total phosphates, expressed as P_2O_5, in meat products is < 8 g/kg [9] or ≤ 5 g/kg of added phosphorus [10].

Chronic non-communicable diseases such as cardiovascular diseases are a national public health problem affecting as much as 51.7% of the general adult population. In Serbia, they constitute the major contributor to the burden of disease in terms of disability-adjusted life years (DALYs) or mortality [11]. Despite the lack of systematic national surveys of assessment of total dietary phosphorus intake, studying the nutritional status as well as lifestyle of the population is fundamental to design national guidelines and public policies. Therefore, the objective of this study was to determine phosphorus content in processed meat products. Based on the analysis results, dietary exposure of the Serbian adult population to phosphorus was then estimated and discussed.

2. Materials and Methods

2.1. Sample Collection and Preparation

A total of 682 samples consisting of 425 cooked sausages and 257 smoked meat products were randomly collected over three years (2018, 2019 and 2020). Samples included domestic and imported products from different local retail markets across the Serbia. Further description of the analytical procedure and equipment for determining phosphorus content in the sampled food commodities can be found in previous work conducted by Koricanac et al. [2].

2.2. Exposure Assessment and Risk Characterization

The dietary intake levels of phosphorus (mg/kg BW/day) from processed meat products was estimated on the basis of the concentrations of the phosphorus in examined samples and on the basis of average *per capita* consumption data obtained by Household Budget Survey [12]. Total phosphorus EDI was compared with the ADI proposed by EFSA [7].

2.3. Statistical Analysis

Data were analysed using Minitab 17 Ink statistical software. Phosphorus contents in groups of the studied samples of processed meat products were expressed in the form of descriptive statistics and their distributions.

3. Results and Discussion

The contents and distributions of phosphorus in each group of processed meat products are summarized in Table 1 and Figure 1. The EDI data for phosphorus in processed meat products are shown in Table 2.

Data obtained in this study indicate low phosphorus content in processed meat products. As shown in Table 1, the mean phosphorus (P_2O_5) content in finely minced cooked sausages was 4.68±0.88 with a range of 1.12 to 9.22 g/kg. According to national regulation, only two (0.5%) of the 425 finely minced cooked sausages were above regulatory limit (< 8 g/kg), reaching a content of 9.22 g/kg P_2O_5. Our results are in line with data obtained in previous studies [2, 13]. Regarding smoked meat products, phosphorus (P_2O_5) content ranged from 2.32 to 10.64 g/kg with a mean value of 6.05±1.30 g/kg. In our study, seven (2.72%) of the 257 smoked meat products was above the regulatory limit, reaching a content of 10.64 g/kg P_2O_5.

The average EDI of phosphorus for the Serbian adult population ranged from 1.115 to 1.441 mg/kg BW/day (Table 2). Our results showed that the processed meat products were a minor contributor to the dietary intake of phosphorus (3.08%). The ADI of 40 mg/kg BW/day corresponds to an intake of 2.8 grams of phosphorus per day for an average adult weighing 70 kg [7]. Hence, exposure to phosphates

from the meat products in our study is considered to be far below this ADI. The main contributor to the dietary intake of phosphorus could differ by region depending on the habitual diet of the study population. However, data for children's exposure was not taken into consideration during the study period.

Table 1. Mean levels and ranges for phosphorus (P_2O_5) in processed meat samples

Food Group	N	Mean±SD (g/kg)	Min-Max (g/kg)	Above MPL N (%)
Finely minced cooked sausages	425	4.68±0.88	1.12-9.22	2 (0.5)
Smoked meat products	257	6.05±1.30	2.32-10.64	7 (2.72)
Total	**682**	**5.19±1.24**	**1.12-10.64**	**9 (1.32)**

N – total number of analysed samples; MPL – maximum permitted level (< 8 g/kg) [9].

Table 2. Estimated dietary intake of phosphorus and risk characterization of phosphorus intake

Food Group	Mean±SD (g/kg)	ADC (g/day)	EDI (mg/kg BW/day)	Contribution to ADI (%)	ADI (mg/kg BW/day)	AI (mg/day)
Finely minced cooked sausages	2.04±0.38	38.2	1.115	2.79	40	550
Smoked meat products	2.64±0.57		1.441	3.60		
Total	**2.27±0.54**		**1.233**	**3.08**		

P content (43.64% of P_2O_5); ADC – Average Daily Consumption of meat products [12]; EDI – Estimated Daily Intake; Default body weight value for adults was 70 kg [14]; ADI – acceptable daily intake [7]; AI – adequate intake [15].

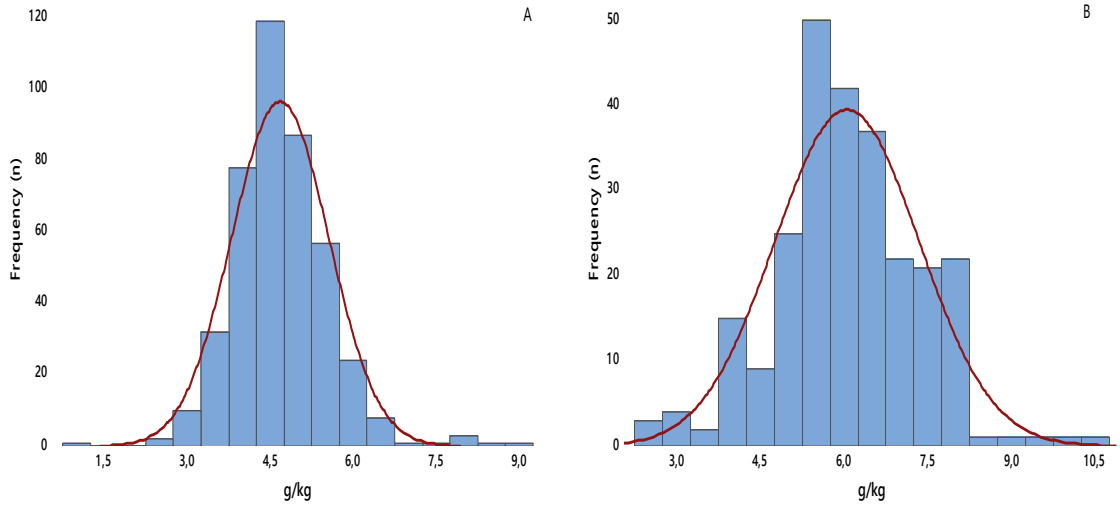

Figure 1. Distribution of phosphorus content (P_2O_5) (g/kg) in finely minced cooked sausages (A) and smoked meat products (B).

4. Conclusion

The results presented in this paper indicate there is no health concern for phosphorus intake by adults in Serbia consuming meat-based products. This study highlights the need for additional total diet studies on the dietary intake of phosphorus in different population groups.

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The influence of modified atmosphere packaging on shelf life of ćevapčići

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The influence of modified atmosphere packaging on shelf life of ćevapčići

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Abstract. The aim of this study was to determine the microbiological, sensory and chemical changes of modified atmosphere packaged ćevapčići in a gas mixture consisting of 70% O_2 and 30% CO_2. Packaged ćevapčići were stored for 10 days at 3ºC. Microbiological examination comprised determination of pathogenic microorganisms (*Salmonella* spp., coagulase positive staphylococci, *Escherichia coli* and *Listeria monocytogenes*) as well as indicators of hygiene and spoilage (total viable count, psychrotrophic bacteria, *Enterobacteriaceae*, lactic acid bacteria and *Brochothrix thermosphacta*). Using quantitative-descriptive test with grading scales from one to five, sensory properties of ćevapčići were assessed (color and odor in raw condition and odor, texture and taste after roasting) on the 1st, 4th, 6th, 8th and 10th days of storage. Regarding the chemical parameters, every day during the storage, pH was examined and on 4th and 8th days, acid value and peroxide number were established. On the basis of the results obtained and the recommended total viable count, which should not be higher than 7 log cfu/g, and taking into consideration sensory properties, we can conclude that ćevapčići packed in the modified atmosphere containing 70 % O_2 and 30% CO_2 had a shelf life of seven days.

1. Introduction

Due to its specific chemical composition and high water content, meat is one of the most perishable groceries. Spoilage can be defined as any change that makes food unacceptable for the consumer from a sensory point of view [1]. An unpleasant odor and taste can occur as a result of the growth of microorganisms, whereby intense sensory changes are associated with degradation of nutrients in meat and the emergence of undesirable volatile metabolites.

In fresh meat, about 7 log cfu/g of bacteria is responsible for the appearance of an unpleasant odor with a milky note, while a putrid odor is created by the decomposition of free amino acids when the bacteria population reaches 9 log cfu/g [2]. Under aerobic conditions, *Pseudomonas* spp. are the dominant species of microorganisms that cause meat spoilage, even at chill temperatures [3]. The ability of *Brochothrix thermosphacta* to grow in aerobic and anaerobic conditions classifies it in the group of microorganisms responsible for the appearance of unpleasant odors [4]. Bacteria of the genera *Serratia*, *Enterobacter*, *Proteus* etc., belonging to the family *Enterobacteriaceae*, as well as lactic acid bacteria, contribute to the spoilage process of chilled meat [5, 6]. Although microorganisms play a significant role in the occurrence of meat spoilage, the final assessment of changes is based on sensory analysis [3]. In assessing the shelf life of meat and meat products, it is necessary to apply a combination of microbiological, chemical and sensory analysis.

Modified atmosphere packaging (MAP) is used as an effective way to extend the shelf life and preserve the quality of fresh and minced meat [7, 8, 9, 10, 11, 12]. MAP is a type of packaging from

which the air is completely removed, after which the newly formed vacuum is replaced with a single gas or mixture of gases. The most commonly used gases in a modified atmosphere are carbon dioxide (CO_2), oxygen (O_2) and nitrogen (N_2) [13]. They are used in various combinations, in which each of them has a role to play. Because of its ability to significantly extend the shelf life of the meat, MAP has become, over the last two decades, a significant and increasingly popular technology in the field of meat packaging for retail [7].

Color, oxidative changes of lipids and growth of microorganisms are the most important quality criteria to be evaluated regarding the storage of fresh meat. Packing meat in a modified atmosphere should prevent discoloration and oxidation of meat lipids, and at the same time, it should slow down the growth of microorganisms responsible for meat spoilage. That is why the most common modified atmosphere for packaging fresh meat consists of 70–80% O_2 and 20–30% CO_2 [14].

Use of high concentrations of oxygen inside packaging preserves meat's bright red color, which is the most important sensory characteristic of fresh meat in retail, but at the same time it can bring an increase in oxidative reactions and the occurrence of rancidity of fat in the meat, which causes an unpleasant odor and taste. Zakrys et al. (2008) [15] monitored the effect of packaging in a modified atmosphere on sensory properties of beef, and came to the conclusion that the meat packaged in a modified atmosphere with 50% O_2, 30% N_2 and 20% CO_2 has more desirable sensory properties than when other gas mixtures were used.

Carbon dioxide is a major antimicrobial factor in MAP, especially against Gram-negative bacteria, such as *Pseudomonas* spp. The effectiveness of this gas depends on its original and final concentration in packaging, the storage temperature and initial microbiota of the meat [7]. Higher CO_2 concentrations in gaseous mixtures favor the growth of lactobacilli, and reduce growth of *B. thermosphacta* [16].

In retail stores in Serbia, ćevapčići are very common as part of the range of meat products. They belong to the group of shaped minced meat products. However, ćevapčići packed in a modified atmosphere are rarely found on the Serbian market. The goal of this work was to establish the shelf life of packaged ćevapčići in a gas mixture consisting of 70% oxygen and 30% carbon dioxide. The microbiological, sensory and chemical characteristics of ćevapčići were monitored during cold storage.

2. Materials and Methods

Ćevapčići were made from beef and pork meat that were minced to a granulation of about 4 mm, with the addition of water, table salt, spices, emulsifying salts (sodium polyphosphate) and antioxidants (ascorbic acid). After shaping, ćevapčići were packaged in a modified atmosphere, ten pieces in one package. A Traysealer T-350 Multivac packaging machine was used, containers were Cryovac LidSys (Sealed, Air, USA) and top foil F-Type, Lid HB-S (manufactured by Spektar, Gornji Milanovac) with the following characteristics: degree of oxygen permeability (<15 cm3/m2/day, 20°C, 65% RH), moisture permeability (<50 g/m2, 38°C, 90% RH). Packages were filled with a ready-made mixture of gases from the MESSER TEHNOGAS (70% O_2 and 30% CO_2). The amount of gas mixture in the package was 100–200 ml per 100 g of product. The same day, packaged ćevapčići were transported under cold chain conditions to the laboratory at the Institute of Meat Hygiene and Technology, where they were stored at 3°C for ten days.

2.1. Microbiological analysis

Samples of ćevapčići were microbiologically examined by the following methods:

a) Total number of aerobic mesophilic and psychrophilic bacteria was determined by the SRPS EN ISO method 4833: 2008 (PCA, Merck) [17]

b) Number of bacteria in the family *Enterobacteriaceae* was determined by the method SRPS ISO 21528-2: 2009 (VRBG, Merck) [18]

c) The presence of *Salmonella* spp. was determined by the method SRPS ISO 6579: 2008 (BPW, MKTTN, RVS, XDL, Merck) [19]

d) The number of *E. coli* was determined by the SRPS ISO method 16649-2: 2008 (TBX, Oxoid) [20]

e) The number of lactic acid bacteria was determined according to method ISO 15214: 1998 (MRS, Merck) [21]
f) The number of coagulase-positive staphylococci was determined according to method SRPS EN ISO 6888-2: 2009 (ETGP, Merck) [22]
g) The number of *Brochothrix thermosphacta* was determined by the method ISO 13722: 1999 (STAA agar, Oxoid) [23]
h) The number of *Listeria monocytogenes* was determined by the method SRPS EN ISO 11290: 2010 (Fraser broth base, Palcam agar, Oxoid) [24]

2.2. Evaluation of sensory properties

Sensory properties were evaluated using a quantitative descriptive test (SRPS ISO 6658, 2002) [25], with a scale from 1 to 5 (Table 1) (color and odor before thermal treatment, smell, texture and taste after thermal treatment) on the 1st, 4th, 6th, 8th and 10th days of the study. A group of five evaluators evaluated the sensory properties of the samples. Evaluators were previously chosen and trained by tests for determining the sense of taste (SRPS ISO 3972, 2002) [26] and training assessors in detection and recognition of odors (SRPS ISO 5496, 2002) [27].

Table 1. Quantitative-descriptive scale used for evaluation of sensory properties of ćevapčići

Numerical score	Descriptive score
5	Exceptionally good
4	Very good
3	Acceptable
2	Barely acceptable
1	Unacceptable

2.3. Chemical analysis

The pH was determined by a standard method, SRPS ISO 2917/2004 (pH meter Cyber Scan 510) [28]. The pH of the samples was analyzed daily. The acid value and peroxide numbers were determined on the fourth and eighth days of the study, and the following methods were used:

a) SRPS ISO 660, 2000 – Oils and fats of vegetable and animal origin - Determination of acid number and acidity [29];
b) SRPS ISO 3960, 2001 – Oils and fats of vegetable and animal origin - Determination of peroxide number [30].

2.4. Statistical analysis

Test results (mean, measures variations, analysis of variance) were statistically processed using Microsoft Excel 2007.

3. Results and Discussion

Changes in the numbers of tested microorganisms in samples of ćevapčići are shown in Figure 1.

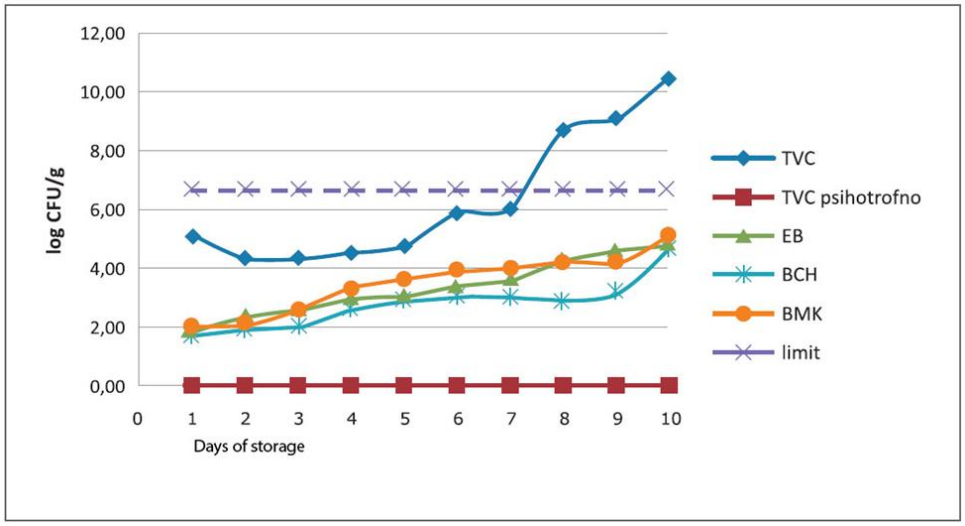

Figure 1. Changes of counts of various microorganisms in ćevapčići during storage
TVC – Total count of aerobic mesophilic microorganism; TVC – Total count of aerobic psychrophilic microorganisms; EB – Bacteria of the *Enterobacteriaceae* family; BCH – *Brochothrix thermosphacta;* BMK – Lactic acid bacteria

In ćevapčići during the storage period, no pathogenic microorganisms or growth of these organisms were detected (coagulase-positive *Staphylococcus*, *E. coli*, *L. monocytogenes*, *Salmonella* spp.). During storage of packaged ćevapčići at 3°C for ten days, the number of aerobic mesophilic bacteria showed linear growth trend of 0.66 log cfu/g/day, where on the eighth day of storage, the growth became exponential and numbers exceeded the recommended eligibility limit of 7 log cfu/g (ICMSF, 1986) [31]. For *Enterobacteriaceae*, lactic acid bacteria and *B. thermosphacta*, gradual growth by the tenth day of storage was found. The total number of aerobic psychrophilic bacteria during the storage period did not exceed 1 log cfu/g. Ozlem et al. (2011) [12] found that modified atmosphere with 70% O_2 and 30% CO_2 favors the growth of lactobacilli and bacteria from the *Enterobacteriaceae* family in minced beef, and inhibits the growth of *Pseudomonas* spp. and *B. thermosphacta*. Ercolini et al. (2006) [32] studied growth of *Pseudomonas* spp., *B. thermosphacta,* lactobacilli and *Enterobacteriaceae* in fresh beef and found weaker growth of these microorganisms in samples packed in a modified atmosphere with 60% O_2 and 40% CO_2 relative to the samples stored in the air.
The results of the sensory evaluation of ćevapčići are shown are in Figure 2.

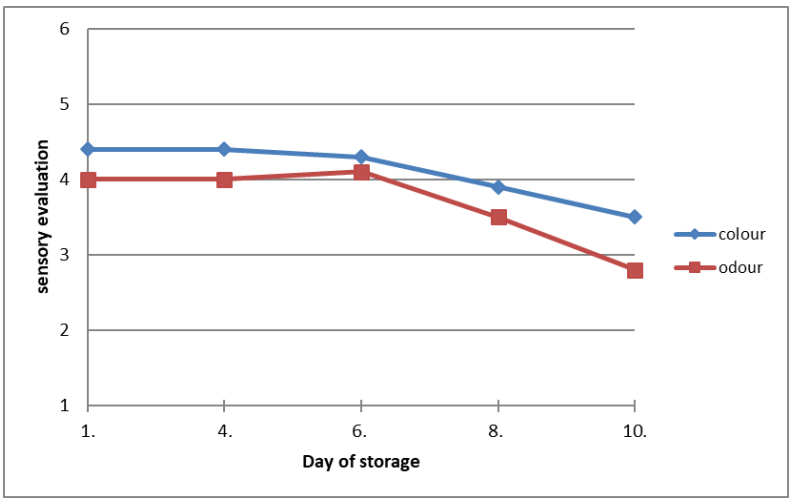

Figure 2. Changes of color and odor in ćevapčići during storage

The color and smell of ćevapčići were rated as very good (4.40 ± 0.22 and 4.00 ± 0.35, respectively) at the start of storage. The smell of ćevapčići on the last day of storage was rated as acceptable (2.80 ± 0.22) while the color was rated higher (3.50 ± 0.41). Results of sensory evaluation of odor, texture and taste of ćevapčići after the thermal treatment are shown are in Figure 3.

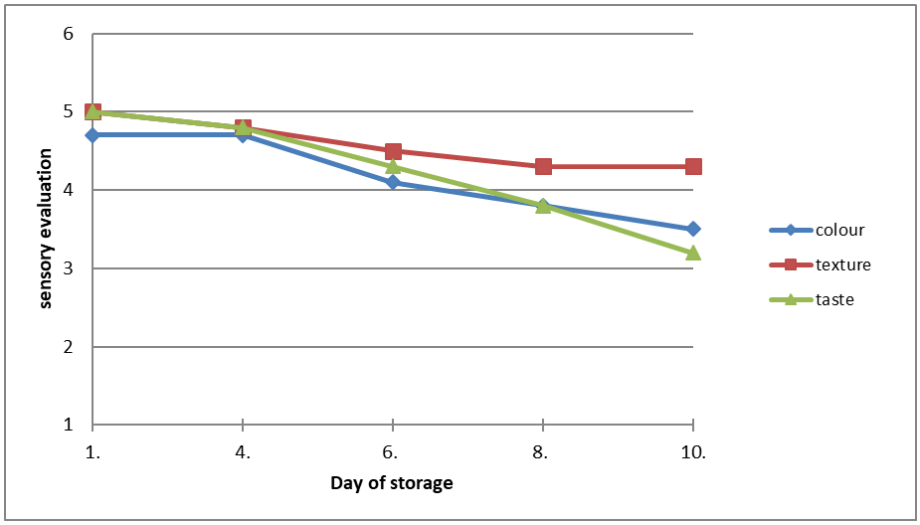

Figure 3. Changes of color, texture and taste in ćevapčići during storage

On the first and fourth day of the study, average odor grades after the thermal treatment were 4.70 ± 0.27 and 4.70 ± 0.22, respectively. The odor was rated as exceptionally good. During the storage, the average odor grade decreased after the thermal treatment so that on the last day of storage, it was 3.40 ± 0.27 and was rated as acceptable. After the thermal treatment on the first, fourth and sixth days of storage, the texture of ćevapčići was rated high (5.00 ± 0.00, 4.80 ± 0.27 and 4.50 ± 0.35, respectively), while on the eighth and tenth days of storage, slightly lower grades were assigned for texture (4.30 ± 0.44 and 4.30 ± 0.41, respectively). The taste of ćevapčići on the first and on the fourth days of storage was assessed as exceptionally good (5.00 ± 0.00 and 4.80 ± 0.27, respectively), and on the sixth and eighth days, the taste scores were lower (4.30 ± 0.27 and 3.80 ± 0.27, respectively). On the last day of storage, although rated lower (3.20 ± 0.25), the taste was still acceptable. Since the applied quantitatively descriptive scale defined grade 2.00 as the limit of acceptability, the taste scores obtained show the ćevapčići retained the desired sensory properties on the tenth day of storage.
The average pH of ćevapčići during the storage was 5.57 ± 0.08 with a coefficient of variation of 1.45%. Changes of pH in ćevapčići during the storage are shown in Figure 4.

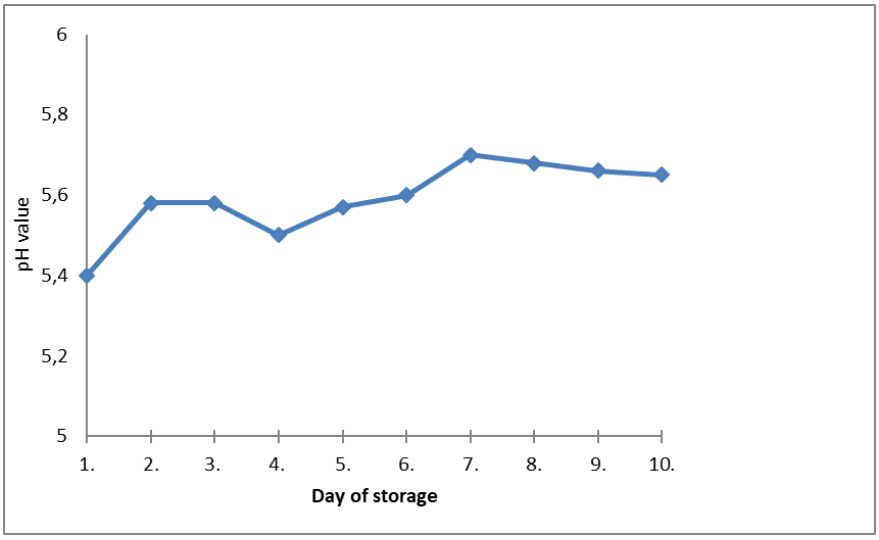

Figure 4. Changes of pH of ćevapčići during storage

In contrast to our results, Yilmaz and Demirci (2010) [10], for kebabs packaged in a modified atmosphere with 65% N_2 and 35% CO_2, found pH declined during the entire storage period.
Acid value and peroxide number changes are shown in Table 2.

Table 2. Acid value and peroxide number

Examination day	Acid value	Peroxide number
4th day	3.67 mg KOH/g	0.00 mmol/kg
8th day	3.74 mg KOH/g	0.00 mmol/kg

On the eighth day of storage, the acid value indicated that no hydrolytic changes of lipids had occurred in samples relative to the fourth day of testing, while peroxide values showed that no oxidative changes had occurred in lipids. Ozlem et al. (2011) [12] concluded that packaging of minced beef in modified atmosphere with 50% O_2, 30% CO_2 and 20% N_2 achieves the lowest degree of lipid oxidation. Jakobsen and Bertelsen (2000) [33] found that temperatures below 4°C in fresh meat packed in a modified atmosphere prevent lipid oxidation, which is in accordance with our results.

4. Conclusion

The total number of aerobic psychrophilic bacteria during the storage period of ten days did not exceed 1 log cfu/g. Gradual growth of bacteria from the family *Enterobacteriaceae*, lactic acid bacteria and *B. thermosphacta* was found up to the tenth day of storage. Based on acid values and peroxide number, it can be concluded that no hydrolytic or oxidative changes of lipids occurred in ćevapčići during storage. Also, sensory evaluation did not identify any changes in terms of rancidity, and the ćevapčići were acceptable in terms of color, smell, taste and texture throughout storage. Based on the results, and especially based on the recommended total number of aerobic mesophilic bacteria, which should not exceed 7 log cfu/g, and on sensory characteristics, ćevapčići packaged in a modified atmosphere with 70% oxygen and 30% carbon dioxide were still acceptable after seven days' cold storage.

Acknowledgement

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Detection and quantification of hepatitis E virus genome in pig liver samples originating from Serbian retail establishments

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Detection and quantification of hepatitis E virus genome in pig liver samples originating from Serbian retail establishments

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Abstract. Hepatitis E is considered an emerging human viral disease with a zoonotic nature, and domestic and wild pigs are the main reservoirs of hepatitis E virus (HEV) among animals. Pork liver is the target tissue of this virus. This study aimed to investigate the presence of HEV in commercial pig liver samples. Sixty samples were collected during one year from different retail outlets in Serbia. Furthermore, the collected samples were separated by four seasons, and every season included three months. The presence of HEV in the livers was examined by molecular analysis using RT-qPCR. The overall prevalence of the virus in analysed pig livers was 5%. HEV was detected in three livers, two in the first season and one in the second, while in the third and fourth season, no positive livers were detected. However, there were no statistically significant differences between the surveyed seasons. HEV was quantified in positive livers. Among positive livers, HEV concentrations ranged between 8×10^1 and 1.9×10^4 genome copies of the virus per gram. The presence of HEV in commercial pig livers indicates a potential risk for consumers. Appropriate heat treatment of meals during preparation is essential to eliminate the potential risk of developing the illness.

1. Introduction

Hepatitis E virus (HEV) is a pathogen that causes Hepatitis E disease. In addition to humans, HEV infection has been confirmed in several animal species, while the prime route of transmission is faecal-oral [1]. Furthermore, zoonotic HEV transmission was also confirmed by Meng et al. [1]. This epidemic is increasing worldwide and presents public health concerns in developing countries and developed countries [1]. During the previous decade, an increase in HEV confirmed cases were recorded in developed countries [3].

This virus is a small, non-enveloped icosahedral virus with a single-stranded, positive-sense RNA genome approximately 6.6-7.2 kb in size. It belongs to the *Hepeviridae* family and the *Orthohepevirus* genus[4]. Further, HEV has been classified as the *Orthohepevirus A* species, encompassing seven genotypes[4]. Only the first four (1-4) can infect humans[5]. Generally, it has been recognised that HEV genotypes 1 and 2 are specific for waterborne transmission, while genotypes 3 and 4 are common to humans and other animal species[6]. Endemic regions for genotypes 1 and 2 are Asia, Africa, and Central America, where the water is a fundamental reservoir of the virus.

On the contrary, in the developed countries, HEV transmission primarily occurs through food. The domestic pig (*Sus scrofa domestica*), wild boar (*Sus scrofa*) and deer (*Cervidae*) are considered the essential animal reservoirs of HEV[7]. Meat consumption is increasing worldwide due to rapid population growth and urbanisation[8][9]. Consequently, the risk of contamination increases since meat,

tissues and meat products from these animal species are the primary sources of HEV[7]. In the modern world of hedonism, "new" food or locally specific food characteristic of specific regions is increasingly attractive [10]. For instance, *Figatellu*, a customary type of sausage made from raw pork liver, was the primary source of some acute hepatitis E cases[11]. The liver is the target organ for HEV, and because of that, this organ and its products are the most apparent sources of the virus. The presence of HEV in liver and liver products has been confirmed by many studies, while the prevalence of HEV has varied from study to study[12][13][14]. In Serbia, according to Milojević et al., HEV was detected in 34% of liver samples of pigs younger than three months, while in retail establishments, it was not detected[14].

The food-borne route is specific because most pigs infected with HEV do not have visible symptoms and enter the abattoir as healthy animals[15]. As a result, their tissues and meat go into production, posing a risk to human consumers. Usually, humans infected with HEV are asymptomatic[16]. However, after an incubation period between 2 and 8 weeks, a certain percentage of patients have symptoms such as abdominal pain, vomiting, icterus with nausea, fever and hepatomegaly[1]. Approximately 2% of human cases are lethal[1]. Like other diseases, immunocompromised persons are particularly at risk, especially pregnant women, with fatal outcomes rising to 25%[17].

Given these facts, this study's objectives were to detect and quantify HEV from pig livers originating from retail establishments on the territory of Serbia.

2. Materials and methods

2.1. Commercial pig liver samples

The sampling was performed between January and December 2019 from various retail establishments in Serbia. The one-year period was divided into four seasons, while each season included three months. A total of 60 pig livers were sampled, enclosed in sterile containers, stored in an insulated icebox and transferred in the shortest possible time to the laboratory. All pig liver samples were then collected in sterile 50 mL Falcon centrifuge tubes and stored in a deep freezer at -20°C until further processing.

2.2. RNA extraction

An amount (100 mg) of each liver sample was homogenised in 1 mL of Trizol (Invitrogen, USA) and 600 µg of zirconia beads using a BeadBeater homogeniser (Biospec, USA). Next, 200 µL of chloroform was added. The mixture was vortexed for 2 min, incubated for 10 min at room temperature, and centrifuged at 12,000×g for 10 min at 4°C. Phase separation was facilitated using phase-lock heavy gel tubes (5 Prime, Germany). The upper aqueous phase was collected and stored at -70°C until RNA extraction. Total viral RNA was extracted from samples using RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions.

2.3. Detection of HEV RNA

A real-time PCR (RT-qPCR) assay was developed to detect HEV3 genotype, using previously published but modified primers and probe[18]. Primer HEV3-f was partially changed with the replacement of guanine (G) with the degenerate nucleotide "R" at position 5323 (nucleotide position determined based on the HEV genome registered in GenBank under accession number AF060669). The HEV3-r primer and HEV3 probe were not changed. Primers and probe were developed in the highly conserved, overlapping ORF2/3 region of the HEV genome. TaqMan probes were labelled with the fluorophore and quencher molecules FAM/Blackhole Quencher 2 (Microsynth, Switzerland). TaqMan RT-qPCR (RNA UltraSense One-Step Quantitative RT-PCR System, Invitrogen, USA) was performed in 20 µL reaction volumes (Table 1) and 5 µL of total RNA was extracted from each sample.

Table 1. TaqMan RT-qPCR master mix

Ingredients	Volumes
5× Reaction Mix	5 µL
Enzyme mixture	1.25 µL
Forward primer	0.5 µM
Reverse primer	0.9 µM
Probe	0.25 µM

The reactions were carried out in 96-well optical reaction microplates (Agilent, USA) in an AriaMX RT-qPCR machine (Agilent, USA). RNAs were reverse transcribed and amplified according to the following program: 1 cycle at 55°C for 60 min and 95°C for 5 min, followed by 50 cycles of 95°C for 15 s, 60°C for 60 s and 65°C for 60 s. Positive and negative controls were included in each run. All samples with a cycle threshold value (Ct) for detecting HEV lower than 40 were interpreted as HEV-positive, and all other samples were interpreted as HEV-negative.

2.4. Quantification of HEV in the positive samples

To quantify genome copies of the virus per gram (g.c./g) in the positive liver samples, a synthetic molecule (RNA transcript) was constructed. Complementary DNA to hepatitis E virus (GenBank acc. No. MG051653), 71 nucleotides long, was cloned into pEXA2 vector (Eurofins, Germany), then cloned into *E. coli* One Shot aTOP10F (Invitrogen, USA). The target sequence was used to generate the standard curve; curves with a slope lying between -3.1 and -3.6 and an R2 ≥ 0.98 were used for quantification.

2.5. Statistical analysis

Statistical analysis of the performed experiment was done in the statistical package GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, California, USA), www.graphpad.com, and MS Excel.

3. Results and discussion

During a one-year period, sixty liver samples from different retail stores were examined for the presence of HEV using RT-qPCR (Figure 1). Over the year, the prevalence of HEV in all liver samples examined was 5 % (3/60).

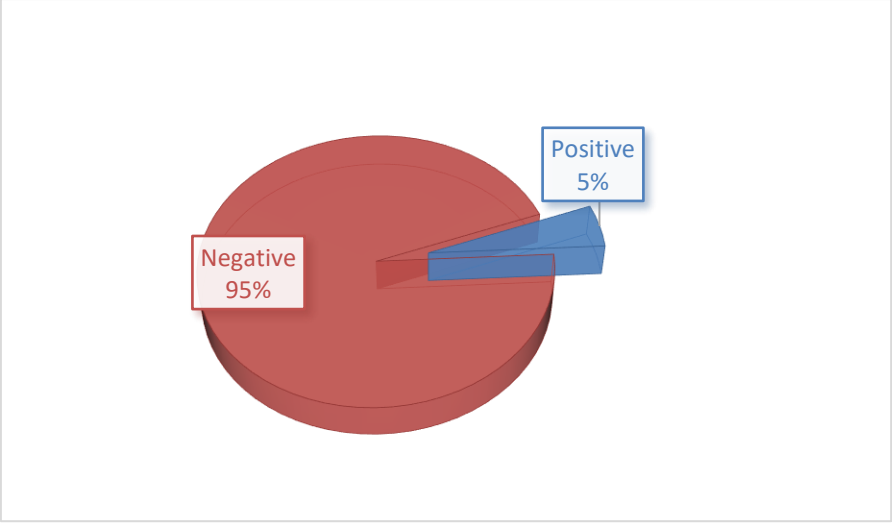

Figure 1. Percentage of HEV-positive pig livers in the examined samples

When we analysed the obtained data by seasons, we determined that during the first season, of the 15 samples tested, two (13.33%) were positive (Figure 2). Among 15 liver samples examined in the second season, 6.67% (1) was HEV-positive. During the third and fourth seasons, HEV was not detected in the examined pig livers. Therefore, statistically significant differences in HEV prevalence between surveyed seasons were not confirmed. Furthermore, HEV was quantified in the three positive pig livers. Among positive pig liver samples, the HEV concentration ranged between 8×10^{1} and 1.9×10^{4} HEV g.c./g.

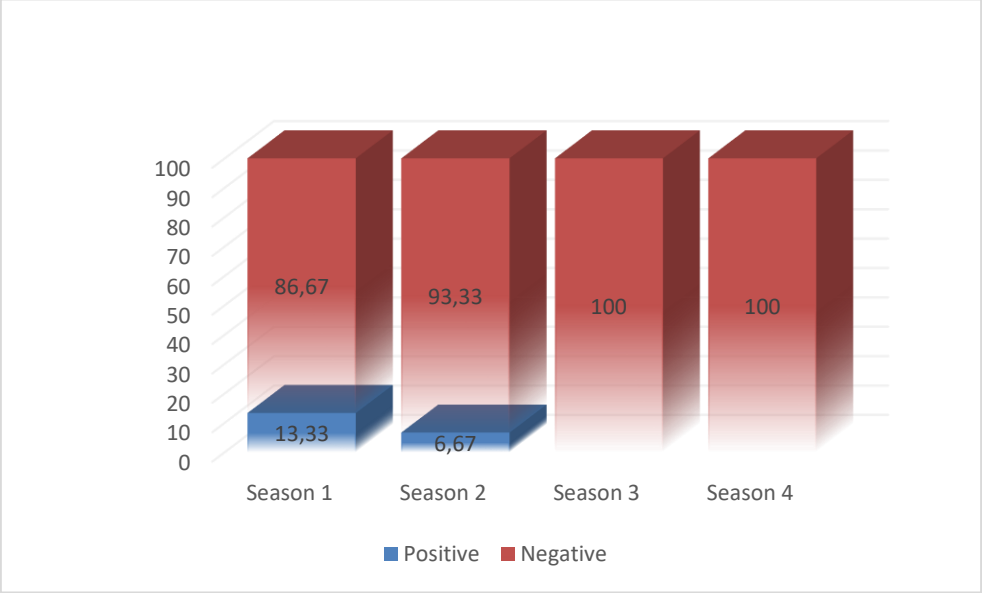

Figure 2. Percentage of HEV-positive pig liver samples according to annual season

The percentage of HEV-positive pig livers originating from Serbian retail establishments in this research was similar to the results of testing commercial liver samples published so far. Wenzel et al. published results wherein the HEV prevalence in liver samples from German retail stores was 4% [19]. Also, in Germany, approximately the same prevalence (5%) for HEV in commercial pig liver samples was reported [19]. Similarly, results obtained by Bouwknegt et al. showed that among tested commercial liver samples in the Netherlands, 6.5% of them were positive for this virus[20]. Besides that, in the United Kingdom, among 80 tested liver samples, HEV was not detected [21]. In Canada, a group reported that 10.5% of tested pork products were HEV positive[22]. Overall, the HEV prevalence was approximately the same in all studies, regardless of country. However, some differences in prevalence were observed for some studies (countries) compared to others. These differences in HEV prevalence between studies can be because of a different national prevalence of HEV among pigs or because of differences in detection procedures. For example, differing RT-qPCR protocols can have different sensitivity levels. Still, in most of the studies, HEV was detected in pig-origin samples, and HEV presence in commercial pig livers was confirmed.

The exact infectious dose of HEV required to infect humans has not yet been determined; however, according to the French Agency for Food, Environmental and Occupational Health and Safety (ANSES), the infective dose for the oral route in humans is approximately $10^{5.5}$ g.c**Error! Reference source not found.**. The results obtained in our research show that the average HEV concentration in positive pig livers was 6.7×10^{3} g.c./g. Feurer et al. published similar results to ours, in which the average HEV

concentration in HEV-positive pig liver samples was 1.3×10^5 g.c./g[23]. Boxman et al. reported HEV concentrations in positive pork sausages ranged between 2.1×10^2 and 1.2×10^6 g.c./g[24]. Quantitative data are an essential parameter in risk assessment, especially in ready-to-eat products. For raw meat products that are cooked, adequate heat treatment is the most critical process to reduce HEV numbers, because heating for 5 min at 71 °C is enough for to inactivate the virus. This type of heat treatment will reduce the risk and enable safe food to be consumed.

4. Conclusion

The presence of HEV in liver samples from retail establishments can be an essential risk to food safety for consumers, especially for immunocompromised groups, including pregnant women as the primary at-risk group. The results of this study warrant the start of monitoring to follow HEV RNA levels in pig livers and pork products over time for risk assessments and risk management purposes. Routine coordinated surveillance of viral epidemics and surveillance of HEV in food products, combined with systematic of typing strains, and joint expertise of veterinarians, food and clinical microbiologists is recommended to help study source and identify risk prevention measures. Furthermore, strict implementation of good hygienic practices at all stages of the food chain and hazard analysis and critical control point (HACCP) procedures is required to decrease the HEV concentration in food and prevent further dissemination of this pathogen. In regions with high HEV prevalence among pigs, vaccination of pig herds could be an excellent measure in combating the spread of this zoonosis.

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The feasibility of animal source foods' color measurement using CVS

The feasibility of animal source foods' color measurement using CVS

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Abstract. Color assessment of animal source foods was investigated using a computer vision system (CVS) and a traditional colorimeter. With the same measurement conditions, color readings varied between these two approaches. The color measured by CVS was highly similar to the actual color of animal source foods, and ranged from 75.0%-100.0% of actual colors, whereas colors read by a Minolta colorimeter showed non-typical appearances. The CVS-obtained colors were more similar to the color of food visualized on the monitor, compared to colorimeter-generated color chips. Considering these results, it could be concluded that the CVS is a superior alternative for replacing traditional devices by providing better accuracy.

1. Introduction

Animal source foods provide numerous essential compounds in human nutrition [1-3]. Regardless, color is still the most significant sensory attribute when it comes to consumers' food decisions [4]. It is a vital tool in marketing, especially in food marketing [5].

As far as meat color is considered, darker color is less preferred by customers, who connected it with a lack of quality [6]. This physical attribute can be a measure of some imperfection in milk, such as adulteration [7] or spoilage [8] and can indicate long-term storage conditions [9]. Moreover, consumers desire yellow-orange egg yolk rather than off-white yolk [10-11].

Objective color evaluation is crucial for food technology. Currently, the most common color measurement devices are Minolta colorimeters [12, 13]. These devices offer simple and fast food color analysis, and moreover, they are easy to handle and calibrate. Each colorimetric instrument has various settings influencing food color parameters such as color system, illuminant, observer and port size. However, only a few researchers reported all the procedures and technical parameters used for meat and milk color determination, as stated by Tapp *et al*. [12] and Milovanovic *et al.* [13]. The majority of papers reported using illuminant D65, 8 mm aperture size and 10° standard observer for instrumental color measurement of milk and meat.

However, the colorimeter has numerous drawbacks regarding inability to capture broad spectral information related to internal characteristics of subjects [14] and a requirement for subjects with homogenous color [15]. In general, these color devices require homogeneous and uniform samples to achieve consistent analysis [16]. To overcome shortcomings of colorimeters, a new, alternative method, the computer vision system (CVS), has been developed. By applying the CVS, the color readings can be determined for each pixel of a sample image, and the technique is rapid, cost-effective and simple [17]. Additionally, CVS has been widely used for color measurement of animal source foods [18-22].

2. Materials and Methods

Sample preparation, color measurement devices used, sensory tests by a trained panel and statistical analysis performed were all explained in previous publications [18-22].

3. Results and Discussion

3.1. Meat and meat products

Instrumental color data (L*a*b*, hue and chroma) for meat and meat products were significantly different when collected by two methods (CVS and colorimeter) [18-21].

Regarding poultry meat, chicken and turkey (lighter colored poultry meat) color acquired by CVS had higher L*, a* and b* coordinates as compared to the colorimeter measurements. Moreover, the color of duck and goose (darker colored poultry meat) had a lighter, more green and blue (with the exception of duck) appearance when read by colorimeter than when acquired by the CVS. The total color difference was 18.50 (chicken) and 22.04 (turkey), so it can be concluded that the two methods accessed the color of these two meats as significantly different and even contrasting. However, the total color differences between the two color devices for goose and duck were half the differences calculated for chicken and turkey [18].

As far as game meat is concerned, wild boar and deer meats (darker colored game meat) as acquired by CVS were darker, redder and yellower (with the exception of deer meat) than the color measured by the colorimeter. On the other hand, quail, pheasant and rabbit meat (lighter colored game meat) color acquired by CVS had higher lightness than the colorimeter readings. All redness values were much higher when acquired by CVS compared to colorimeter measurements, meaning the colors acquired by CVS were more red (or less green). Total color differences ranged from 9.7 (pheasant) to 19.0 (rabbit) [19].

In the study of pork, high lightness, less redness, and relatively high yellowness indexes of pork meat were measured using colorimeter compared to the CVS-acquired colors. In the case of pork meat and fat parts, total color differences were 16.7 and 10.8, respectively [20].

Considering beef meat, the color attributes of a*, b*, and chroma values acquired by the CVS were higher than those measured by colorimeter. Total color difference was 15.1 (beef meat parts) and 13.0 (beef fat parts), indicating that the colors assessed by the two methods were opposite [20].

Regarding meat products, uniformly-colored meat products, when color was acquired by CVS, had a lighter appearance (except beef prosciutto), or were more red and less yellow (except for pork prosciutto and raw sausage) than the color measured by colorimeter. Furthermore, when acquired by CVS, bi-colored meat products [21] had a darker color for meat segments (except for Mortadella) and brighter color for fat segments compared with the colorimeter measurements. The variance between the two systems was in line with total color differences (which in meat segments ranged from 7.3 to 14.8 and in fat segments ranged from 7.4 to 12.9). The color results of non-uniformly colored meat products also showed the large differences between the two devices. The highest total meat-parts color difference (20.3) was observed for beef fermented sausage, and maximum total fat-parts color difference (35.3) was observed for fermented pork sausage [21].

3.2. Milk and milk products

The color coordinates of milk and milk products were statistically different when determined by the two devices (CVS and colorimeter) as reported by Milovanovic *et al*. [22].

In terms of milks, the samples seemed lighter and redder when CVS was used than when the colorimeter was used. On the other hand, all milks had higher b* readings, resulting in more yellow milk appearance, when read by the colorimeter than when acquired by CVS. According to the color difference scale, these two different devices provided easily perceptible total color difference, from 4.3 (cows' milk and goats' milk) to 5.6 (sheep's milk) [22]. The color parameters of raw milks read by colorimeter were in line with the literature color data reported by Milovanovic *et al*. [13].

Overall, dairy products with a dominant white color read by colorimeter had higher L* (brighter color), lower a* (greener color) and higher b* readings (yellower color) as compared to the CVS-acquired colors [22].

The color of white cheeses assessed by colorimeter was lighter than color acquired by CVS. White cheeses were closer to the red and blue region when color was acquired by CVS as compared to the green and yellow region read by the colorimeter. Color differences ranged from 11.3 to 11.8 [22].

As regards liquid fermented dairy products, all L* and b* readings read by colorimeter were higher than by CVS, whereas a* readings were more in the redness region when color was acquired by CVS compared with colorimeter-produced color. The color variations were in line with the color differences and ranged from 5.8 (yoghurt) to 6.6 (kefir) [22].

Color determinations using the two devices for color detection of sour cream and heat processed cream were significantly different. Moreover, using the colorimeter resulted in a brighter, greener and yellower appearance as compared to the color acquired by CVS. The total color differences ranged from 6.7 (heat treated cream) to 11.0 (sour cream) [22].

When it comes to the skim milk powder, there was a significant difference between colorimeter and CVS color readings. L* measured by colorimeter had higher values than L* acquired by CVS. On the contrary, all a* values acquired by CVS were higher (more red) than those measured by the colorimeter. Yellowness values measured by the colorimeter were higher (yellower appearance) than those acquired by the CVS [22].

With regard to the lightness observations of kajmak spread, the colorimeter produced higher values (brighter appearance) than the CVS [22]. All a* values acquired using CVS were less green, in contrast to the colorimeter-measured color, whereas all the b* values indicated a more yellow color with the colorimeter, in comparison to the CVS. The overall color difference was 9.5, indicating the difference in suggested color would be perceptible at a glance.

Dairy products with a dominant yellow color, on color acquisition by the CVS, showed darker (apart from Grana Padano), more red and more blue appearance [22], as compared to the colorimeter. All yellow cheeses, except Grana Padano, on color acquisition by the CVS, showed darker color than was measured with the colorimeter. Regarding a* observations, the CVS resulted in more red appearance, or colors obtained by the colorimeter were less green. The total color difference ranged from 6.0 for pasta filata to 14.9 for processed cheese, indicating large color differences [22].

Regarding butter color, a* values acquired by CVS were higher than those measured by the colorimeter, indicating a less red appearance. In contrast, yellowness data were higher with the colorimeter than by CVS. There was a great total color difference, 11.8 [22].

Apricot fruit yoghurt had different color data as read by the colorimeter and the CVS [22]. Colorimeter-generated color was lighter in terms of L* value. Furthermore, the redness parameter was higher with the CVS than with the colorimeter. Yellowness was higher with the colorimeter than with the CVS, denoting a more yellow appearance of this fruit yoghurt.

The color of whey powder as acquired by the CVS was significantly darker, more red and less yellow compared with colorimeter-measured appearance. The total color difference was 17.1, indicating a large color difference [22].

3.3. Eggs

The color parameters of eggshell measured by the two approaches were statistically different with some exceptions (L* reading for quail's eggshell and WI for turkey's eggshell). The color of eggshell gathered through the colorimeter depicts a brighter, less red and more yellow appearance than the CVS-acquired color.

Regarding the color of egg yolk, the colorimeter produced a lighter (except goose egg's yolk), more green and less yellow color, whereas the CVS indicated the appearance of albumen as lighter (except quail's egg albumen), more red and less yellow than the colorimeter.

4. Conclusion

Even if the same subjects and parameters for color evaluation were studied, significant differences were observed in the color properties measured by the two systems. The colorimeter was less representative and less precise for measuring the color of animal source foods. The reason for this was light penetration, the amount of which related to the device used. In the CVS, the lamps are placed 50 cm above the subject and the light hits the surface and only penetrates a few mm into the subject, whereas the colorimeter is positioned onto the subject surface, and the light penetration through the food matrix must be higher than for CVS. Therefore, the CVS should be seriously taken into account as an effective and more powerful alternative to the colorimeter and as a non-contact tool for measuring the color of animal source foods.

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Chemical quality parameters (water, protein, fat, NaCl ash and nitrites) in fermented sausage with the addition of *Yersinia enterocolitica*

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Chemical quality parameters (water, protein, fat, NaCl ash and nitrites) in fermented sausage with the addition of *Yersinia enterocolitica*

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Abstract. The connection between diet and health was known to the oldest civilizations. Meat processing has influenced the increase of meat consumption, protein utilization and its energy value. Fermented sausages which are prepared in a safe way are rich in animal proteins and are an important part of the diet. Meat and adipose tissue are the basic ingredients in the production of fermented sausages, along with spices and salts, and other additives depending on the specifics of the product. Ripening is a process that separates raw sausages from all other types of sausages, and the quality of raw materials and their treatment directly affects the quality of the finished product, because the process of fermented sausage production is dominated by biological and biochemical processes. Different types of foods can cause food-borne illnesses, one of which is zoonotic yersiniosis caused by *Yersinia enterocolitica*. Due to the importance of *Yersinia* in meat, the European Food Safety Authority (EFSA) recommended that in addition to controlling the presence of *Salmonella*, it is mandatory to examine pork carcasses for the presence of *Y. enterocolitica*.

1. Introduction

The genus *Yersinia* belongs to the family *Enterobacteriaceae*. In this genus, 3 of the 12 species are pathogens, among them *Y. enterocolitica*, for which 6 biotypes are recognised (5 pathogenic and 1 non-pathogenic). This Gram negative, facultative anaerobe grows in a wide range of temperatures: optimal 28-29°C, minimum -2°C. *Y. enterocolitica* can thrive in cool temperatures as well as warmer ones, so it can grow easily in refrigerated environments. This bacterium has the ability to growth from -2°C to 42 °C. The sources of this pathogen include contaminated water and the intestinal tracts of any infected humans or animals. It is frequently found in pigs. The foods associated with this pathogen are water, unpasteurized milk, meat (especially pork), and seafood. Disease symptoms can last for 1 to 3 weeks [1-3].

To avoid exposure to *Y. enterocolitica*, a number of procedures should be followed. Be sure to wash your hands after handling raw meat, especially pork. Avoid cross-contamination, and make sure you always drink water from a clean source. Avoid drinking milk that has not been pasteurized. Coming in contact with the bodily liquids of any infected animal can also spread the bacteria, so you should be careful and wash your hands after disposing of animal waste. Also, be sure to cook any meat products thoroughly to a safe temperature. [4-6].

2. Fermented sausages

Some meat products are very stable and do not need to be stored in refrigerators. Such are fermented products, in which the growth of microorganisms is inhibited by a very low moisture content. This type of meat product is not subject to heat treatment and is, therefore, often referred to as raw fermented sausages [1,3]. They are still of great importance in regions and conditions where it is difficult to provide a cold chain for transport and storage [1]. The production of dry, fermented sausages in Europe dates back to ancient Rome, and from the Mediterranean area [7,8], from where it spread to Germany, Hungary and other countries including the USA, Argentina and Australia [7]. In our region, dry fermented sausages arrived at the beginning of the 19th century from Italy, across the Pannonian plain [2,3].

Traditional production of fermented raw sausage in households in Serbia takes place under uncontrolled conditions (temperature, humidity, and fermentation) during colder seasons [8]. This process relies on the activity of fermentative bacteria that are naturally present in the meat and the environment of the production area [7,9-13]. Preservation of traditional production and authentic products can help small producers and local economies, but it is also important to preserve the traditional knowledge, cultural heritage and regional identity in what are often small and underdeveloped areas [5,8,10,14,15].

Today, in industrial production, these conditions are provided in air-conditioned plants and production is no longer conditioned by climatic conditions.

3. Basic chemical parameters of quality properties (water, protein, fat, NaCl, ash and nitrites) in fermented sausage with inoculated *Yersinia enterocolitica*

In Central and Northern Europe, the reduction of pH in fermented products is much more important for conservation, while in Mediterranean countries, the reduction of water activity (a_w) is more significant [16,17]. In foods which have a neutral pH, stored at a temperature of 5 ºC, there is a possibility that the number of *Y. enterocolitica* will increase in a short period of time [12]. The presence of organic acid reduces the ability of *Y. enterocolitica*. *Y. enterocolitica* has the potential to multiply during storage of meat and meat products. *Y. enterocolitica* can grow at refrigerator temperature, in vacuum packaging or in modified atmosphere packaging. Foods that are rich in protein can be spoiled with bacteria which create hydrogen peroxide [18].

During ripening, the content of protein, fat, ash and salt increases due to drying (due to the loss of moisture). The content of nitrates and nitrites constantly decreases due to bacteria that reduce them to nitrogen monoxide. The influence of table salt on the microbiological processes in sausage is related to the influence of salt on the amount of water available to microorganisms, and also salt is used as a carrier for nitrite and sodium nitrite [1,3]. The amount of table salt added to fermented sausages is from 2.4 to 3.0% [3], and usually is between 2.8 and 3.2% [2]. In addition to the direct effect on the taste of the product, the table salt participates in the aromatic properties of other meat ingredients, colour and affects the microbiological processes in sausage [1-3]. The chemical composition of sausage fillings is on average: water content 55.36%, fat 25.71%, protein 15.45%, ash 3.54% and water 30.1% [15].

Regarding the fats, much attention is given to the relationship between meat consumption and cardiovascular diseases, cancer, diabetes and obesity [6]. However, adipose tissue is a traditional and necessary ingredient of sausages, because it helps to bind various ingredients and participates in creating the specific taste of sausage. Adipose tissue as a component of sausage stuffing affects the quality of fermented sausages. If the adipose tissue is fresher, with a lower content of polyunsaturated fatty acids,

the products have a better sensory quality [2]. Sausages that are produced with fatty tissue with a higher content of polyunsaturated fatty acids have less weight loss after drying [2,3]. During the long-term ripening of sausages, chemical changes can occur that can lead to rancidity of sausages. [18]. The development of rancidity can be slowed down by proper selection of fat for production [1-3]. Peroxides, formed by the oxidation of fatty acids, oxidize myoglobin and prevent the formation of nitrosyl-myoglobin, a pigment which is responsible for the stable colour of fermented sausages [3]. It is believed that for the production of home-made sausages, the optimal amount of adipose tissue is from 10 to 20% [19]. The composition of fats of different animal species gives a characteristic aroma and taste of these products [16].

Inactivation of pathogenic bacteria during the ripening of sausages requires the control of their growth, so ripening is a key step in the production of fermented sausages [20,21]. In a study on fermented sausages that were experimentally contaminated with *Y. enterocolitica* [15], during maturation, this bacteria was not detected in control sausages. In narrow-diameter sausages that were contaminated with *Y. enterocolitica*, this pathogen was detected by the 7th day while in the wider diameter sausages it was detected by the 18th day of maturation [15]. The control group of sausage with wider and narrower diameters showed similar values of the chemical parameters as the sausages without the addition of starter culture (statistically significant difference $p < 0.01$). The water content of dry fermented sausages is always below 35%, but also less than 30% in many cases, which corresponds to an a_w of 0.90; these low a_w levels prolong the shelf life of the product [1,3]. At the end of the fermented sausage production process, no significant differences were found between the examined chemical quality parameters of sausage for both narrower and wider diameter sausages. Testing of the basic chemical composition of the sausages was done on day 0 and at the end of the production process, and standard analytical methods for testing were used. ISO reference methods were used to test the basic chemical composition and physicochemical properties.

3.1. Basic chemical quality parameters of the narrower diameter sausages

The chemical composition of the sausages of narrower diameter: the average water content of sausages of narrower diameter is from 28.19 ± 0.37% to 29.34 ± 0.13%, fat from 40.03 ± 0.34% to 40.87 ± 0, 52%, protein from 25.42 ± 0.23% to 25.67 ±, 0.19%, ash from 5.17 ± 0.031% to 5.28 ± 0.045%, NaCl from 3.98 ± 0.036% to 4.09 ± 0.055%, nitrite from 0.05 ± 0.001% to 0.054 ± 0.001%. No statistically significant differences were found between the average values of the examined chemical quality parameters of the sausages of narrower diameter (difference between the average values is statistically significant at $p < 0.05$).

3.2. *Basic chemical quality parameters of the wider diameter sausages*

The chemical composition of the sausages of wider diameter: the average water content of sausages of wider diameter was from 28.45 ± 0.20% to 29.96 ± 0.19%, fat from 39.51 ± 0.178% to 39.92 ± 0.23 %, protein from 25.33 ± 0.145% to 25.38 ± 0.17%, ash from 5.198 ± 0.10% to 5.29 ± 0.07%, NaCl from 3.99 ± 0.83% to 4.08 ± 0.07%, nitrite from 0.05 ± 0.001% to 0.054 ± 0.001%. No statistically significant differences were found between the average values of the examined chemical quality parameters of the sausages of wider diameter (difference between the average values is statistically significant at $p < 0.05$).

4. Conclusion

During the production of the fermented sausages which are produced without heat treatment during ripening and drying, controlled conditions are used that stop the growth of pathogenic bacteria due to the simultaneous actions of several factors. There are numerous data in the literature on the chemical composition of fermented sausages [14,19,22-30]. The chemical composition of fermented sausages depends on the choice of raw materials, the proportions of muscle and fat tissue and their relationship. Dry fermented sausages should contain up to 35% water, meat protein content should be at least 20%, and collagen in meat proteins at most 15%, with the exception of poultry sausages and sausages placed on the market under a different trade name [31].

Our results show that all examined groups of sausages from this experiment meet this condition. In many European countries, increased demand for traditional products has been observed. However, the composition of fermented sausages differs between products and regions [32]. These products are foods with strong regional characteristics and origin, which should be protected and promoted as a part of the national traditions.

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Effect of α-tocopherol, rosemary extract and their combination on lipid and protein oxidation in beef sausages

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Effect of α-tocopherol, rosemary extract and their combination on lipid and protein oxidation in beef sausages

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Abstract. This study focuses on the oxidative changes in lipids and proteins of beef sausages with incorporated α-tocopherol, rosemary extract or their combination during 3 months' storage at 4°C. For this purpose, sausages were formulated with no antioxidant (Control, C), 200 ppm α-tocopherol (T), 200 ppm rosemary extract (R), and 100 ppm α-tocopherol + 100 ppm rosemary extract (TR). To observe oxidative changes in lipids; peroxide value, thiobarbituric acid reactive substances (TBARS), and total oxidation value (TOTOX), in proteins; sulfhydryl and carbonyl contents were measured. Use of antioxidants and storage time significantly affected oxidative stability of sausages ($P<0.05$). Antioxidants, individually or in combination, retarded lipid peroxidation and improve the oxidative stability of sausage during storage. The antioxidant combination showed synergistic effect on protein oxidation, as the lowest carbonyl contents were found in TR samples. As a result, a combination of antioxidants with different effect mechanisms could be the better option to prevent oxidative changes in meat products.

1. Introduction

Oxidation is the main non-microbial phenomenon in meat and meat products which creates undesirable changes in product quality, limits the shelf life and produces adverse health effects due to the development of toxic secondary oxidation products. Lipid and protein oxidations occur through free radical reactions, and due to their high amount of lipids and high concentration of prooxidants, meat products are highly susceptible to oxidative changes [1-3].

Use of antioxidants is one of the main strategies for preventing oxidation in meat products. Apart from synthetic antioxidants, natural compounds with antioxidative effect are proposed since they are safe, cheaper and consumers prefer natural sources to synthetic compounds [4]. Antioxidative effects can be achieved by different mechanisms, such as chelation of metals, scavenging free radicals, breaking chain reactions, and inhibition of lipid peroxidation [5].

α-tocopherol, an oil-soluble natural antioxidant, is widely used in the meat industry due to this compound acting as an electron donor and breaking the chain reactions. Previous reports showed the high antioxidative effect of α-tocopherol in meat products [6-8]. Rosemary (*Rosmarinus officinalis L.*) extract is an effective natural antioxidant for meat products due to its high amount of phenolic diterpenes and phenolic acids [9,10], and due to phenolic hydroxyl groups, rosemary extract can scavenge free

radicals and prevent lipid oxidation [11]. Previous studies have demonstrated the effects of using antioxidants individually in meat product formulations. However, the use of a combination of antioxidants with different action mechanisms could be a better way prevent lipid and protein oxidation.

To the best of our knowledge the use of α-tocopherol and rosemary extract in combination in beef sausages has not been studied yet. Therefore, the aim of this study was to investigate the effects of individually or combinative use of α-tocopherol and rosemary extract on lipid and protein oxidation in beef sausages.

2. Material and methods

Four different beef sausage formulations were prepared (Table 1). Minced beef and beef fat were purchased from a local butcher, α-tocopherol, and rosemary extract were obtained from Kimbiotek (İstanbul, Turkey). Minced beef, curing ingredients, and half of the ice were homogenized and ground for 1 min in a cutter (Alpina, Switzerland). Beef fat, α-tocopherol, and/or rosemary extract (depending on formulation), other ingredients, and the remaining of the ice were added to the meat mixture, and batters were homogenized for 3 minutes to obtained sausage emulsion. Emulsions were stuffed into casings and smoked at 40°C for 2 hours (Afos, England) then heat treated in a boiling vessel until the core temperature reached 70°C. Once cooking was completed, sausages were cooled, vacuum packaged, and stored at 4°C for 3 months. Oxidative changes of lipids were determined in terms of peroxide values [12], thiobarbituric acid reactive substances (TBARS) [13], and total oxidation value (TOTOX) [14]. Protein oxidation was investigated by the determination of sulfhydryl [15] and carbonyl groups [16]. The effects of antioxidants and storage period were investigated by using two-way ANOVA analysis. Means were compared by using Duncan's Post-Hoc tests in the SPSS 23 software.

Table 1. Formulation of sausage samples

Sample	Beef (g)	Beef fat (g)	Ice (g)	α-Tocopherol	Rosemary extract
C	3000	1000	1000	-	-
T	3000	1000	1000	200 ppm	-
R	3000	1000	1000	-	200 ppm
TR	3000	1000	1000	100 ppm	100 ppm

* Sample denomination: C (Control group, without antioxidant), T (Sample with 200 ppm α-tocopherol), R (Sample with 200 ppm rosemary extract), TR (Sample with 100 ppm α-tocopherol and 100 ppm rosemary extract).
**Additives added to product formulation as 5000 g of products: 1.5% Salt, 0.15% sugar, 0.15% sodium tripolyphosphate, 0.09% ascorbic acid, 150 ppm sodium nitrite, 3% powdered milk, %3 sodium caseinate, %4 starch, 1.5% bread crumbs, spices: 0.9%

3. Results and discussion

As seen in Table 2, antioxidant and storage period showed a significant effect on peroxide values (PV), TBARS, and TOTOX of beef sausages ($P<0.05$). PVs of sausages were between 4.03 – 5.21 meqO_2/kg at the beginning of storage, but 8.11 – 14.11 meqO_2/kg at the end of storage (Table 3). Although some fluctuations were recorded during storage, in general, the impact of the storage period on PVs was significant ($P<0.05$). During storage, PV of C treatment increased continuously, PV of T sausages rose until month 2, and then significant decrement was observed ($P<0.05$). At month 3, the highest PV was recorded in C treatment ($P<0.05$). Antioxidant addition, whether individual or in combination, had a significant effect on peroxide content ($P<0.05$). Similar results were observed by Georgantelis et al. [17] wherein both α-tocopherol and rosemary extract were used in fresh pork sausages. In all cases, PVs of sausages were lower than 25 meqO_2/kg, which is described as the limit for fatty foods [18].

Table 2. Analysis of variance on the effect of antioxidants and storage time on lipid and protein oxidation of beef sausages (F-values of independent variables and interactions)

	Source of variances		
Parameter	A	B	A x B
Lipid oxidation			
Peroxide value	84.333*	4.726*	4.147*
TBARS	30.339*	13.396*	3.541*
TOTOX	87.182*	5.263*	4.261*
Protein oxidation			
Sulfhydryl	55.115*	11.178*	7.260*
Carbonyl	55.989*	505.020*	5.152*

A: antioxidant, B: Storage time.
*$p < 0.05$

TBARS values of sausages are given in Table 3. Initial TBARS values ranged between 0.15 – 0.42 mg MA/kg; the highest TBARS values were found in control treatment and R sausages, while T and TR sausages showed similar TBARS values ($P>0.05$). All samples showed an increased in TBARS value until month 3. C treatment had the highest TBARS value at the end of storage time. TBARS values of R, T and TR sausages increased up to month 2, then a significant reduction was observed, probably due to the decomposition of aldehydes ($P<0.05$). The final values in month 3 ranged between 0.35 – 0.75 mg MA/kg, with TR sausages having the lowest oxidation rate ($P<0.05$). Throughout the storage, TBARS values of all sausages were lower than 2.0 mg MA/kg, which is described as the limit of TBARS values in meat and meat products [13]. Similar to our results, Azizkhani and Tooryan [19] reported that using tocopherols and rosemary extract as a combination showed the greatest antioxidative effect in beef sausage storage during 3-month storage. Georgantelis [17] reported that TBARS values of fresh pork sausages formulated with chitosan and its combinations with either α-tocopherol or rosemary also showed the most intense antioxidative effect.

TOTOX, total oxidation value, is described as 2 x peroxide value + TBARS value [14]. Initial TOTOX values of sausage were between 8.24 and 10.61, and significantly increased during storage (Table 3). TOTOX values of sausages showed a similar trend as PV. The highest TOTOX value was observed for T sausage in the month 2. However, at the end of storage, C sausage had significantly higher TOTOX values compared to sausages with antioxidants. According to Decker et al. [20], the TOTOX value for food should be lower than 26; in this case, all of our sausages except C sausage in month 3 (28.98) were within the limit.

Table 3. Lipid oxidation results of sausages

	Storage time (months)			
Sample	Initial	1st	2nd	3rd
Peroxide value ($meqO_2/kg$)				
C	$4.03^{b,Z}\pm0.32$	$5.75^{a,YZ}\pm0.77$	$8.99^{a,Y}\pm1.87$	$14.11^{a,X}\pm3.14$
T	$5.21^{a,Z}\pm0.23$	$3.67^{b,Z}\pm0.38$	$11.31^{a,X}\pm2.47$	$8.67^{b,Y}\pm1.05$
R	$4.72^{ab,Y}\pm0.64$	$4.09^{b,Y}\pm1.02$	$9.44^{a,X}\pm0.75$	$9.62^{b,X}\pm0.72$
TR	$4.04^{b,Z}\pm0.10$	$2.68^{b,W}\pm0.58$	$8.45^{a,Y}\pm1.23$	$9.99^{b,X}\pm0.40$
TBARS (mg MA/kg)				
C	$0.43^{a,Y}\pm0.08$	$0.56^{a,XY}\pm0.05$	$0.54^{a,XY}\pm0.18$	$0.75^{a,X}\pm0.16$
T	$0.19^{b,Z}\pm0.05$	$0.40^{b,Y}\pm0.04$	$0.63^{a,X}\pm0.01$	$0.54^{ab,XY}\pm0.15$
R	$0.32^{a,Y}\pm0.06$	$0.42^{b,Y}\pm0.02$	$0.70^{a,X}\pm0.12$	$0.45^{b,Y}\pm0.04$

TR	$0.15^{b,Z}$±0.03	$0.41^{b,XY}$±0.02	$0.48^{a,X}$±0.05	$0.35^{b,Y}$±0.04
TOTOX				
C	$8.49^{b,Z}$±0.62	$12.07^{a,YZ}$±1.49	$18.54^{a,Y}$±3.86	$28.98^{a,X}$±6.19
T	$10.61^{a,Z}$±0.43	$7.73^{bc,Z}$±0.75	$23.25^{a,X}$±4.96	$17.88^{b,Y}$±2.01
R	$9.75^{ab,Y}$±0.61	$8.61^{b,Y}$±2.02	$19.59^{a,X}$±1.52	$19.69^{b,X}$±1.41
TR	$8.24^{b,Z}$±0.31	$5.78^{c,Z}$±1.14	$17.39^{a,Y}$±2.51	$20.33^{b,X}$±0.85

All values are means ± SD of three replicates.
Means within the same column with different superscripts (a-d) are different
Means within the same row with different superscripts (X-W) are different

Similar to lipid oxidation, oxidative change in proteins is believed to proceed via free radical chain reaction [21]. The use of antioxidant and storage time had significant effects ($P<0.05$) on protein oxidation of sausages in terms of sulfhydryl and carbonyl contents (Table 2). Initial sulfhydryl contents of sausages were between 1.39 – 2.25 nmol/mg protein, while sulfhydryl contents of sausages were between 1.77 – 2.76 nmol/mg protein at the end of storage (Table 4). Sulfhydryl contents of sausages increased in month 1, then decreased significantly during storage ($P<0.05$). The sulfhydryl reduction in sausages could be explain by the formation of disulphide-crosslinks [22].

Carbonyl contents of samples were between 1.16 – 1.81 nmol/mg protein at the beginning of storage (Table 4). The highest carbonyl content was found in C sausage while the lowest carbonyl content was observed in TR sausage ($P<0.05$). During storage, significant increments were observed in all sausages ($P<0.05$). Control sausages showed the highest formation rate of carbonyl groups of all the treatments after 3 months' storage. Using α-tocopherol and rosemary extract combination showed a synergistic effect on carbonyl contents of sausages, and TR sausages had the lowest carbonyl content among the treatments at the end of storage time ($P<0.05$).

Table 4. Protein oxidation results of sausages

	Storage time (months)			
Sample	Initial	1st	2nd	3rd
Sulfhydryl groups (nmol/mg protein)				
C	$1.39^{b,Y}$±0.18	$4.86^{a,X}$±0.72	$2.09^{b,Y}$±0.06	$2.27^{ab,Y}$±0.52
T	$2.02^{a,Z}$±0.34	$4.17^{a,X}$±0.73	$3.47^{a,XY}$±0.57	$2.76^{a,YZ}$±0.50
R	$2.12^{a,Y}$±0.46	$4.12^{a,X}$±0.58	$3.62^{a,X}$±0.39	$1.77^{b,Y}$±0.33
TR	$2.25^{a,Y}$±0.12	$2.92^{b,X}$±0.09	$1.51^{b,Z}$±0.36	$1.87^{b,Z}$±0.09
Carbonyl groups (nmol/mg protein)				
C	$1.81^{a,W}$±0.15	$2.78^{a,Z}$±0.08	$3.38^{a,Y}$±0.11	$5.46^{a,X}$±0.21
T	$1.51^{ab,W}$±0.26	$2.08^{b,Z}$±0.02	$3.12^{b,Y}$±0.03	$4.89^{ab,X}$±0.27
R	$1.34^{bc,W}$±0.15	$2.72^{a,Z}$±0.16	$3.30^{a,Y}$±0.02	$4.43^{bc,X}$±0.40
TR	$1.16^{c,W}$±0.06	$1.69^{c,Z}$±0.01	$2.38^{c,Y}$±0.04	$3.83^{c,X}$±0.52

All values are means ± SD of three replicates.
Means within the same column with different superscripts (a-d) are different
Means within the same row with different superscripts (X-Z) are different

4. Conclusion

Use of antioxidants individually in meat products is one of the effective ways to inhibit oxidative changes. However, using antioxidant combinations which have different effect mechanisms to limit oxidation reactions could be a better option. The present study indicates that using α-tocopherol, rosemary extract or their combination prevents oxidative changes in beef sausages during 3-month cold

storage. At the end of storage, sausages with antioxidants had significantly lower TBARS and TOTOX values. TOTOX value of C sausage in month 3 was higher than the limit value. Moreover, the α-tocopherol and rosemary extract combination showed a synergistic effect on carbonyl contents of sausages. In conclusion, the use of antioxidant combinations could be a novel approach to delay oxidative changes in meat products.

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Biosensors for animal health and meat safety monitoring: farm-to-slaughterhouse continuum

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Biosensors for animal health and meat safety monitoring: farm-to-slaughterhouse continuum

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Abstract. The meat supply chain needs to be managed for sufficient levels of consumer protection. Healthy animals are an essential precondition for a safe food supply, since zoonotic diseases, including meatborne pathogens, are a major threat to public health. Information about the livestock's general health, animal welfare and prevalence of major meatborne hazards such as *Salmonella*, *Campylobacter*, STEC and *Listeria monocytogenes* is of utmost importance for effective biosecurity control on farm. Early detection of these hazards in faecal samples, monitoring blood levels of metabolites relevant for animal welfare (hormones) and animal health (acute phase proteins) can provide high-level control in the animal farming industry. Multiplex biosensors for pathogens and metabolites in the farm-to-slaughterhouse continuum constitute a practical and cost-efficient tool for early detection of signs related to meat safety. Point-of-care multiplex biosensors are an advantage versus commonly used methods ELISA and RT-PCR, since they provide possibilities for early detection and do not require expensive equipment, trained personnel or significant time for sample transfer and analyses. Biosensors can improve meat inspection and meat safety controls, and can serve as a primary tool for monitoring food safety parameters and contribute to the modernization of veterinary inspection and risk-based meat safety assurance system.

1. Introduction

Meat safety is always at the forefront of public health and social-economic concerns [1]. Major meat safety challenges are associated with hazards that can be considered as traditional, new or emerging. This involves increased virulence and/or low infectious dose with antimicrobial resistance or resistance to other food-related stresses [1]. These hazards enter the meat chain in multiple points along the farm-slaughterhouse continuum. On the other hand, current, traditional meat inspection protocols (ante-mortem and post-mortem), based on visual inspection, palpation and incision, were not changed since the end of the nineteenth century, and were not fully efficient in terms of the current needs for consumer protection [2,3,4,5], since these protocols are intended for detection of traditional hazards (e.g. *Trichinella* spp., *Brucella* spp., *Mycobacterium bovis*, *Bacillus anthracis* and *Taenia solium/bovis* - cysticercosis) and can even increase cross-contamination due to palpation and/or incision procedures. The emerging hazards affecting safety of raw meat and poultry are bacterial pathogens such as Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and non-O157 [6], *Salmonella, e.g.* the big five: *S.* Typhimurium, *S.* Enteritidis, *S.* Infantis, *S.* Virchow, *S.* Hadar [7], *Campylobacter jejuni, Yersinia enterocolitica* and *Toxoplasma gondii*, while *Listeria monocytogenes* remains a concern in ready-to-eat (RTE) processed meat products [1,8]. These hazards cannot be detected by traditional meat inspection methods, and therefore, there is a growing need for development of on-site, user-friendly and rapid

testing and pathogen detection methodologies in the farm-to-slaughterhouse continuum and with sufficient sensitivity and specificity, as are biosensors as point-of-care (PoC) devices. These biosensors are devices that have the potential to detect and quantify physiological, immunological and behavioural responses of livestock and multiple animal species [9]. PoC solutions are an advantage in comparison to the commonly used methods ELISA and RT-PCR, as well as other sensors available on the market that do not provide possibilities of early detection and require expensive equipment, trained personnel and significant time for sample transfer and analyses.

Novel biosensing methodologies offer highly specialised monitoring devices for the specific measurement of individual and/or multiple parameters covering an animal's physiology as well as monitoring an animal's environment. In addition to that, information on animal welfare and general animal health status are valuable to supplement the implementation of harmonized epidemiological indicators (HEI) and food chain information (FCI) flow, from farm to slaughterhouse (bottom-top) and backwards, from slaughterhouse to farm (top-down), setting up the foundation for effective implementation of risk-based meat safety assurance system (RB-MSAS).

2. Biosensor application for animal health and meat safety control: current status

Biosensors in livestock farm management provide significant benefits and applications in animal health and welfare monitoring, including detection of reproductive cycles [9, 10]. With the development of integrated systems and the internet of things (IoT), the continuously monitoring sensing devices are expected to become affordable. The data generated from integrated livestock monitoring should assist farmers to improve animal productivity. A biosensor is a device that recognizes a target biomarker characteristic for a particular pathogen and/or animal welfare or animal health molecules (indicators), via an immobilized sensing element called a bioreceptor (monoclonal antibody, RNA, DNA, aptamer, glycan, lectin, enzyme, tissue, whole cell) (Figure 1). The bioreceptor is an essential component as its biochemical characteristics assure high sensitivity and specificity of the biomarker detection and allow avoidance of interferences with other microorganisms or molecules present in the tested sample [11, 12]. It is challenging to provide high levels of sensitivity and specificity of biosensors for quantitative detection of biomarkers (pathogen, animal welfare & animal health indicator) in complex media such as are the matrixes collected from the production environment on farm and at slaughterhouse (e.g. faeces, saliva, blood, serum). Therefore, there is a need for PoC and/or automatic reliable detection and quantification tools that can foresee when disease is likely to occur before that any clinical sign appears in animals [11].

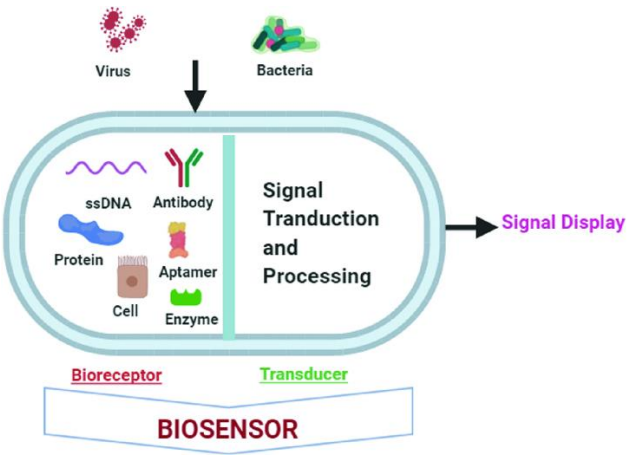

Figure 1. Biosensor mode of action [12]

2.1. Biosensors on farm

Biosensors can provide accurate and real-time detection for a wide range of conditions related to animal health and welfare on farm [9], such as: lameness in solipeds from acceleration data provided by ear tags, automatic 3D vision locomotion monitoring for cows, monitoring of physiological and behavioural stress in animals, monitoring of vocalisation sounds to assess response of broilers to environmental variables, cattle and pig cough monitoring as indicator of respiratory disease and environmental conditions, drinking behaviour of animals, automatic detection of health (body temperature) with a video-based infrared thermography camera, evaluating hormone profiles to improve automated oestrus detection [9] and faecal shedding of food borne pathogens [10].

2.1.1. Sensor for detection of metabolites

Mechanical sensors (pressure sensors). Designed to be used specifically in pastures and stables. The noseband and an electronic interface are connected to record, analyse and store data at 20 Hz at computer [13]. For example, the jaw movement is identified as a pressure peak through the transmission of the movement to the halter and the change in the tube pressure. The software can identify bites and/or chews [13].

Acoustic sensors. Used for the analysis of the jaw movement and grazing behaviour, precisely identifying chewing and biting to enable the estimate of the food intake in cattle [14].

Acceleration sensors. Used for monitoring of jaw movement and feeding behaviour. The static acceleration due to gravity and dynamic acceleration due to animal movement are measured [15].

Breath analyses biosensors. Enable non-invasive method used for disease diagnostics by detection and characterization of volatile organic compounds (VOCs) [9]. VOCs can be found in breath, blood, faeces, skin, urine and vaginal fluids of animals and humans [9]. Breath metabolites encompass gasses, (e.g. hydrogen and methane) and fatty acids, all of which can be used as specific biomarkers for detection of metabolic and pathologic processes. For example, a high level of glucose in blood is detected by presence of specific VOCs, e.g. ketones, ethanol and methanol [16]. In livestock, these biosensors can accurately identify bovine respiratory diseases (BRD) [17], brucellosis [18], bovine tuberculosis [19], Johne`s diseases [20], ketoacidosis [21] and even foot and mouth (FMD) disease [22].

Perspiration metabolite biosensors. They have been developed mostly for human health monitoring, such as analysis of sweat for sodium concentration and lactate levels [23]. Such sensors can be also adapted to be used for animal welfare control, e.g. physical stress.

Tear fluid biosensors. The level of certain metabolites in tears can provide information about the concentration of these metabolites in blood. For example, a glucose sensor has been developed [24].

Progesterone analyses biosensor. This sensor was developed by integrating a selected aptamer specific for its binding properties with progesterone [9].

Salivary detection of metabolites. The metabolites detected in saliva can provide valuable information on animal welfare and disease. This is a non-invasive method where biomarkers in saliva are used instead of taking the blood samples. For example, a high level of uric acid in saliva could be connected with a metabolic syndrome, renal syndrome or physical stress, or salivary cortisol, which reflects the level of animal stress, can be monitored [25].

2.1.2. Sensors for detection of animal diseases

Bovine Respiratory Disease (BRD). This PoC biosensor is made to be sensitive and specific to anti-IgE present in commercial anti-BHV_1 (bovine Herpes Virus-1, the cause of BRD) and in real serum samples from cattle [26].

Bovine Viral Diarrhoea (BVD). The sensor can detect BVD antibodies in serum of cattle [27]. The detection time is 8 min, with detection limit of 10^3 CCID/ml in BVD samples.

Avian Influenza virus (AIV). The sensor is based on detection of immobilised H7N1 antibodies, providing low level of detection [28].

Foot and Mouth Diseases (FMD). The developed sensor includes a lateral flow immunochromatographic platform for the detection of antibodies against FMD proteins [29].

Mastitis. An indirect on-line sensor system based on the automated California mastitis test (CMT) in milk has been developed [30], or the recently developed sensor for detection of mastitis based on haptoglobin (Hp) [31].

Other. There are also other developed biosensors enabling PoC detection of ketosis and porcine reproductive and respiratory syndrome (PRRS) virus,

2.2. Biosensors in slaughterhouses

There is no wide commercial and routine use of biosensors in slaughterhouse for the purposes of meat safety monitoring, so far. On the other hand, several biosensors for detection of food(meat)borne pathogens are available. For example, lateral flow aptamer-based biosensors for PoC detection of *Salmonella* Enteritidis and *Escherichia coli* O157:H7 were developed with sensitivity level of 10^1 CFU/ml and 10 CFU/ml, respectively [32, 33]; DNA-based sensor for detection of *Campylobacter* in meat (poultry) samples with detection level of 1.5×10^1 CFU/g [34]; Cell-based sensors which have mammalian cells as sensing elements to detect the pathogens or toxins of *Clostridium perfringens* [35]; Antibody-based biosensors for detection of *Escherichia coli* [36], or; conductometric-based biosensors for *E. coli* at detection level from 1 to 10^3 CFU/mL [37]. However, the performance and detection limit of above mentioned biosensors were mainly tested with enriched bacterial suspension (in vitro) with scarcity of data when using a matrix from the production environment (e.g. straw, faeces, blood).

2.3. Biosensors in environmental control (slaughterhouse wastewater)

Slaughterhouses generate a substantial amount of wastewater, and the treatment protocols and post-treatment purity level of waste waters are regulated. Biochemical oxygen demand (BOD) is a widely used parameter to describe the level of organic pollution in water and wastewaters. Just recently, BOD biosensors were developed, based on detection of refractory compounds [38]. Namely, natural waters and slaughterhouse wastewaters have several specific refractory compounds that microorganisms are not able to use and degrade within the biosensor's short measuring time. A semi-specific biosensor is manufactured that uses *Aeromonas hydrophila* P69.1 to estimate BOD in high fat and grease content wastewaters, while a universal biosensor is manufactured that uses non-specific *Pseudomonas Fluorescens* P75. Service life of *A. hydrophila* and *P. Fluorescens* biosensors are 110 and 115 days, respectively. The measuring time is 20 minutes, and the biosensor based on *A. hydrophila* proved to be more accurate in measuring the fat content of the meat industry wastewaters [39].

3. Need for development of biosensors for use in the farm-slaughterhouse continuum (F2SC)

For the purposes of meat safety monitoring and control in the meat supply chain continuum, there is a need for development and optimization of multiplex biosensors which will be effectively used as PoC devices in F2SC [10]. Namely, these biosensors should preferably detect and quantify several key target molecules relevant for farm biosecurity (e.g. selected pathogens), animal welfare (e.g. selected hormones) and general animal health (e.g. acute phase proteins) and, thus, serve as excellent food safety management tools to improve the consumer protection (Figure 2). The major challenges are related to the functionalization and increase of biosensors' sensitivity, together with optimization of sampling protocols to enable accurate detection of key molecules in matrixes available in production environments (farm, slaughterhouse).

Healthy animals are an essential precondition for a safe food supply, since zoonotic diseases are a major threat to public health. Concerning livestock, early information about the prevalence of major health hazards of bacterial origin is of utmost importance for effective control on the farm. Early detection of *Salmonella*, *Campylobacter,* STEC O157, *Listeria monocytogenes* etc. in faecal samples, as well as monitoring blood levels of metabolites relevant for animal welfare and animal health, can provide high-level control in the animal farming and meat industry. Integration of such multiplex biosensors for pathogens and metabolites in the F2SC is a practical and cost-efficient tool for early detection of signs that meat safety is jeopardized.

The availability of relevant information, together with HEI for cattle, pigs, poultry (as well as other meat producing animals) will contribute to the FCI flow from farm-to-slaughterhouse (bottom-up) and vice versa, from slaughterhouse-to-farm (top-down) and enable implementation of a RB-MSAS [4].

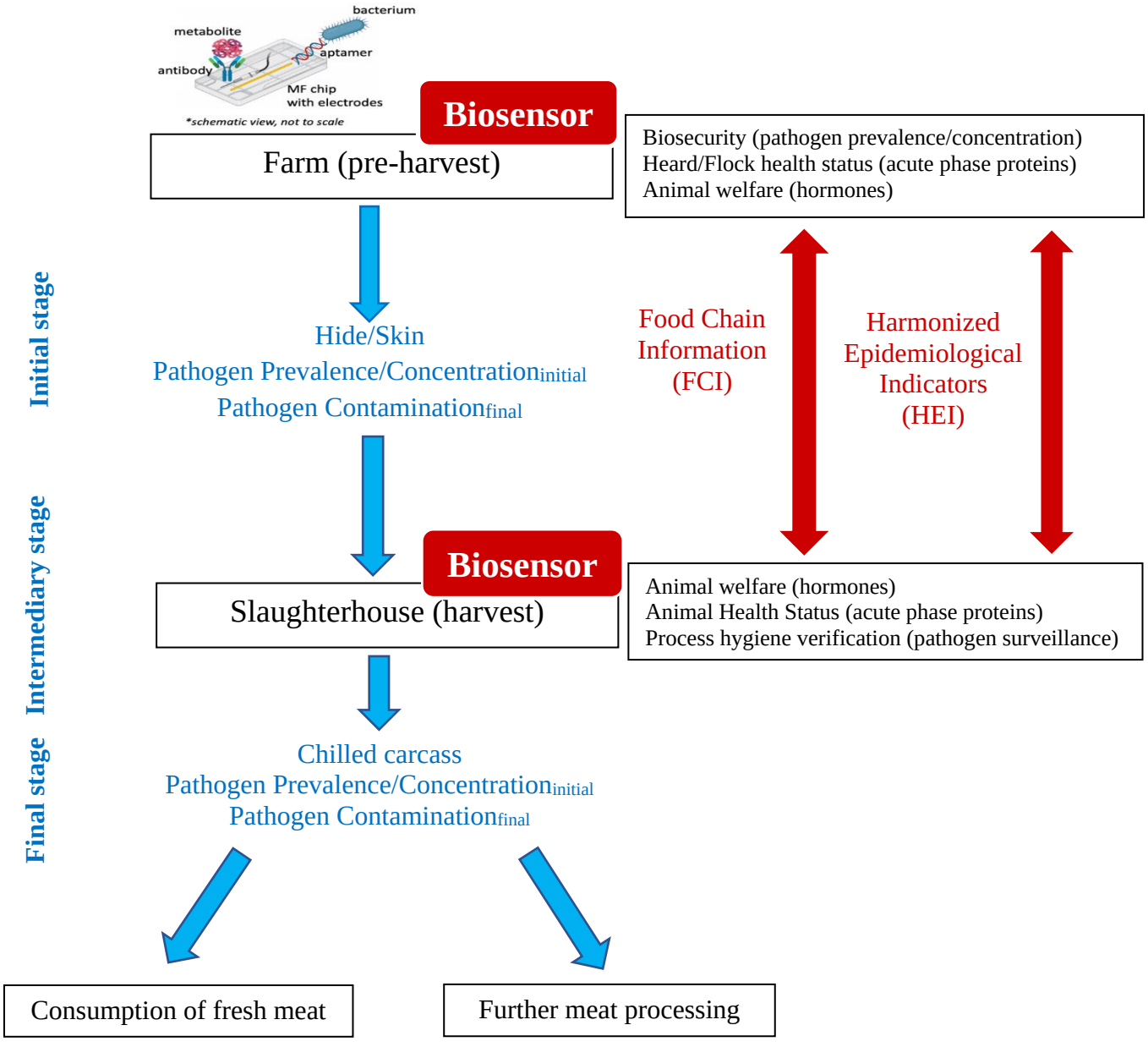

Figure 2. A model for practical implementation of multiplex, point-of-care biosensor in the farm-to-slaughterhouse continuum for animal health, animal welfare and meat safety monitoring

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Antimicrobial activity of thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare)* essential oils against *Listeria monocytogenes* in fermented sausages

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Antimicrobial activity of thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare)* essential oils against *Listeria monocytogenes* in fermented sausages

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Abstract. The aim of this study was to investigate antibacterial effects of oregano and thyme essential oils on *Listeria monocytogenes* in fermented sausages and their effect on the sensory characteristics of these sausages. For testing purposes, sausages contaminated with *L. monocytogenes* were produced. Changes in the microbiological status of fermented sausages and physicochemical properties were monitored during ripening. Essential oils exhibited antibacterial activity against *L. monocytogenes*, and in the groups with a high concentration (0.6%) of oregano or thyme essential oils (KLO2 and KLT2), the number of *L. monocytogenes* was below the detection threshold on day 14 of ripening, with a stronger effect of oregano. In groups with 0.3% essential oil of oregano or thyme added, the number of *L. monocytogenes* was reduced to below the detection threshold on day 21 of ripening. During the ripening, the a_w and pH of all test groups of fermented sausages decreased. Experimental sausages with 0.3% thyme essential oil had acceptable smell and taste, while in other experimental groups, sausage smell and taste were very intense, uncharacteristic and unacceptable.

1. Introduction

Foodborne listeriosis is one of the most serious and severe foodborne diseases, caused by the bacterium *Listeria monocytogenes.* This pathogen has been isolated from various ready-to-eat (RTE) food products, including fermented dry and semi-dry sausages [1]. The number of *L. monocytogenes* decreases during fermentation and drying of sausages because of the set of hurdles created in the manufacturing process (low pH and water activity (a_w) and high salt concentration). However, this microorganism can be isolated from fermented sausages because of its ability to adapt to its environment and because of its presence in raw meat [2]. The manufacturing process is not effective enough to reduce or eliminate this microorganism from the finished product [3, 4].

Because of the growing popularity of natural and organic food, there has been a consumer shift away from chemical preservatives in food, as these compounds exhibited many adverse effects [5]. There is a new trend in the meat industry, where there is no place for artificial preservatives with possible

carcinogenic and toxic properties [6, 7, 8]. Essential oils obtained from a variety of plant materials impart distinctive flavours, exhibit antimicrobial activity in meat products and possess antioxidative properties [9]. Oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris)* are aromatic plants with important antioxidant and antimicrobial properties. Oregano essential oil is known to possess antibacterial, antiviral, antifungal, antiparasitic and antioxidant activities. Thyme has been used medicinally for thousands of years. Beyond its common culinary application, it has been recommended for a myriad of indications, based upon proposed antimicrobial, antitussive, spasmolytic and antioxidant activity [10]. Carvacrol and thymol are the two main phenols that constitute oregano and thyme essential oils, as well as the monoterpene hydrocarbons *p*-cymene and γ-terpinene [11].
The aims of this study were to investigate antibacterial effects of thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*) essential oils on *L. monocytogenes* in fermented sausages and to examine their effect on the sensory properties of these sausages.

2. Materials and Methods

2.1. Sausage manufacture

Five different formulations of sausages were prepared: one of control sausages with no essential oil, two formulations with thyme (0.3% and 0.6%) and two formulation with oregano (0.3% and 0.6%) essential oil. In half the sausages with essential oil and in control sausages, an inoculum of *L. monocytogenes* was added. The other half of the sausages were *Listeria*-free, and they were used for sensory analysis. Fermented sausages were manufactured in the experimental laboratory at the Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, University of Belgrade, following the traditional formulation. All batches were prepared with the same raw components: 60% lean pork meat, 40% pork backfat, 5% water, 2% sodium chloride, 1% lactose, 0.05% sodium ascorbate, 0.015% sodium nitrite, 0.03% potassium nitrate, 0.05% black pepper and 0.05% white pepper. The meat and fat were minced at 4 °C through a 6 mm grinder plate (Mainca PM-98, Equipos cárnicos S.L., Granollers, Barcelona, Spain) and mixed with the other ingredients. This paste was stuffed in synthetic sausage casings of 33-35 mm diameter with a Mainca EM-12 (Equipos cárnicos S.L., Granollers, Barcelona, Spain) stuffing machine. Thereafter, sausages were fermented for 24 h (22-24 °C, 94–98% RH) followed by 28 days of dry-curing (16–18 °C, 80–85% RH) in a controlled dry-cured chamber (Tarré, STA-15-HXA, Noain, Navarra).

2.2. Essential oils

The essential oils extracted by the steam distillation method (year 2012) were purchased from the manufacturer Herba doo (Belgrade, Serbia). Essential oils were kept in dark glass bottles at 4 °C.

2.3. Detection and isolation of L. monocytogenes

Samples were taken from each batch after 0, 7, 14, 21 and 28 days of production. For *Listeria* enumeration, 25 g of fermented sausages was weighed out aseptically and transferred into sterile Stomacher bags and homogenized with 225 ml of buffered peptone water (BPW; Merck, Germany) that was added to each sample. The bag contents were homogenized in a Stomacher blender (Stomacher 400 Circulator, Seward, Worthing, UK) for 2 min. Serial decimal dilutions were prepared and 1 ml or 0.1 ml of appropriately diluted suspension was inoculated directly on the surface of the appropriate media for *L. monocytogenes* enumeration. Inoculated fermented sausages were analysed for *L. monocytogenes* on days 0, 7, 14, 21 and 28 of maturation (ripening of sausages). *L. monocytogenes* was enumerated on the Agar Listeria acc. to Ottaviani and Agosti (ALOA, Oxoid, Hampshire, UK) and plates were incubated for 24-48 h at 37°C according to ISO 11290-1:2017. After incubation, plates were examined visually for typical colony types and morphological characteristics associated with each growth medium, the number of colonies was counted, and results were recorded as colony forming units per gram (cfu/g).

2.4. pH and a_w of sausages

The pH of sausages was measured using the portable pH meter (WTW 340i, Germany) according to methods recommended by International Organization for Standardization (ISO 2917:2004). Means of three measurements are presented. Water activity (a_w) of sausages was determined using aw-Wert Messer, GBX Scientific Instruments, Fa-St/1, according to ISO 21807:2004E, at a constant temperature of 20°C.

2.5. Sensory evaluation

A panel consisting of seven trained members performed sensory evaluation (ISO 8586-2:2008) in the laboratory that was designed according to the requirements of standard SRPS EN ISO 8589:2012. The panellists were asked to evaluate the dry fermented sausages for the following characteristics: external appearance and/ or condition of the packaging, appearance and composition of cut surface, colour and stability of colour, smell and taste and texture and/or juiciness. Evaluations were performed according to a 5-point scale descriptive system, from 1 to 5, where each mark represented a certain level of quality. The overall sensory quality of sausages was multiplied by an appropriate coefficient of significance (external appearance of sausage x2 + appearance and composition of cut surface x5 + colour and colour maintenance on the cutting x3 + door and taste x7 + texture and juiciness x3).

2.6. Statistical analysis

Statistical analysis of the results was carried out using GraphPad Prism v6 (GraphPad, San Diego, CA, USA) software. Since the data were homogeneous (coefficient of variation <30%), groups were compared using two-way ANOVA with repeated one factor measures followed by Tukey's multiple comparison tests.

3. Results and Discussion

Approximately equal numbers of *L. monocytogenes* were added to the stuffing of fermented sausages of all groups at the beginning of production (day 0). In the groups with the addition of a higher concentration (0.6%) of oregano or thyme essential oils (KLO2 and KLT2), *L. monocytogenes* numbers were below the detection threshold on day 14 of ripening, with a stronger effect of oregano. In sausage groups with 0.3% oregano or thyme essential oil, the numbers of *L. monocytogenes* were below the detection threshold on day 21 of ripening. In the control group (KL), the number of *L. monocytogenes* decreased below the detection threshold (<2) after day 28 of maturation (Table 1). Analysing the number of *L. monocytogenes* in the experimental groups of fermented sausages during ripening, no significant differences were observed on day 0 ($p>0.05$). On day 7 of maturation, the lowest number of *L. monocytogenes* was found in KLO2 (0.6%) (2.51 ± 0.01), while the highest number was found in the control group KL (5.01 ± 0.01). On day 7 of ripening, there was a statistically significant difference between all examined groups ($p <0.01$).

Table 1. Number of *L. monocytogenes* (log CFU/g) during the ripening and maturation of sausages

Group	Day of during the ripening and maturation of sausages				
	0	**7**	**14**	**21**	**28**
KL	6.22±0.01	$5.01±0.01^{ABCD}$	4.22±0.01	2.15±0.01	< 2
KLO1 (0.3%)	6.26±0.01	$4.04±0.05^{AE}$	2.02±0.01	< 2	< 2
KLO2 (0.6%)	6.26±0.01	$2.51±0.01^{BEFG}$	< 2	< 2	< 2
KLT1 (0.3%)	6.23±0.01	$4.23±0.01^{CF}$	2.44±0.01	< 2	< 2
KLT2 (0.6%)	6.23±0.01	$3.73±0.92^{DG}$	< 2	< 2	< 2

Statistical significance is presented in the same letters: a - p<0.05; A - p<0.01

KL - Control group - fermented sausages without added essential oils + L.m.

KO1 - fermented sausages with 0.3% oregano essential oil

KLO1 - fermented sausages with 0.3% oregano essential oil + L.m.

KO2 - fermented sausages with 0.6% oregano essential oil
KLO2 - fermented sausages with 0.6% oregano essential oil + L.m
KT1 - fermented sausages with 0.3% thyme essential oil
KLT1 - fermented sausages with 0.3% thyme essential oil + L.m
KT2 - fermented sausages with 0.6% thyme essential oil
KLT2 - fermented sausages with 0.6% thyme essential oil + L.m

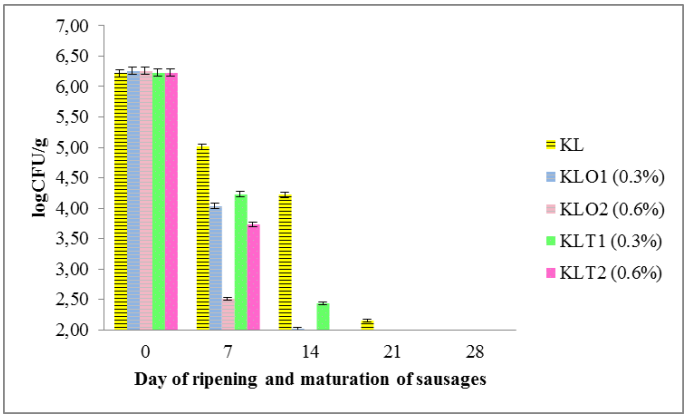

Figure 1. Number of *L. monocytogenes* (log CFU/g) during the ripening and maturation of sausages

Examination of the pH on day 0 of ripening showed the highest pH was in the control group with added oregano essential oil KO1 (0.3%) (5.71 ± 0.01), and the lowest was in the control group with thyme essential oil KT2 (0.6%) (5.64 ± 0.02). Further analysis revealed significant differences in almost all examined groups except KLO1, KLO2, KT1 (0.3%), ($p < 0.05$; $p < 0.01$). By day 7 of ripening, the highest pH was in KO1 (0.3%) (5.43 ± 0.02), and the lowest in KT2 (0.6%) (5.30 ± 0.02). On day 14, the highest recorded pH occurred in group KLO1 (5.40 ± 0.00), while the lowest was in the group KT2 (0.6%) (5.27 ± 0.02). On day 21 of ripening, the control group with oregano essential oil KO1 (0.3%) (5.42 ± 0.02) had the highest pH, and the lowest pH occurred in the group with thyme essential oil KT2 (0.6%) (5.31 ± 0.02). Also, at the end of ripening (day 28), the highest pH was found in the control group (5.43 ± 0.01), and the lowest in the group with thyme essential oil KT2 (0.6%) (5.32 ± 0.01). Statistically significant differences occurred within all examined groups ($p < 0.05$; $p < 0.01$).

Table 2. pH values during the ripening and maturation of sausages

Group	Day of during the ripening and maturation of sausages				
	0	**7**	**14**	**21**	**28**
KL	5.67±0.02	5.37±0.02^{ABCD}	5.38±0.01^{ABCD}	5.41±0.01^{AB}	5.43±0.01^{AB}
KO1 (0.3%)	5.71±0.01^{aABb}	5.43±0.02^{AEFGHIJ}	5.38±0.01^{EFGH}	5.42±0.02^{abCD}	5.41±0.01^{CDEF}
KLO1	5.67±0.02	5.41±0.01^{KLMNOP}	5.40±0.00^{IJKLM}	5.41±0.01^{EF}	5.42±0.01^{GH}
KO2 (0.6%)	5.66±0.02^{a}	5.34±0.02^{EKa}	5.35±0.01^{INO}	5.37±0.02^{aG}	5.38±0.03^{CI}
KLO2	5.67±0.02	5.34±0.02^{FLb}	5.36±0.01^{aPQ}	5.37±0.01^{bH}	5.38±0.01^{DJ}
KT1 (0.3%)	5.67±0.01	5.32±0.01^{BGM}	5.32±0.02^{AEJR}	5.40±0.02^{IJ}	5.41±0.02^{KL}
KLT1	5.65±0.03^{A}	5.31±0.02^{CHN}	5.32±0.02^{BFKa}	5.39±0.03^{K}	5.40±0.02^{Ma}
KT2 (0.6%)	5.64±0.02^{B}	5.30±0.02^{DIOab}	5.27±0.02^{CGLNPR}	5.31±0.02^{ACEGHIK}	5.32±0.01^{AEGIJKM}
KLT2	5.66±0.01^{b}	5.33±0.01^{JP}	5.30±0.01^{DHMOQ}	5.35±0.01^{BDFJ}	5.35±0.01^{BFHLa}

Statistical significance is presented in the same letters: a - p<0.05; A - p<0.01

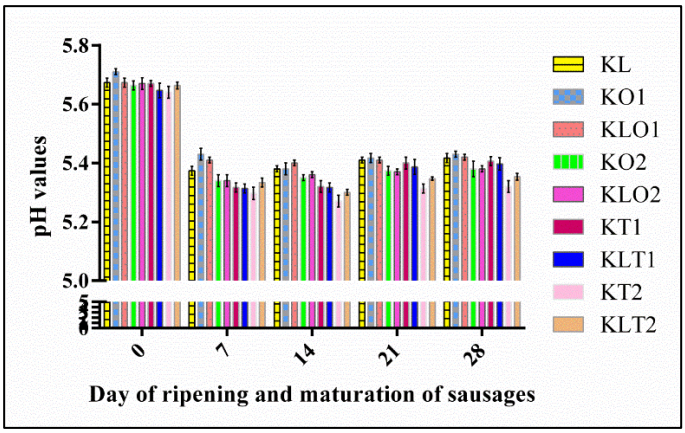

Figure 2. pH values during the ripening and maturation of sausages

Statistical analysis of the a_w values in the experimental groups of fermented sausages during ripening revealed the same or almost the same mean a_w on all examined ripening days. Significant differences within the examined groups were not found on days 0, 7 or 14 of ripening ($p > 0.05$), but they were recorded on day 21 and 28 of ripening within all examined groups ($p < 0.05$; $p < 0.01$).

Table 3. a_w values during the ripening and maturation of sausages

Group	Day of during the ripening and maturation of sausages				
	0	**7**	**14**	**21**	**28**
KL	0.96±0.002	0.94±0.002	0.92±0.002	0.89 ± 0.002^{A}	0.89 ± 0.002^{ABC}
KO1 (0.3%)	0.96±0.002	0.94±0.001	0.92±0.002	0.90 ± 0.002^{BCD}	0.90 ± 0.002^{DEFG}
KLO1	0.96±0.002	0.94±0.002	0.92±0.002	0.90 ± 0.002^{EFG}	0.89 ± 0.002^{aHIJ}
KO2 (0.6%)	0.96±0.002	0.94±0.02	0.92±0.002	0.90 ± 0.005^{H}	0.89 ± 0.002^{bKLM}
KLO2	0.96±0.001	0.94±0.001	0.91±0.002	0.89 ± 0.007^{I}	0.90 ± 0.002^{NOPR}
KT1 (0.3%)	0.96±0.001	0.94±0.01	0.92±0.001	0.89 ± 0.001^{BEJK}	0.88 ± 0.001^{DabN}
KLT1	0.96±0.002	0.94±0.00	0.92±0.001	0.89 ± 0.001^{ACFHIL}	0.88 ± 0.001^{AEHKO}
KT2 (0.6%)	0.96±0.002	0.94±0.002	0.92±0.002	0.90 ± 0.001^{DGM}	0.90 ± 0.002^{BFILP}
KLT2	0.96±0.002	0.94±0.001	0.92±0.001	0.90 ± 0.002^{JKLM}	0.88 ± 0.002^{CGJMR}

Statistical significance is presented in the same letters: a – p<0.05; A – p<0.01

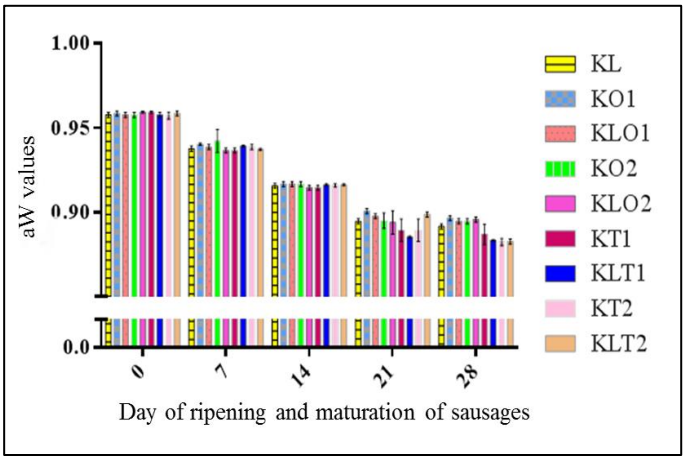

Figure 3. a_w values during the ripening and maturation of sausages

In sensory examination of experimental groups of sausages to which oregano or thyme extracts were added in concentrations of 0.3% and 0.6%, external appearance, cut surface appearance and composition, texture and juiciness and colour and stability of colour were highly rated in all groups

(from 4.2 to 4.8). However, the smell and taste were only acceptable only in the experimental group with 0.3% thyme (3.2), while in the other experimental groups it was very intense, uncharacteristic and unacceptable (grade 2 and lower).

Table 4. Sensory evaluation of sausages

Group	External appearance	Cut surface appearance and composition	Colour and stability of colour	Smell and taste	Texture and juiciness	Overall sensory quality
KO1 (0.3%)	4.8 ± 0.3	4.5 ± 0.0	4.3 ± 0.3	3.2 ± 0.3	4.3 ± 0.3	80.3 ± 6.0
KO2 (0.6%)	4.8 ± 0.3	4.4 ± 0.2	4.2 ± 0.4	2.0 ± 0.3	4.2 ± 0.4	70.6 ± 4.7
KT1 (0.3%)	4.7 ± 0.4	4.3 ± 0.4	4.4 ± 0.2	1.9 ± 0.2	4.3 ± 0.3	70.3 ± 4.5
KT2 (0.6%)	4.7 ± 0.3	4.4 ± 0.2	4.2 ± 0.4	1.3 ± 0.3	4.2 ± 0.4	65.7 ± 5.2

4. Conclusion

The technological process of production of fermented sausages, without added essential oils of oregano or thyme leads to a decrease in the number of *L. monocytogenes* to below the detection level after day 28 of ripening. However, some concentrations of these essential oils reduce numbers or completely eliminate *L. monocytogenes* before the end of the technological process. With the addition of a higher concentration (0.6%) of essential oils, the number of *L. monocytogenes* was below the detection threshold on day 14 of ripening, with a slightly stronger effect of oregano. In groups with 0.3% essential oil, after day 21, *L. monocytogenes* was below the detection threshold. Examination of the sensory properties of fermented sausages to which essential oils were added showed that only sausages with 0.3% thyme essential oil are acceptable.

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Categorization of animal feed according to microbiological quality - preferable improvement in the food chain

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Categorization of animal feed according to microbiological quality - preferable improvement in the food chain

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Abstract. Given the fact that the law on animal feed in Serbia has long been expected and that the current Regulation on the quality of animal feed, which includes microbiological criteria, requires improvement over the years, it is time to choose the best new solutions. The recommendable change that would bring the categorization of animal feed according to more objective and comprehensive criteria is based on the use of the VDLUFA (*Verbands Deutscher Landwirdschaftlicher Untersuchungs und Forschungsanstalten*) method. In several European countries, it has become routine, due to the great interest of feed producers and animal breeders in good knowledge of microbiological quality of feed as a guarantee of a wholesome final product. It involves determination of the contents of moulds, yeasts and bacteria while taking into account their potential pathogenicity. Based on the number of microorganisms, divided into seven groups, feed and feedingstuffs are classified into four categories. Classes I to III can be placed on the market, while class IV is not suitable for animal nutrition. More precise, regular determination of microorganisms would also provide a better insight into other common feed-born problems, such as, for instance, the possibility of mycotoxin occurrence.

1. Introduction

The link between safe food and feed is now well recognized. In particular, the modern approach to food safety identifies measures to reduce and prevent the entry of hazards in the early stages of the production chain, including primary feed production. It is already well known that a large number of different microorganisms could be present naturally in feed, or could occur as its contaminants [1]. Some microbes are useful and can contribute to feed utilization and animal productivity, or some are purposely added to fight harmful pathogens (examples are probiotics). Nevertheless, feed can also contain undesirable organisms able to affect animal health: bacteria, fungi, viruses, prions, parasites, or their adverse metabolites (toxins and mycotoxins) [2, 3, 4]. EFSA's Panel on Biological Hazards has identified *Salmonella* spp. as a major hazard for microbiological contamination of animal feed, while *Listeria monocytogenes, Escherichia coli* O157:H7 and *Clostridium* spp. are other possible threats, but animal feed is a far less important source in this regard. However, in terms of transmission of antimicrobial-resistant bacteria or microbial-resistant genes, feed must not be neglected [5].

On the other hand, serious attention must be given even to the presence of saprophytic microorganisms, especially if they are found in large numbers. They can cause organoleptic changes due to the utilization of carbohydrates, and the decomposition of lipids and proteins, thereby reducing the nutritional value of feed. In such situations, changes in the taste and smell of feed are usually evident, and digestive problems occur. Clinically, there is a decline in productivity and impairment of the general health of animals, due to decrease of immunity and weakening of resistance to other hazards [6].

However, complete microbiological analysis is needed to adequately assess feed safety and quality. The Serbian Regulation on the quality of feed [7] prescribes microbiology conditions for feedingstuffs and compound feed in Serbia. However, many comments and questions have emerged since it was published. Due to several professional objections, there has long been a need to amend it. In the European Union, the criteria for microbiological quality of feed are based on the principles of the Codex Alimentarius [8] and are in line with Regulation (EC) No 183/2005 laying down requirements for feed hygiene [9] and Regulation (EC) no 767/2009 [10]. The purpose is to establish a feed safety system for food producing animals that covers the entire food chain, taking into account relevant aspects of animal health and the environment, in order to minimize risks to consumers' health. The HACCP system in the feed industry and the application of good practices contribute to feed safety. However, national regulations can vary, and there are several European countries that have raised the hygienic standards of animal feed based on the VDLUFA (*Verbands Deutscher Landwirdschaftlicher Untersuchungs und Forschungsanstalten*) method. It has become routine, due to the great interest of feed producers and animal breeders in good knowledge of microbiological quality of feed, as a guarantee of a wholesome final product [11, 12]. Therefore, this method could be a guideline in the modernization of feed hygiene and feed legislation in Serbia. In addition, mentioning the category of the microbiological quality within the feed declarations would increase the competitiveness of the products on the market.

The aim of this paper is, based on the European experience, to give a description and advantages of the categorization of animal feed according to microbiological quality using the VDLUFA method, as a proposal for improving the Serbian feed system and current feed regulation.

2. VDLUFA (*Verbands Deutscher Landwirdschaftlicher Untersuchungs und Forschungsanstalten*) method description

According to the official VDLUFA website, it represents an association of German agricultural analytical and research institutes established with the aim of achieving harmonised professional work, bringing together analytical and research institutes (LUFA), centres for dairy science and vocational education, university institutes, federal and other research and testing centres. VDLUFA's focus is both on the development of methods and on quality assurance in the field of agricultural sciences, in addition to providing a platform for applied agricultural research. The Department of Microbiology of Animal Feed at VDLUFA, since 1981, governed research on a systematic approach to determine microbiological quality according to the contents of moulds, yeasts and bacteria. The method for such categorization of feed is described in Methods book III. Detailed instruction is given within the four standard operating procedures (SOPs):

- SOP No 28.1.1 for enumeration of microorganisms using solid culture media [13] describes media and their recipes. In the main, they are identical to those in ISO standards. The difference is that the sample for bacteria count should be cultured on tryptose agar with triphenyltetrazolium chloride (TTC). After three days of incubation at 30°C, depending on their metabolic activity, bacteria reduce TTC to red formazan. In this way, otherwise colourless colonies become yellow, orange (saprophytes), red or pink (most often contamination indicators). The number of moulds is determined on nutrient agar, dichloran-rose-bengal-chloramphenicol agar (DRBC) and dichloran 18% glycerol agar (DG 18). In contrast to ISO standards, in this method both nutrient agars are used, irrespective of the water activity (a_w), while taking into account the counts from the agar on which more colonies of yeasts and moulds are grown. This SOP gives basic procedural rules to determine germ numbers (colony forming

units = cfu) in samples of feed additives, premixtures, single and compound feedstuffs. It describes quality requirements for working equipment and general methodical steps.

- SOP No 28.1.2 for enumeration of bacteria, yeasts and *Dematiaceae* moulds (blackness fungi) in feeds [14] prescribes the counting technique, which is done as described in ISO 21527-1:2011 [15] and ISO 21527-2:2011 [16] and ISO 4833:2014 [17]. Detected colonies can be diagnostically differentiated as indicator microorganisms. In this case, the method provides data with regard to the description of feed quality and unspoiltness as defined by legal terms.
- SOP No 28.1.3 is for identification of bacteria, yeasts and moulds as product-typical or spoilage indicating microorganisms (IM) [18]. The microorganisms, distinguishable on the basis of their colony morphology, microscopic features, and further tests, are identified as 19 product-typical or spoilage-indicating indicator microorganisms. Indicator microorganisms with the same microbiological significance are allocated to seven microorganism groups.
- SOP 28.1.4 for microbiological quality assessment using orientation values [19] describes fundamental rules directing the analysis and evaluation of germ numbers (cfu) of bacteria, yeasts, moulds and *Dematiaceae* (blackness fungi) in feed. It shows whether a feed is unspoiled or has developed signs of degradation in gradual stages compared with normal quality.

3. Current vs. VDLUFA approach

Microorganisms naturally colonize feed in various ways. Certain species can be found on plant materials dominating at the time of harvest (collective term: field flora or primary flora). Feedingstuffs of animal origin, on the other hand, show relatively low germ numbers (relict flora) because of processing. Current Serbian regulation on the quality of feed [7] regulates certain pathogenic bacteria in Article 102: *Salmonella, Clostridium botulinum, Clostridium perfringens, Staphylococcus* and a category named "other microorganisms" which is not clear and not precise enough. Article 101 gives maximal permitted levels of saprophytic bacteria and yeasts and moulds in feedingstuffs and compound feed. In feedingstuffs of plant origin, the maximum allowed number of mesophilic aerobic bacteria is 12,000,000 cfu/g and yeasts and moulds 200,000 cfu/g. Feedingstuffs of animal origin are also mentioned, although there is another regulation for the hygiene of processed by-products [20], so this information is confusing to interpret. Regarding mash compound feed, regulation [7] provides only partial possibility to distinguish microbiological quality, without any determination of microbial species and based on the number of microorganisms in feed for two categories of animals: "young" (mesophilic aerobic bacteria 3,000,000 cfu/g and yeasts and moulds 50,000 cfu/g) and "adult" (mesophilic aerobic bacteria 5,000,000 cfu/g and yeasts and moulds 200,000 cfu/g). Special values are also prescribed for pelleted mixtures (mesophilic aerobic bacteria 2,000,000 cfu/g and yeasts and moulds 20,000 cfu/g).

The VDLUFA method categorizes feed in accordance with its microbiological quality in a more objective and comprehensive manner, taking into account the type of feed and what animal category it is intended for. It also includes whether the feed mixture is subjected to heat treatment (pelleting) and, most importantly, what species of microorganisms are found in the mash. Therefore, it is crucial whether there are saprophytes or indicator microorganisms, since orientation values for pollution indicators (especially yeasts and *Mucorales* moulds) are lower than for saprophytic microorganisms. Table 1 shows orientation values (OV) by the VDLUFA method [19] as are included in the Croatian regulation on feed safety [21] and previously published by Nesic et al. [22].

Table 1. VDLUFA orientation values (OV) for the Quality Class I feed (desirable quality) [19, 21, 22]

	Mesophilic aerobic bacteria x 10^6 cfu/g			**Moulds x 10^3 cfu/g**			**Yeasts x 10^3 cfu/g**
Groups of indicator microorganisms (IM)	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
	Orientation values (OV)						

Feedingstuffs							
Flour and grits from oilseeds extraction	1	1	0.1	10	20	1	30
Oil cake from compression of oilseeds	1	1	0.1	10	20	2	30
Meal and branches, except wheat and rye branches	5	1	0.1	50	30	2	50
Wheat and rye branches	8	1	0.1	50	50	2	80
Corn (grain and wholemeal)	2	0.5	0.05	20	30	5	60
Wheat and rye (grain and wholemeal)	5	0.5	0.05	30	20	2	30
Barley (grain and wholemeal)	20	1	0.05	40	30	2	100
Oat (grain and wholemeal)	50	1	0.05	200	50	2	200
Hay	30	2	0.15	200	100	5	150
Straw	100	2	0.15	200	100	5	400
Silage	0.4	0.2	0.03	5	5	5	1000
Haylage	0.2	0.2	0.01	5	5	5	200
Mash compound feed for							
broilers	3	0.5	0.1	30	20	5	50
laying hens	5	1	0.1	50	50	5	50
piglets	5	0.5	0.1	30	20	5	50
breeding and fattening pigs	6	1	0.1	50	50	5	80
calves	2	0.5	0.1	30	20	5	50
dairy cows and breeding and fattening cattle	10	1	0.1	50	50	5	80
Pelleted compound feed for							
broilers	0.5	0.1	0.05	5	5	1	5
laying hens	0.5	0.5	0.05	5	10	1	5
piglets	0.5	0.1	0.05	5	5	1	5
breeding and fattening pigs	1	0.5	0.05	5	10	1	5
calves	0.5	0.5	0.05	5	5	1	5
dairy cows and breeding and fattening cattle	1	0.5	0.05	5	10	1	5
horses	0.5	0.5	0.01	2	6	1	5
rabbits	0.2	0.2	0.01	1	3	1	2

Quality Class I (desirable quality) includes feed for which it is determined that the number of indicator microorganisms (IM) does not exceed the established orientation value (OV) as given in Table 1. Quality class II (reduced quality) includes feed in which detected number of IM is up to five times greater than the established OV. Quality class III (poor quality) includes feed for which determined number of IM is 5 to 10 times above the established OV. Quality class IV (not acceptable for animal feeding) includes feed for which it is established that the number of IM is 10 times higher than the OV set out in Table 1. Feed placed on the market and used in animal nutrition must meet the criteria for classification into class I to III, in accordance with the parameters laid down in Table 1. Animal feed that is based on parameters from this table listed in grade IV will not be suitable for animal feeding.

Table 2. VDLUFA groups of indicator microorganisms (IM) [18]

Group	Significance	IM group	Indicator microorganisms (IM)
Aerobic, mesophilic bacteria	Product-typical	1	Yellow pigmented bacteria *Pseudomonas/ Enterobacteriaceae*, other bacteria (e.g. coryneform bacteria)
	Spoilage indicating	2	*Bacillus* spp. *Staphylococcus/Micrococcus*
		3	*Streptomycetes*
Moulds and *Dematiaceae* (blackness fungi)	Product-typical	4	*Dematiaceae* *Verticillium* spp. *Acremonium* spp. *Fusarium* spp. *Aureobasidium* spp. Other product-typical moulds
	Spoilage indicating	5	*Aspergillus* spp. *Penicillium* spp. *Scopulariopsis* spp. *Wallemia* spp. Other spoilage-indicating moulds
		6	*Mucorales* moulds (*Mucor* spp., *Rhizopus* spp.)
Yeasts	Product-typical and spoilage indicating	7	All yeasts (*Candida, Rhodontorula*)

According to the VDLUFA method, indicator microorganisms (IM) are divided into seven groups depending on the extent to which they can affect the animal health, as shown in Table 2. Thus, in groups 1 and 4 are microorganisms specific for contamination in the field and the number of which decreases with storage. In groups 2 and 5 are microorganisms that multiply in storage, while in groups 3 and 7 are those which might impair animal health. In a separate group are *Mucorales* moulds, which produce plum mycelium in larger quantities than other genera, thus, inhibiting the growth of other moulds, which has to be taken into account in assessing microbiological quality [12].

Although good knowledge of microorganisms growing on different media for their proper identification and distribution into appropriate groups is needed, the advantages of the VDLUFA categorization are encouraging. It is a far more detailed approach than in current Serbian regulation, and which gives a more complete picture of the actual microbiological situation. However, whether such a feed could harm the health of animals or of consumers, or whether it represents a danger to the natural balance in addition to the reduction in quality, can only be examined by a risk analysis for the specific case under review. This could involve, for instance, further examinations, e.g. for pathogens or toxic substances (e.g. mycotoxins). The examination for specific pathogenic microorganisms, such as e.g. *Salmonella, Escherichia coli, Listeria* and *Clostridium perfringens*, is therefore not the subject of this procedure and has to be done additionally. The assessment of a feed with regard to a risk-free feeding is principally not subject of this operating procedure, but is an indicator of its microbiological quality.

4. Conclusion

Microbiological categorization of animal feed based on VDLUFA method, according to the years of experience within several European countries, could also be beneficial for the assessment of feed in Serbia. It can provide a benchmark in the modernization of feed hygienic standards and feed legislation, while the data on the category of microbiological quality, if mentioned on feed declarations, would increase the competitiveness of the products on the market. This approach also offers the possibility to

reduce and prevent the entry of hazards in the early stages of the food production chain. More precise, regular determination of microorganisms could provide a better insight into other common feed-borne problems, such as the possibility of mycotoxin occurrence. All in all, it would be a positive step forward, therefore, advisable and preferable.

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Influence of polyphenols on sensory properties of fermented sausages

Influence of polyphenols on sensory properties of fermented sausages

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Abstract. In the current research, the use of polyphenols in the production of fermented sausages as a natural preservative and their influence on the sensory characteristics of these products were investigated. Polyphenols could have antimicrobial and antioxidant roles in meat products, but also a range of positive biological effects on consumers. The results of the research showed that the addition of polyphenols did not significantly affect the sensory properties (colour, cross-section appearance, consistency, odour and flavour) of the three groups of sausages (control fermented sausage and two sausage variants, one with nitrite and one without nitrite), and that sausages were highly rated during most of the storage period. In addition, all tested sausages were evaluated as acceptable until the end of the entire storage period, i.e., throughout the 280-day period after sausage production.

1. Introduction

Polyphenols are secondary metabolites of plants that play physiological roles in leaves by protecting them from microorganisms and ultraviolet irradiation. These compounds include flavonoids (anthocyanidins, flavonols, flavanols, isoflavonoids, flavones and flavanones) and phenolic acids. It has been proven that polyphenols can exert a range of positive biological effects on consumers including antioxidative, anticarcinogenic, anti-inflammatory and antimicrobial ones, so they could play an important role as a functional ingredient in meat products, providing them the properties of functional food. Furthermore, some recent research indicates polyphenols could be used as natural preservatives in meat products [1]. Fermented sausages are meat products which, after being stuffed into casings, are preserved by fermentation and drying, i.e., ripening, with or without smoking. Salts, spices, additives etc. can be added to the stuffing of these sausages [2]. Since the fermented sausages are not heat treated during production, so their nutritive and bioactive ingredients keep mainly unchanged during production, they have great potential to be designed as functional foods [3] and to be carriers of polyphenols as a functional ingredient.

As prescribed by Serbian Regulation [2], the sensory properties of fermented sausages must meet certain criteria: the surface of sausages must not be deformed, the casing should fit well with the filling, the texture should be firm, the cross section should have the appearance of a mosaic composed of pieces

of meat and fat which are evenly distributed and well connected, the presence of cavities is not allowed on the cross sections, the colour should be stable, and the odour and flavour pleasant and characteristic. Sensory properties of fermented sausages are influenced by the quantity and quality of meat, but also by other ingredients incorporated in the stuffing [4]. The colour is a very important characteristic of meat products and is frequently a deciding factor for consumers when purchasing fermented sausages [5]. The typical aroma of dried fermented sausages results from the accumulation of volatile substances, such as alcohols, ketones, aldehydes, esters, terpenes, aliphatic hydrocarbons and furans, as well as non-volatile substances such as amino acids, peptides, sugars and nucleotides, which come from the basic ingredients of sausages (meat, spices and additives) or are formed by their enzymatic degradation during ripening [6].

In this study, the influence of polyphenols, added during the production of fermented sausages, on the sensory properties of fermented sausages during the entire storage period was examined.

2. Materials and methods

Three groups of fermented sausages were produced. The first group, control (C), comprised sausages of the usual composition: 35 % beef, 35 % pork and 27 % fatty tissue in which 2.2 % nitrite curing salt was added as a preservative. The second group of sausages (N+P) was of the same basic composition as the first one, but with 0.17 % added polyphenol preparation (grape skins and seeds powder), and the third group (P) was produced without the addition of nitrite, but with 0.17 % added polyphenol preparation as a natural preservative. All sausages also contained added: 0.2 % sugar, 0.2 % spice mix and starter culture.

The sausage stuffing was packed into collagen casings with a diameter of 55 mm, after which the sausages were subjected to the processes of smoking, drying and ripening, under the following conditions: tempering at room temperature for 12 hours; two days of fermentation at 26°C and relative air humidity (RH) 90 %; smoking occasionally for three days at 22°C to 24°C; drying and ripening at 15°C and RH which gradually decreased from 90 % to 75 % over 35 days. After ripening, the products were stored at 15°C. The sensory properties of all the produced fermented sausages were assessed using a quantitative descriptive test (ISO 8586-2: 2008 [7]; ISO 6564: 1985 [8]), by the evaluation of the following properties: colour, cross-section appearance, texture, odour and flavour. These parameters were evaluated on 0, 30, 70, 100, 130, 190, 220, 250 and 280 days of storage, according to a five-point score system in which 5 points meant ‘excellent’ and 1 point denoted ‘unacceptable’. Scores were grouped according to sausage types and average scores were calculated. Sausages with scores of 2.0 and higher for each test trait were considered acceptable.

3. Results and discussion

The results of the sensory test showed that the cross-section appearance of all products was very similarly evaluated. In the first 70 days of storage, the cross-section appearance of P sausages received slightly lower grades (4.8 ± 0.3) than C sausages and N+P sausages (5.0 for both) did; this difference was not statistically significant (P=0.15). From day 100 until the end of storage, the ratings for the appearance of the cross-section reduced, so that at the end of the storage period, P sausages were rated with an average grade of 3.3 ± 0.3, and C and N+P sausages 3.0 ± 0.0 (P=0.03).

During the storage, the average colour grade of P sausages was significantly lower during the first 30 days (4.4 ± 0.5), compared to the other two groups (5.0 ± 0.0 in both) (P=0.03) because of a weaker developed red colour in the central parts of the sausage as well as the presence of a 5-10 mm wide peripheral ring. In this case it was not a “dry edge” as a consequence of over drying of the sausage surface, but the colouring of peripheral parts of sausages due to migration of diluted grape polyphenol pigments together with moisture which diffuses towards the periphery of the sausage during drying [9]. In the further stages of ripening and storage, this ring gradually disappeared, most likely as a consequence of the equalization of the colour of the sausage in the cross-section during the formation of stable forms of reduced myoglobin in the mature product, as stated by other authors [10]. Based on the above, the appearance of a darker coloured ring in sausages with polyphenols can be considered a

normal transient phenomenon, as also shown by colour ratings in the period between days 70 and 130, where the colour of P sausages was given better scores (4.5 ± 0.3 to 4.6 ± 0.3) than at the beginning of storage and there were no statistically significant differences (P=0.83) in colour grades between the three groups of sausages (C=4.6 ± 0.2 and N+P=4.6 ± 0.2). After 130 days of storage, the average colour grades of all sausage groups gradually decreased, so that on day 280 the average grade was 3.0 in all of them.

In terms of texture, all product groups were evaluated identically (P>0.99). By day 70 of storage, the texture of all products was given 5.0 points, after which the grades gradually reduced so that on day 190, they averaged 4.3 ± 0.3 and at the end of storage, they were 3.0 in all groups of sausages.

The odour of all groups of sausages was evaluated with an average grade of 5.0 until day 30, regardless of the method of storage (Figures 1, 2 and 3), after which odour grades gradually decreased so that on day 100 the average grade was 4.3 ± 0.6 in P sausages, and 4.4 ± 0.5 in C and N+P sausages, whereby these differences were not statistically significant (P=0.95). The odour of P sausages was again slightly worse (3.0) on days 220 and 250 than of C (3.2 ± 0.3) and N+P sausages (3.3 ± 0.3), but this difference was not statistically significant (P=0.4 and P=0.7, respectively). At the end of storage, the odour of all sausages was evaluated with the same average grades (3.0). The acceptable odour of sausages with polyphenols at the end of storage can be attributed to the antioxidant role of polyphenols, described by some other authors [1] and confirmed by lower lipid oxidation parameters we determined (data not shown).

The taste of P sausages (4.80 ± 0.4) was worse in the first 30 days compared to C and N+P sausages (5.0), but this difference was not statistically significant (P=0.46). From day 70, the grades gradually reduced, and all sausages were identically rated for taste. At the end of sausage storage, the taste of C sausages (2.0) was evaluated as the worst in comparison with N+P (2.3 ± 0.4) and P (2.4 ± 0.4) sausages, without statistically significant differences (P=0.2 and P=0.4, respectively).

Sausages enriched with polyphenols were acceptable throughout the entire storage period of 280 days, with an average score of 3.0 for all the tested sensory properties, with control sausages being the lowest rated due to a poor and slightly rancid aroma, which received a score of 2.0. Moreover, all sausage groups were rated very highly even on day 190 of storage, which is important given that fermented sausages, according to some authors [11], are usually stored for no longer than 180 days.

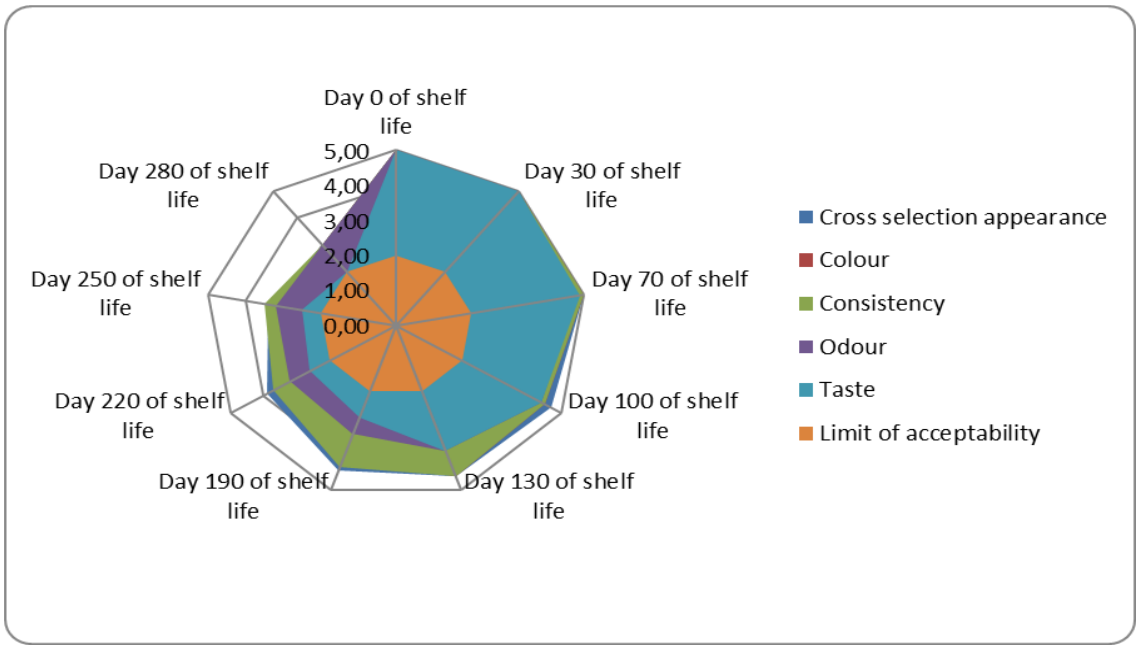

Figure 1. Ratings of cross-section appearance, colour, consistency, odour and taste of sausages in the control sasusages (C) during storage

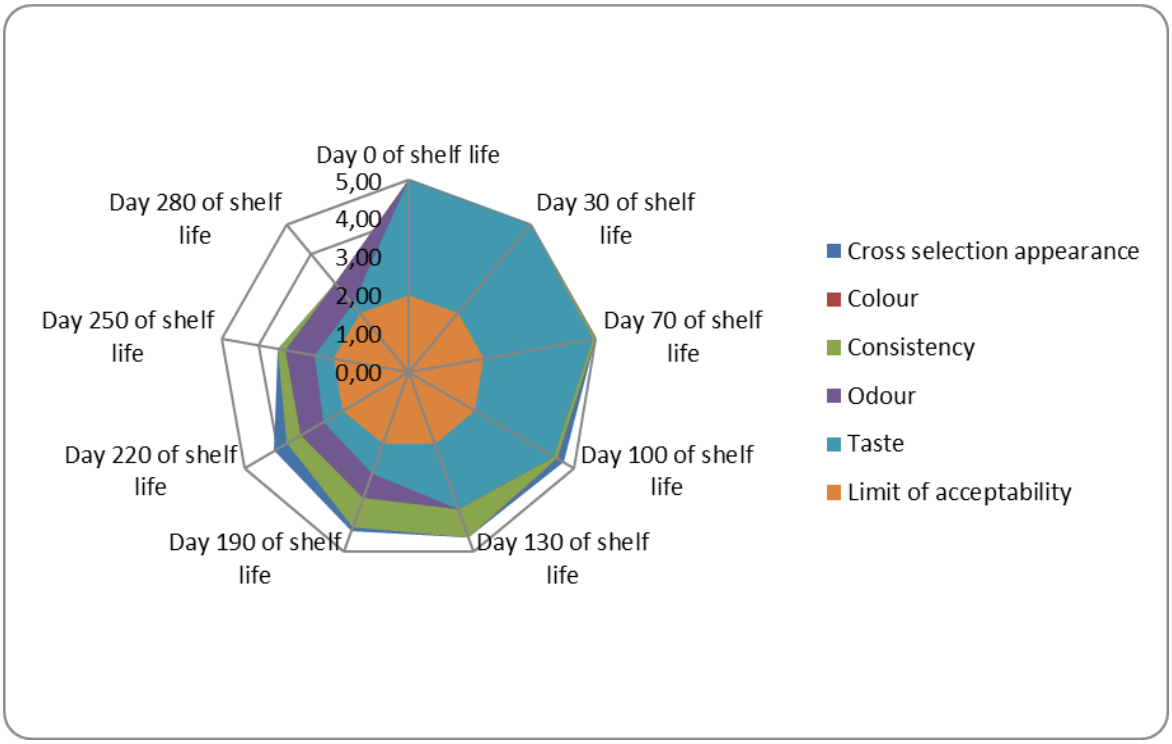

Figure 2. Ratings of cross-section appearance, colour, consistency, odour and taste of sausages with added nitrites and polyphenols (group N+P) during storage

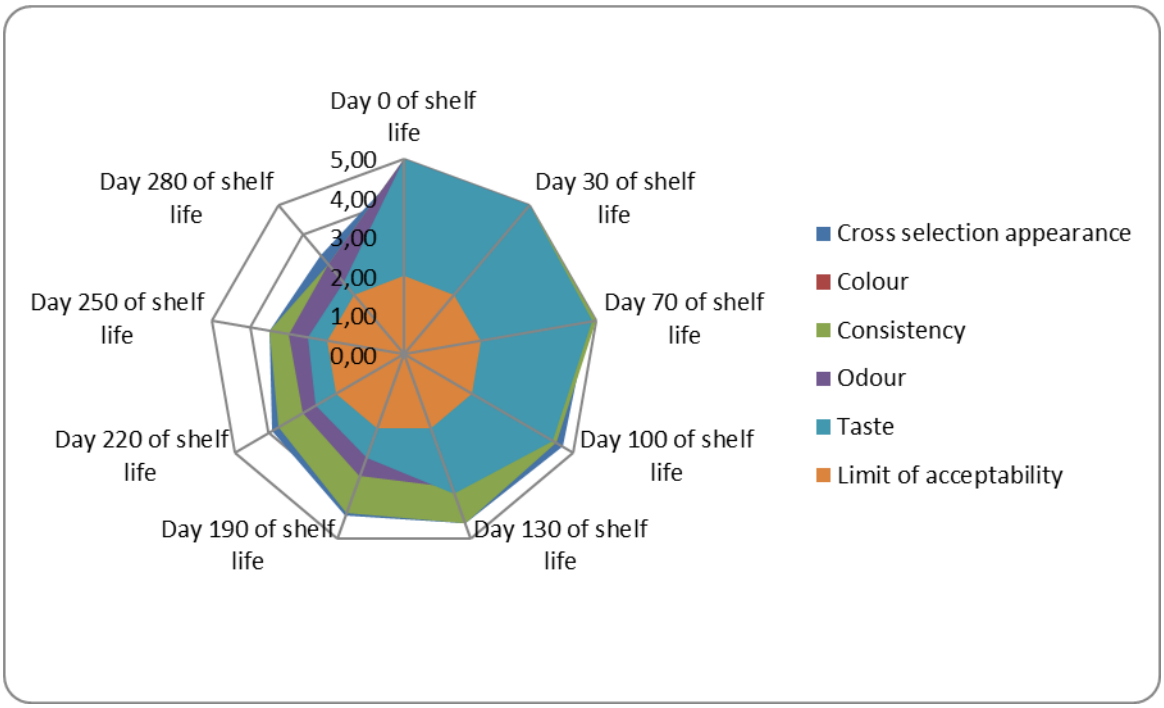

Figure 3. Ratings of cross-section appearance, colour, consistency, odour and taste of sausages with added polyphenols, but without nitrites (group P) during storage

4. Conclusion

The sensory characteristics of all the three groups of fermented sausages were evaluated as approximately the same, in all phases of storage. The decrease in grades for all parameters, especially after day 130 of storage, can be attributed to oxidative changes that endangered primarily the smell, taste, colour and appearance of the sausage cross-sections. Thus, this study confirms the possibility of achieving a prolonged shelf life for fermented sausages enriched with polyphenols, even those produced without nitrite.

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Can yellow gentian (*Gentiana lutea*) be useful in protection against foodborne mutagens and food contaminants?

Can yellow gentian (*Gentiana lutea*) be useful in protection against foodborne mutagens and food contaminants?

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Abstract. *Gentiana lutea* is a wellknown and respected medicinal plant that is used in many pharmacopoeias, mainly against different gastrointestinal disorders. The plant is under protection regimes in its natural habitats and for that reason is grown on plantations. In addition, it could be cultivated *in vitro*. The plants grown on plantation and in *in vitro* conditions were used to prepare methanolic and 50% ethanolic extracts of root and leaf/shoot, which were tested for antigenotoxic and antibacterial properties, against foodborne mutagens (heterocyclic aromatic amines PhIP and IQ) and food contaminants, respectively. The results obtained pointed out the excellent genoprotective effect (up to 78% inhibition of PhIP/IQ genotoxicity) based mostly on the antioxidative potential. The antibacterial effect was mainly weak; only the extracts of *in vitro* grown plant induced moderate activity against *Listeria monocytogenes* and *Staphylococcus aureus* (MICs ranged 0.15-5 mg/ml). In addition, the extracts' potential to prevent biofilm formation by *L. monocytogenes* was very high (up to 90% inhibition). Taken together, the results obtained encourage further research that would be directed to the formulation of potent antigenotoxic and antibiofilm agents based on *G. lutea*.

1. Introduction

The need for safe and high-quality food is an imperative for the world population, and the reasons for that are numerous. Among others, ending hunger and poverty and promoting good health and well-being seem to be the most important [1]. To provide sustainable food processing, producers are inevitably faced with numerous problems that have to be resolved, in order to provide consumers with high quality food that complies with safety and security requirements, and simultaneously supports health [2]. The growth of microbial contaminants and the presence of mutagens in food are considered as among the most pronounced problems that need solutions.

2. Characterization of the problems: concepts of microbial contamination and food mutagens

Concerning microbial contamination, despite strict food protection measures implemented in developed countries, this problem still exists and the necessity to investigate new food preservatives, which are efficient and do not induce side effects on consumers' health, is evident. Furthermore, the problem of foodborne illness is disproportionately higher among populations of developing, low- and middle income countries than in wealthy countries. The need for cheap and efficient means of food preservation in less wealthy nations is pronounced. As an illustration of the need to continuously combat this problem, the WHO report pointed out that annually, 600 million people became ill by ingestion of contaminated food, and among them as many as 420,000 casualties were estimated [3].

According to these facts, there is a serious need for the upgrading of food preservation techniques in order to provide green food processing that would consequently promote health [4]. Furthermore, being aware of side effects that could arise from synthetic additives/preservatives, the search for the potent antimicrobials of plant origin that could be used in foods is strongly advised [5].

Another important question that should be resolved considers the biological activity of food ingredients. Many of them are embedded with health-promoting properties – it is well known that bioactive constituents of fruits, vegetables and spices possess antioxidative, antimicrobial, antiviral,

anti-inflammatory, antirheumatic, lipid-lowering, antidiabetic, anticancer, hepato- and nephroprotective, and other beneficial effects [6]. However, some of foods' bioactive compounds could induce harmful effects; there are also literature data indicating herbs' toxicity and mutagenicity [7,8]. This paper will pay special attention to food mutagens.

Substances that can induce DNA damage and consequently lead to formation of mutations are designated as mutagens or genotoxic agents. Genotoxicity is defined as the ability of an agent to induce damage to genetic material, i.e. DNA molecule, or cellular components associated with the functionality and behavior of chromosomes [9]. By inducing genotoxicity, mutagens contribute to different genetic disorders and degenerative diseases, including hepatic, cardiovascular and neurodegenerative conditions, diabetes, chronic inflammation, arthritis and cancer. Genotoxic substances can also be found in food; some of them accidentally occur in food (such as aflatoxin, as a result of mould contamination), but the others are intentionally added, like food additives (such as boric acid and sunset yellow) [10-12]. Both of these food mutagen groups could be avoided by strict prevention of mould contamination, or by careful revision of permitted and prohibited food additives, which is periodically realized. However, there is one more group of food mutagens – the ones that are formed in foodstuffs during food processing, i.e. foodborne mutagens. They include three subgroups: polycyclic aromatic hydrocarbons, nitrosamines and heterocyclic aromatic amines (HAA) [12]. Although strict implementation of some codes of practice and standards, recommended by Codex Alimentarius Commission, could contribute to the reduction of foodborne mutagen levels, their presence in processed food cannot be completely avoided [13]. For that reason, the alternative strategy of using natural products with antigenotoxic properties in nutrition is recommended. There are numerous compounds of natural origin, mainly from edible and medicinal plants, that are well known for their antigenotoxic/genoprotective potential and could be designated as phyto-antimutagens [14,15].

3. Yellow gentian – use, biological activities, sources and chemical composition

Taking into account the above-mentioned facts, the aim was of this study was to explore and identify bioactive agents of plant origin that would be efficient both as antimicrobials and antimutagens. Our efforts directed us to great yellow gentian (*Gentiana lutea*), a medicinal plant which is recognized by many pharmacopoeias. It is used in pharmaceutical, cosmetic and food industries, in production of drugs and cosmetics, and as an additive in beverages and foods [16,17]. Reviews have reported numerous biological activities of this reputable folk remedy, such as antioxidative, antimicrobial, anti-inflammatory, antidiabetic, hepatoprotective, anticancer and radioprotective effects [18,19]. It is used in traditional medical preparations for gastrointestinal and menstrual disorders, treating wounds and arthritis, and as an antihypertensive agent [20,21].

However, due to the high demand for this medicinal plant, *G. lutea* is under protection regimes, both in the European Union and in Serbia [22,23]. Therefore, in order to preserve the plant in its natural habitats, *G. lutea* for commercial uses is cultivated on plantations [24]. Furthermore, with the aim to introduce *G. lutea* into *in vitro* plant tissue culture, preliminary attempts have been previously made [25,26], but cultivation of plants, both root and shoot, has been successfully undertaken by our research team [27].

Previous analysis of chemical composition pointed out that the main pharmacologically active compounds in *G. lutea* organs are secoiridoids, followed by iridoids, xanthones and C-glucoflavones. In addition, plant organs, mainly the ones in aerial plant parts, are rich in polyphenols, including flavonoids [18].

In order to study the antimutagenic and antimicrobial potentials of *G. lutea* substances, we prepared methanolic and 50% ethanolic extracts of root and leaf of the plantation grown plant, as well as methanolic extracts of root and shoot of the *in vitro* grown plant [27,28]. The various extracts (Table 1) and their chemical profiles, obtained by UPLC-PDA MS/MS analysis, and total polyphenols and flavonoids in the extracts (Table 2) are shown. The results presented in Table 2 confirmed the high contents of polyphenols and, among them of flavonoids, especially in the extracts of aerial plant parts (leaf and shoot). Furthermore, the contents of tested constituents did not differ significantly between the methanolic and 50%-ethanolic extracts of the same organs of plantation grown plant, at least for the

majority of the constituents. Iv-Met-S differed from all the other extracts – the high levels of bioactive constituents pointed to the constituents that were intensively produced in aerial parts (secoiridoids gentiopicroside, sweroside and swertiamarin, and the iridoid, loganic acid) remaining there only in the case of *in vitro* cultivation, while in the case of plantation grown plants, they were transported to the roots [27].

Table 1. Descriptive indication of labelled test substances – methanolic and 50%-ethanolic extracts of *G. lutea* grown in plantation and *in vitro*

Plantation grown plants (Pg)				*In vitro* grown plants (Iv)	
50% **Et**hanolic-water extracts (Et)		**Met**hanolic extract (Met)		**Met**hanolic extract (Met)	
Root (R)	**L**eaf (L)	**R**oot (R)	**L**eaf (L)	**R**oot (R)	**S**hoot (S)
Pg-Et-R	Pg-Et-L	Pg-Met-R	Pg-Met-L	Iv-Met-R	Iv-Met-S

Table 2. Chemical profiles of *G. lutea* extracts*

Constituent (%)	**Pg-Et-R**	**Pg-Et-L**	**Pg-Met-R**	**Pg-Met-L**	**Iv-Met-R**	**Iv-Met-S**
gentiopicroside	5.058±0.291 ^{a}	1.141±0.147 ^{b}	4.424±0.339 ^{a}	1.528±0.170 ^{b}	1.949±0.101 ^{b}	11.463±0.098 ^{c}
Sweroside	2.080±0.270 ^{a}	0.207±0.034 ^{a}	1.999±0.052 ^{a}	0.232±0.033 ^{b}	0.184±0.005 ^{c}	3.495±0.125^{d}
Swertiamarin	0.631±0.032 ^{a}	0.301±0.036 ^{b}	0.551±0.017 ^{a}	0.302±0.007 ^{b}	0.046±0.005 ^{c}	1.338±0.056^{d}
Loganic acid	0.710±0.148 ^{a}	1.792±0.096 ^{b}	0.706±0.006 ^{a}	1.789±0.152 ^{b}	0.031±0.005 ^{c}	2.271±0.184^{d}
Mangiferin	/**	0.129±0.023 ^{a}	/	0.049±0.003 ^{b}	/	0.110±0.022^{a}
Isogentisin	0.259±0.086 ^{a}	0.231±0.053 ^{a}	0.136±0.019 ^{b}	0.049±0.002 ^{c}	0.022±0.011 ^{d}	0.039±0.007^{c}
Homoorientin	0.025±0.005 ^{a}	3.999±0.122 ^{b}	0.019±0.002 ^{a}	0.692±0.064 ^{c}	/	0.140±0.020^{d}
Isovitexin	0.013±0005^{a}	3.038±0.487 ^{b}	0.006±0.003 ^{a}	0.739±0.033 ^{b}	/	0.114±0.001^{c}
Total polyphenols	25.8±3.2	53.4±3.5	22.7±3.3	44.7±3.5	23.9±1.7	50.8±0.5
Total flavonoids	1.4±0.1	20.7±1.8	1.12±0.1	18.2±2.7	1.8±0.4	21.6±3.1

*The results are taken from our previous works [27,28]; Statistical significance was determined by comparing all the results and using one-way ANOVA with Tukey's post hoc test. Values with different superscript letters in each row differ significantly ($p<0.05$); **The content is lower than the limit of determination.

4. Genoprotective activity of the *G. lutea* extracts against genotoxicity of selected HAAs

HAAs are considered potent mutagens that are formed during thermal processing and smoking of protein-rich foods, such as meat and fish. After metabolic activation by mammalian liver enzymes, they are converted into mutagenic and carcinogenic agents that play an important role in the etiology of various human cancers. How potent some of them are can be deduced from the observation that active carcinogenic concentrations can be at ng (10^{-9} g) level [12]. The antigenotoxic potential of the extracts was assessed according to activity against the two HAA compounds selected: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3-methyl-3H-imidazo[4,5-f]quinolone (IQ). While PhIP is classified by the International Agency for Research on Cancer (IARC) as a possible carcinogen, IQ is declared as the most likely carcinogen harmful to human health [29].

Screening on this topic was performed by alkaline comet assay, which is routinely used to determine and quantify the DNA damage. Although only the *in vivo* alkaline comet assay is recommended by the OECD for genotoxicity determination, *in vitro* applications on different mammalian cell lines are widespread both in genotoxicity and antigenotoxicity testing [30]. In our investigation, the assay was performed on human hepatoma HepG2 cell line [27]. Preliminary data, concerning genotoxicity testing of PhIP and IQ, as well as of gentian extracts, were provided in order to determine the adequate concentrations of the mutagens, and to define the non-genotoxic concentration ranges of all tested extracts [27,28,31]. Then, co-treatment experiments involving both PhIP/IQ and each of the *G. lutea* extracts were performed; the results obtained indicate the framework of the plant's antigenotoxic activity (Figure 1).

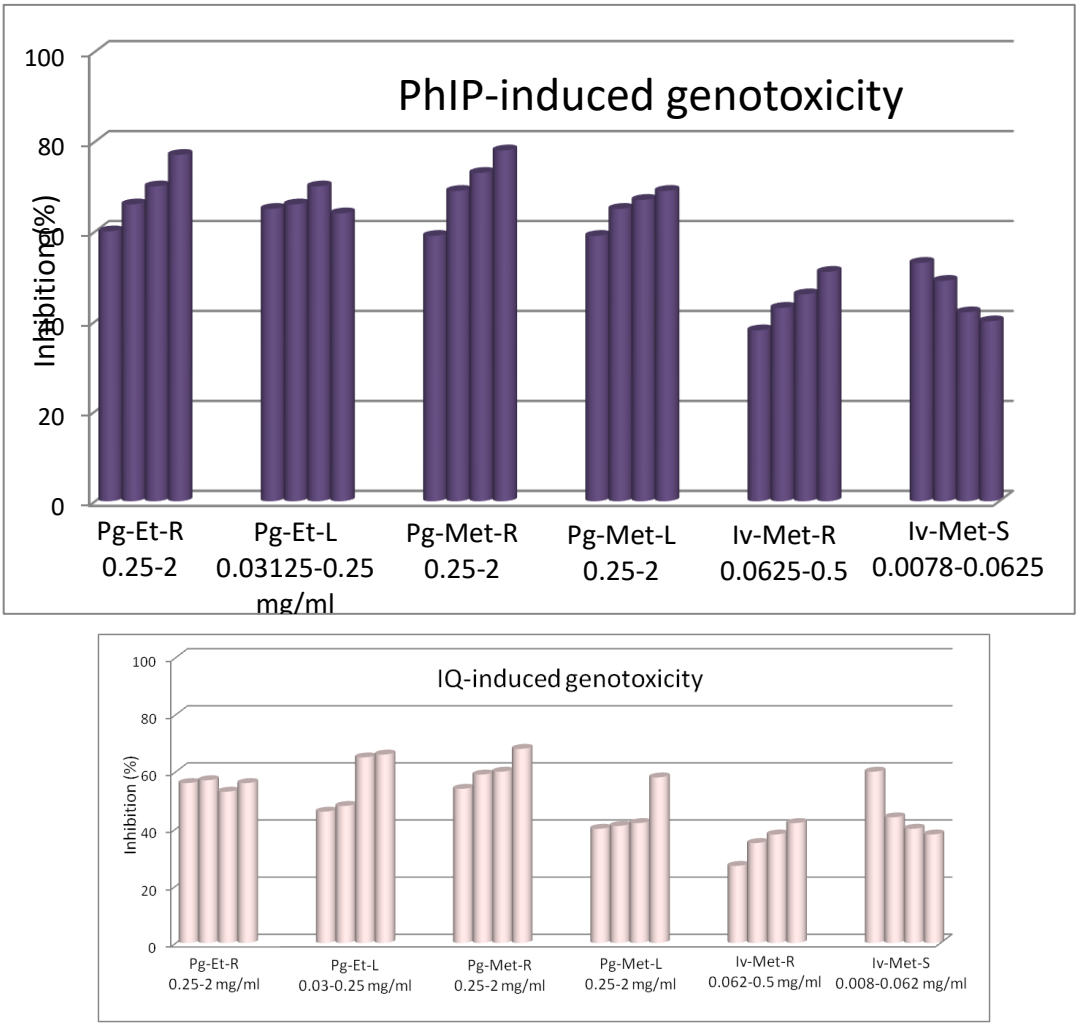

Figure 1. The antigenotoxic effect of *G. lutea* extracts.

The inhibitions (%) of PhIP-induced (above) and IQ-induced (below) genotoxicity were determined in the alkaline comet assay, performed on human hepatoma HepG2 cell line. For all the extracts, antigenotoxic concentration ranges are specified.

As it can be seen from the figures, dose-dependent curves of antigenotoxicity were not determined for all extracts studied. On the contrary, for some of them a J-curve, indicating the highest beneficial effect at the lowest applied concentration, was observed. This detail, together with the fact that in higher concentration ranges all the extracts were genotoxic [27,28], suggests the hormesis phenomenon. The term hormesis refers to the situations characterized by a low-dose response that is opposite in effect to

that seen at high doses [32]. Actually, numerous agents in genotoxicology are defined as "Janus substances, which, applied at low doses act as antimutagens, but applied at higher ones act as mutagens" [33]. The fact that the tested gentian extracts act as Janus agents is cautionary; very careful analysis is required in order to clearly define the active genotoxic concentrations that could be applied for protection against IQ and PhIP mutagenicity.

Furthermore, since both PhIP and IQ mutagenicity is realized, at least partially, by oxidative DNA damage [34,35], we also quantified the extracts' antioxidative potential. Results provided by DPPH assay, which determines the radical scavenging effect, show the extracts of aerial parts, i.e. Pg-Et-L, Pg-Met-L and Iv-Met-S, were embedded with higher capacity to quench radicals [27,28]. In addition, both ethanolic extracts (Pg-Et-L and Pg-Et-R) were confirmed to up-regulate Nrf2 transcriptional factor, being the master factor responsible for the expression of antioxidative enzymes in hepatoma HepG2 cells. Finally, the high antioxidative potential of ethanolic extracts, which was realized in the cells, was confirmed by measuring effects of ethanolic extracts on regeneration of the reduced form of glutathione in the HepG2 cells co-treated with PhIP/IQ and each of the Pg-Et-L/Pg-Et-R extracts [28]. These results pointed to the antioxidative properties of the extracts as being responsible for the observed antigenotoxicity. In other words, due to antioxidative action, the tested gentian extracts protect DNA from oxidative damage induced by IQ and PhIP.

5. Antibacterial and antibiofilm activity of the *G. lutea* extracts

Starting from literature data indicating the strong antimicrobial activity of *G. lutea* extracts [36], and bearing in mind the plant's potential to mitigate gastrointestinal disorders, in further work, we screened for the antibacterial effect of the extracts on a palette of selected food contaminants. However, microdilution assay indicated only weak antibacterial activity which was not evident against all tested bacteria strains. The only exception was effect of the *in vitro* grown plants against *Listeria monocytogenes* and *Staphylococcus aureus*, where moderate antibacterial potential was determined. Table 3 shows the minimal inhibitory and bactericidal concentrations (MICs and MBCs, respectively) determined for the susceptible strains. Taking into account results presented in this Table and the fact that the tested strains of *Escherichia coli* and *Shigella flexneri* were not sensitive at all, the greater effect against Gram-positive than Gram-negative bacteria was evident.

Table 3. Antibacterial effect of *G. lutea* extracts*

	MIC/MBC values (mg/ml)					
	Pg-Et-R	Pg-Et-L	Pg-Met-R	Pg-Met-L	Iv-Met-R	Iv-Met-S
B. subtilis ATCC6633	5/10	5/10	5/10	2.5/5	5/10	1.25/2.5
E. faecalis ATCC29212	5/10	5/10	5/10	2.5/5	nd	5/10
L monocytogenes ATCC19111	10/nd	10/nd	nd	5/10	0.62/1.25	0.31/0.62
S aureus MSSA ATCC 25923	5/10	5/10	nd	5/10	0.62/2.5	0.15/1.25
S aureus MRSA ATCC 43300	nd**	nd	nd	5/nd	5/10	5/10
P. aeruginosa ATCC 15442	10/nd	10/nd	10/nd	10/nd	nd	nd

*Antibacterial activities of the extracts derived from plantation grown plants are taken from our previous work [37]; **nd – not determined in the applied concentration range (0.078-10 mg/ml)

Based on the results of the microdilution assay, we focused on *L. monocytogenes* and *S. aureus* and in further work, tested the extracts' potential to prevent biofilm from being formed by these two bacteria strains. This direction was in accordance with the actuality of the problem of biofilms in food industry [38]. Preliminary unpublished data, provided by crystal violet assay, show that antibiofilm potential against *S. aureus* is not high (the maximum inhibition is 28%), while in the case of *L. monocytogenes*, the potential of the extracts to prevent biofilm formation is multifold higher; the inhibitions determined are up to 90%. Interestingly, the Iv-Met-R and Iv-Met-S extracts, which induced the highest antibacterial

potential in microdilution assay, were moderately active against biofilms and inhibited biofilm formation by maximally 35% (data not shown).

6. Conclusion

This investigation revealed that *Gentiana lutea* methanolic and 50% ethanolic extracts could be considered as the excellent genoprotective agents that protect against the genotoxicity of food borne mutagens, IQ and PhIP. The extracts' antigenotoxic potential is at least partially based on their antioxidative properties. Results concerning antimicrobial effects indicated that some antibacterial potential exists, while the antibiofilm activity against *L. monocytogenes* was high. All data obtained encourage further investigation.

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The potential of the application of *Boletus edulis*, *Cantharellus cibarius* and *Craterellus cornucopioides* in frankfurters: a review

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The potential of the application of *Boletus edulis*, *Cantharellus cibarius* and *Craterellus cornucopioides* in frankfurters: a review

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Abstract. Today, there is increasing demand for the meat industry to produce sausages with partial or complete replacement of commercial antioxidants by natural ones, with minimal or no impact on the quality characteristics. One of the natural additives that are recognized as highly nutritious and rich in antioxidants is mushroom. The three most commonly consumed mushrooms in Serbia are *Boletus edulis*, *Cantharellus cibarius*, and *Craterellus cornucopioides*. This review provides information on the antioxidant and antimicrobial potential of these three mushrooms *in vitro*, and the feasibility of their application in frankfurters. The benefits, limits, and accomplished effects of the addition of mushrooms on lipid peroxidation reduction, microbial inactivation, colour, texture, and sensorial traits are presented with regard to their implementation on the industrial level.

1. Introduction

The contemporary way of life and globalization have resulted in limited time for food preparation and consumption. Meat products belong to a group of perishable foods that can be subjected to bacterial contamination, producing reactions that deteriorate colour, texture, flavour, odour, and sensorial characteristics. Due to increasing demands for meat products that are easy to prepare and consume, there is growing concern about their safety and quality. One of the major problems that can cause deterioration during the storage of meat products is lipid oxidation. This is the oxidation of unsaturated fatty acids when the phospholipid segment of the membrane is oxidized. This oxidation manifests through the formation of numerous advanced lipid oxidation end products (ALEs), which are carriers of rancid odour and taste [1]. Together with the growth of undesirable microorganisms, these changes are recognized as the main factors that influence the shelf life reduction in meat products.

In order to reduce lipid oxidation in meat products and inhibit the growth of microorganisms that can cause deterioration, antioxidant compounds can be added during the process of formulation, incorporated in packaging material, or coated on the surface [2−4]. Generally, there is a well-known trend of the use of synthetic antioxidants such as butylated hydroxytoluene (BHT), that prevent lipid oxidation, simultaneously prolonging shelf life by limiting the formation of free radicals or scavenging

peroxide radicals. However, there is a great concern that the use of commercial antioxidants could be toxic for consumers [5]. In this regard, there are rising demands from customers for the greater usage of antioxidants from natural origins in the meat industry, over synthetic antioxidants [6, 7]. Therefore, plants and their extracts are gaining wide attention in the meat industry for their potential usage as antioxidants and antimicrobials, especially because they are generally recognized as safe (GRAS) [8]. Also, they do not have a negative impact on colour, odour, and flavour and they are easy to apply, stable during storage, and cost-effective. The current review aimed to summarize the use of three natural mushroom decoctions to prevent microbiological deterioration and reduce oxidative changes in frankfurters, without adverse changes in technological properties.

2. Mushrooms as a source of antioxidant and antimicrobial compounds

According to a USAID report and analyses from the annual reports of the Institute for Nature Conservation of Serbia, it is reported that *Boletus edulis*, *Cantharellus cibarius*, and *Craterellus cornucopioides* are the most commonly harvested and consumed mushrooms in Serbia [9]. These mushrooms have long been recognized for their wonderful taste. Also, mushrooms are rich in proteins, vitamins, minerals and fibre [10]. On average, dried mushrooms contain about 22% proteins with most of the essential amino acids, about 5% fat, 63% carbohydrates and 10% minerals, which are a good source of vitamins such as thiamine, riboflavin, niacin, and biotin [11]. When it comes to the fat content, most of the 5% fat is in the form of linoleic acid, an essential fatty acid that cannot be synthesized in the human body. In addition to their good flavour and favourable chemical composition, mushrooms have a low glycaemic index and are high mannitol, which is especially beneficial for diabetics.

Besides these nutritive-beneficial traits, some other compounds can be interesting, both for customers and the food industry. Mushrooms are rich in antioxidants, which can be of great interest to consumers due to their protective role in the human body by reducing oxidative damage without any interference. Also, they lower the risk of cancer, promote immune function, balance blood sugar levels, and detoxicate the human body [12, 13]. Mushrooms' phenolic compounds have proven to be tremendous antioxidants in food systems [14]. The exact mechanism of their activity is not established, but it is assumed that phenolics have ability to chelate metals and scavenge free radicals [15]. High amounts of these compounds occur in *Boletus edulis*, *Cantharellus cibarius*, and *Craterellus cornucopioides* [16−18].

3. Antioxidant and antimicrobial potential of selected mushroom decoctions *in vitro* and in frankfurters

Selected mushrooms (*B. edulis*, *C. cibarius* and *C. cornucopioides*) were tested in the form of decoctions. This is the weakest type of the extraction, as well as the most cost-effective and convenient for industrial application. *B. edulis* and *C. cornucopioides* decoctions expressed excellent antioxidant characteristics when the highest concentrations (10 mg/mL) were tested [19, 20], while good antioxidant properties were obtained from *C. cibarius* (21). On the other hand, antimicrobial properties of the tested mushrooms were also significant. All tested mushrooms expressed antimicrobial activity against *L. monocytogenes* and *Y. enterocolitica*, while *C. cornucopioides* showed even more potential against pathogens, *E. coli* and *S. aureus*. These results, in combination with antioxidant properties, recommended these mushrooms for usage in the production of frankfurters, in order to improve their quality and extend the shelf life of final products. Antioxidant and antimicrobial potentials of selected mushrooms, only in methanolic and acetonic extract, were also proven by Kosanic *et al*. [22, 23].

When it comes to the lipid oxidation of meat products, the addition of selected mushroom decoctions into frankfurters caused significant reductions in comparison to control treatment [24]. Antioxidant potential *in vitro* is expressed in a complex matrix such as frankfurters by the reduction of malondialdehyde content, an indicator of secondary lipid oxidation in meat products. It was due to the antioxidant potential of mushrooms, which is credited to the presence of phenols and its bioactive activity [25].

Regarding microbiological stability of frankfurters, total aerobic mesophilic bacteria (TAMB) is among the issues making food spoiled throughout cold storage and TAMB amounts are frequently used

as an indicator of the storage stability of frankfurters. The usage of *B. edulis* and *C. cibarius* decoctions in frankfurter production resulted in decreased values of TAMB during two months of chilled storage [24], while *C. cornucopioides* decoction reduced these microorganisms for six weeks (compared with controls). These results are very important, especially for frankfurters with *B. edulis* and *C. cibarius* added, since the shelf life of frankfurters on the Serbian market is about 45 days, and these treatments extended it for two weeks. Also, this could have a financial benefit for manufacturers, since without the addition of commercial antioxidants, considerable extension in shelf life is obtained. It is important to point out that the antimicrobial activity of mushrooms is the consequence of the presence of a considerable amount of natural antibiotics [26]. Also, according to Kurćubić *et al*. [27], antimicrobial activity can be attributed to the hydroxylation of the hydroxyl groups on the phenol ring.

4. Quality assessment of frankfurters with the addition of mushroom decoctions

Commonly, the quality of meat and meat products is evaluated through its colour (28). In the research of Novakovic *et al*. (19, 21), *B. edulis* and *C. cibarius* decoction immediately after the production of sausages did not cause a colour change. Similar results for colour parameters were reported by Pil-Nam *et al*. [29] who added shiitake mushroom powder to frankfurters. To be sure how mushroom decoctions in frankfurters affected the colour, ΔE value was calculated. Values can be detected by consumers only when higher than 3 [30]. After two months of cold storage, increased values in ΔE were within the limits that indicated a minor colour change, but it still could be detected by consumers. In comparison the addition of other natural components in sausages, such as grape seed flour [31], or sunflower seed oil [32], caused a considerable colour change of frankfurter colour. Therefore, the addition of *B. edulis* and *C. cibarius* had a trivial influence on the colour characteristics of this product. On the other hand, the addition of *C. cornucopioides* caused immediate changes in the colour of sausages, while after two months these changes were drastic [20]. So, it can be stated that from these three evaluated mushrooms, evident colour changes were obtained on the addition of *C. cornucopioides* that could be considered as a negative influence on the quality of the final product.

Besides the colour, another feature that demonstrates the quality of meat products is the texture. Commonly, the method that measures this feature is texture profile analysis, and it is based on the simulation of the food mastication process in the human mouth. Its performance speed corresponds to the mastication sample in the human jaw [33]. The incorporation of all three mushrooms in frankfurters resulted in increased values for hardness and chewiness [19–21]. This could be explained by the higher amount of proteins in sausages that migrated from mushrooms. The higher amount of protein in formulation, the firmer the texture of the final product, due to the creation of a denser protein matrix that is more resistant to compression [34]. These features are exactly the ones that consumers appreciated the most [35], so it could be concluded that mushroom addition improved the quality characteristic of sausages in terms of texture profile.

In the research of Novakovic *et al*. [19], *B. edulis* did not significantly change the appearance and total score values of sausages, in terms of sensorial traits. When it comes to the addition of *C. cibarius* incorporation in sausages [21], during the first 30 days of cold storage, higher scores were obtained for odour, flavour, and overall quality. This could be the consequence of the presence of various compounds in this mushroom that are characterized as flavour enhancers (lenthionine and monosodium glutamate) [19]. On the other hand, the addition of *C. cornucopioides* resulted in slightly lower scores for sensory evaluation, which would be another negative feature of the quality, when it comes to this type of mushroom.

5. Conclusion

B. edulis, *C. cibarius*, and *C. cornucopioides* expressed good antioxidant and antimicrobial potential *in vitro*, so they were used in the production of frankfurters in order to extend shelf life of the final product. Sausages with *B. edulis* and *C. cibarius* were microbiologically stable throughout 60 days of cold storage, meaning the shelf life was extended by 15 days, considering that the shelf life of frankfurters in Serbia is 45 days. This could be an interesting finding for producers, especially because it could reduce

expenses and increase profits for meat companies. From the aspect of quality, all mushrooms improved the texture of sausages, but the addition of *C. cornucopioides* caused some negative changes in terms of colour and sensorial traits. Therefore, it can be concluded that *B. edulis* and *C. cibarius* have great potential for usage in the meat industry, in order to extend the shelf life and improve the overall quality of the final frankfurter product.

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Health monitoring of wild bears in the Nature Park Skakavac, Canton Sarajevo

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Health monitoring of wild bears in the Nature Park Skakavac, Canton Sarajevo

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Abstract. Many wild animal populations are considered endangered due to anthropogenic activities. Wildlife and nature habitat preservation requires holistic and science based approaches supported by adequate regulations. One of the means for wildlife preservation is undoubtedly heath monitoring and investigation of infectious diseases of the wild animal populations, particularly if spillover effects are considered. Even though the theoretical background is well researched, implementation of disease prevention and control measures in wildlife populations entails more challenges than in domestic animal populations. Immediate signs of health disorders in wildlife often become evident when the infectious agent is well established in an area. Additionally, due to unrestricted and often long-range movement of wildlife, diseases are easily spread across borders. Brown bears, indigenous in Europe, are classified by EU regulations as endangered, almost extinct and rare. The wild bear population in Bosnia and Herzegovina shares a genetic lineage with bear populations of neighbouring Croatia, Serbia and Montenegro and is one of the few remaining fragments of bear populations in Europe. The aim of this paper is to describe implemented activities for health and telemetric monitoring of wild bears in the Nature Park Skakavac, Canton Sarajevo, Bosnia and Herzegovina.

1. Introduction

According to many international reports, growing numbers of wild and indigenous animal populations are becoming endangered due to mainly anthropogenic activities, thus posing great risk for global biodiversity [1,2]. Similarly in Bosnia and Herzegovina (BiH), many wild animal populations, particularly large animals, are decreasing in size for reasons such as non-harmonized and lacking hunting management, deterioration and decrease of natural habitats and outdated and loosely implemented regulations [3]. The process of modernizing wildlife management plans and harmonizing with EU standards includes establishing wildlife population sizes and distributions alongside analysis of health status, particularly if spillover effects (emerging zoonotic diseases) are considered.

In Europe, the population of brown bears in the last few decades has rapidly decreased, and the species is classified by EU regulations as endangered, almost extinct and rare [4, 5]. The wild bear

population in BiH shares genetic lineage with bear populations of neighbouring Croatia, Serbia and Montenegro and is one of the few remaining fragments of bear populations in Europe [6]. Nature Park Skakavac (Canton Sarajevo, BiH) is a specific, peri-urban natural habitat for several large wildlife species, some of which are considered close to extinction according to the IUCN/CITES classification (International Union for Conservation of Nature/Convention on International Trade in Endangered Species of Wild Fauna and Flora).

The aim of this paper is to describe implemented activities for health and telemetric monitoring of wild bears in Nature Park Skakavac, Canton Sarajevo, BiH. Besides its scientific contribution, this study falls in line with demands and recommendations of national and international regulations, conventions, strategies and action plans for environment protection including the Plan and Program for Wildlife Preservation in the Canton Sarajevo.

2. Materials and Methods

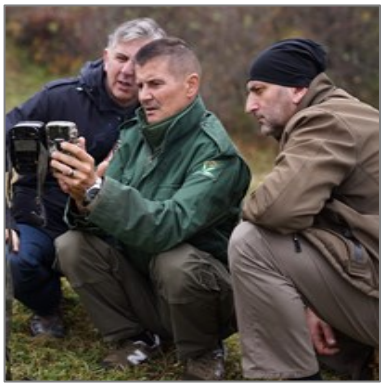
2.1. Study area

The study area was the Nature Park Skakavac, established in 2002 as the first natural heritage site in the Canton Sarajevo, managed by the Canton Sarajevo Administration for Protected Natural Areas. The park is located in the eastern part of central BiH within the administrative borders of the Canton Sarajevo (https://www.google.com/maps/@43.9033123,18.3453458,12.14z). The park terrain is mountainous (as a subset of the wider Dinaridi area) and spreads across 1,430.70 ha. The entire area is rich in natural water bodies together with a high degree of flora and fauna biodiversity, indicating beneficial conditions for wildlife to thrive. The climate in the area is moderate-continental with influences of mountain climate. Average annual temperature is around 5°C, ranging from -32.5ºC to 32ºC. One of the landmarks of the Park is the Skakavac waterfall with a height of 98 m.

2.2. Bear monitoring

In this study, different methods of bear monitoring were applied with the aim of acquiring data on population, movement and health of animals in the period 2018 to 2020. These included direct observation (by binoculars), tracking (paw prints, faeces, signs of scratching, food remains and hair), prey analysis (killing method and patterns of carcass consumption), photo traps, telemetric collars, observation of mortality and other health parameters (depending on collected samples: feces, urine, saliva, hair, tissues/blood) (Figures 1, 2 and 3)

An Aldrich photo trap was used with an incorporated SIM card and transmitter, enabling real time notification to the investigators about captured recordings. In a selected locality within the park, based on tracking results and photo trap recordings, an animal was fitted with a telemetric collar that collected data on bear movement from late spring to autumn. To capture the bear for collar fitting, we used tranquilizer guns and blow darting, alongside personal protective equipment for operators, humane immobilization equipment for the sedated animal and standard sample collection equipment.


Figures 1, 2 and 3.: Different methods of employed bear monitoring: tracking paw prints (left) and collection and analysis of photo traps recordings (middle and right)

We used a Vectronic GPS/GSM telemetric collar that enabled satellite monitoring of animal movement and sent data on changes of location via a mobile network (Figures 4 and 5). Vectronic GPS Plus X software was installed and configured by IT staff from the Veterinary Faculty Sarajevo for the purpose of connecting the server and collar, collar configuration and pilot testing before fitting on a bear.

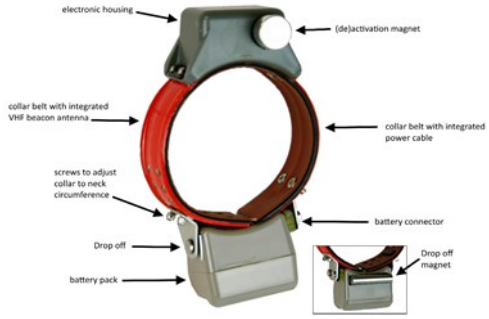


Figure 4. Vectronic GPS/GSM collar (Global Positioning System/Global System for Mobile Communications) used for telemetric monitoring of a bear

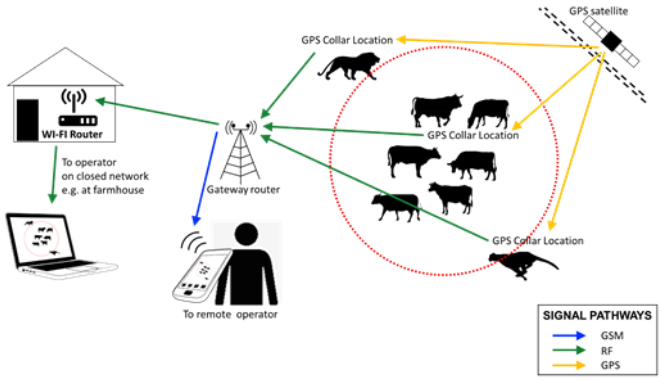


Figure 5. Scheme of the operating principle of the Vectronic GPS/GSM collar used for telemetric monitoring

2.3. Sample collection

During the study period, we collected faecal samples (conserved upon collection in 76% alcohol) for parasitology diagnostics. Samples were examined macroscopically followed by the flotation method to determine the presence of helminths [7] and direct immunofluorescence test (MERIFLUOR® Cryptosporidium/Giardia test (Meridian Bioscience, Inc.) to detect protozoal developing forms [8]. Determination of parasite species was based on morphological characteristics and measurements observed in specimens under the microscope CH20 BIMF200®, (Olympus) and fluorescence microscope BH-2-RFCA® (Olympus), alongside comparison with given parameters specified in the diagnostic method manual [9].

Furthermore, hair and blood samples were collected from the bear fitted with a telemetric collar for dermatologic, hematologic and biochemical testing. All laboratory testing was done in the Laboratory of Parasitology and the Laboratory for Molecular Genetic and Forensic Research of the Veterinary Faculty, University of Sarajevo, which holds BAS EN ISO/IEC 17025:2018 accreditation, and at the Faculty Clinics.

3. Results and Discussion

A telemetric collar was fitted in June 2018 after a photo trap alarm indicated bear location. The animal was sedated using tranquilizer guns followed by blow darting. The sedated animal was given a code (Pasha 003-BH), fitted with a collar, measured and clinically examined (Figure 6). The sedated bear was male, estimated 7 to 8 years of age, in very good body condition. The following body measurements were recorded: weight 220 kg, body length 186 cm, height 130 cm, chest circumference 150 cm, neck circumference 82 cm, head circumference, width and length 88, 24.2 and 42 cm (respectively), tail length 12 cm, canine length 3.2 cm (upper) and 3.1 cm (lower) (no canine/incisor injuries). Body temperature was 40.9ºC, respiration rate 13 per minute and heart beat rate 50 per minute. Clinical examination of collar-fitted Pasha 003-BH bear showed signs of dermatitis on more than 1/5 of the body surface, predominantly in the lumbosacral region, and acute purulent inflammation on the base of the right ear. The animal was treated symptomatically and following photo trap recordings showed improvement in general health (better body and hair coat condition).

Upon completion of examination and data collection procedures, antidote was administered for the purpose of minimal and controlled duration of sedation. The field team for collar fitting was compromised of staff of the Veterinary Faculty from the Canton Administration for Protected Natural Areas and from the local hunting association. The first movement monitoring results were available seven days after collar fitting. The collar-fitted bear's movement was recorded continuously for several months, with GPS coordinates showing location and signal characteristics (length, width and height distance from transmitter to receiver) (Figure 7).

Figure .6 Bear Pasha 003-BH after collar fitting

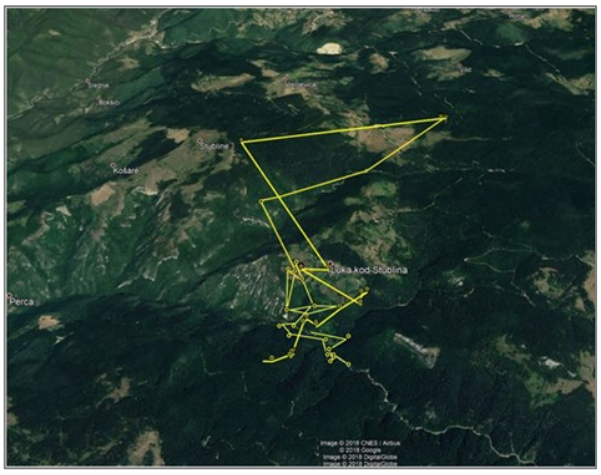


Figure 7. Google Earth view of GPS monitoring of bear Pasha 003-BH's movement

Telemetric and other employed bear monitoring methods revealed increased migration of bears from/to adjacent habitats during spring and autumn with a greater concentration of animals in known den areas. In addition to the collar-fitted bear, we individually identified in the area one more male bear and a female bear with two cubs.

Parasitological investigation of collected faecal samples was negative, except in one sample in which we established eggs of *Baylisacaris transfuga*, a roundworm parasite species commonly found in bears but with high zoonotic potential [10). Clinical manifestation of *B. transfuga* infection in bears is mainly as digestive disorders, while in accidental hosts such as other mammals, birds and humans, ingested eggs that remain active in the environment up to several years can lead to ocular, visceral or neural larvae migrans syndrome [10, 11]. The collar-fitted bear in this study was preventively treated against parasites, with later parasitological investigations being negative.

Haematology and biochemical investigation of collected blood samples did not show significant deviations in parameters compared to referent values. Within normal range, but somewhat low haematocrit and minute deviations of white blood cell lines were observed, indicating a stress type

leucogram probably caused by capture stress [12]. Biochemical analysis revealed high levels of alanine-transferases and phosphorus, indicating nutritional disbalance or kidney insufficiency.

Insight into the health of wild animals provides an option for targeted preventive measures and aids in minimising chances for disease transmission. This is particularly important for zoonotic diseases with wild animals as reservoirs. Infectious agents found in bears, besides compromising health and wellbeing of animal and bear populations in the area, could have zoonotic potential. Reported and planned follow up activities have high impact on promoting animal health and welfare as well as promoting the health of protected natural areas that are animal habitats. Our results serve as a base line for future investigations, wildlife management planning and education of staff and visitors to this site. This study meets the requirements of BiH's international obligations, thus enabling networking with European wildlife and nature protection counterparts alongside conservation of BiH's natural resources for the future.

4. Acknowledgment

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Health lipid indices of dry fermented sausages made of pork meat

Health lipid indices of dry fermented sausages made of pork meat

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Abstract. This research presents the results of a comparison assessment of the cholesterol content, fatty acid profile, and atherogenic (IA) and thrombogenic (IT) health lipid parameters of four dry fermented sausages produced from Mangalitsa and Swedish Landrace pork meat. The highest cholesterol level was found in Sremska sausage prepared from Landrace meat (64.92 mg/100g). Polyunsaturated fatty acid (PUFA) levels were considerably greater in Landrace meat sausages than in other kinds. The main cause of these variations was a higher overall n-6 PUFA concentration. The sausages made from Mangalitsa meat had the highest levels of monounsaturated fatty acid (MUFA) and unsaturated fatty acid (USFA). The highest saturated fatty acid (SFA) level was found in sausages prepared from Landrace meat. Fermented sausages made from Mangalitsa pork meat show better health lipid indices, atherogenic (IA), thrombogenic (IT), and PUFA/SFA ratios.

1. Introduction

Mangalitsa is a fat pig breed, with carcass sides containing 65-70% fat and 30-35% meat [1]. The meat of the Mangalitsa pig was darker colour, its fat was whiter, and its intramuscular fat content and back fat thickness were considerably higher than other pig breeds' meat. Compared to meat from all other fat pig breeds, this meat has a lower saturated fatty acid (SFA) content and a higher percentage of unsaturated fatty acid (USFA) [2, 3].

The level of fat consumed and the fatty acid profile of the diet affect blood cholesterol levels [4]. The impact of fat on cholesterol content can also be seen through the atherogenicity (IA) and thrombogenicity (IT) indexes, which include fatty acids that affect cholesterol changes. Nutritionists advise limiting total fat intake, particularly saturated and trans fatty acids, which have been linked to an increased risk of cardiovascular disease and certain cancers. In addition to decreasing fat intake, nutritionists recommend that consumers increase their intake of polyunsaturated fatty acids (PUFA), particularly n-3 PUFA, at the expense of n-6 PUFA. As a result, the PUFA/SFA and n-6/n-3 PUFA ratios have become significant criteria in determining the nutritional value and healthfulness of foods [5, 6, 7]. In accordance with current trends aiming at revitalizing and expanding traditional food production techniques, autochthonous meat products manufactured from local breeds are gaining popularity [8].

The purpose of this study was to look into the fatty acid profile, cholesterol level, and IA and IT health lipid indices of several dry fermented sausages prepared from Mangalitsa and Swedish Landrace meat. Swedish Landrace was selected as the most frequent commercial meat/fattening pig breed in Serbia, whereas Mangalitsa was identified as the autochthonous Serbian pig breed.

2. Materials and methods

All of the animals were bred at the Institute for Animal Husbandry's test farm (Belgrade, Serbia). Green forages (pasture, clover) were available to all pigs at all times, with the addition of a corn- and wheat-based feed concentrate. At a local slaughterhouse, animals were stunned, slaughtered, and exsanguinated. Meat was processed and cooled for 24 hours after slaughter.

The investigated Kulen and Sremska sausage variants were produced at the Institute for Animal Husbandry's processing plant. Kulen sausage was made with meat that had little fat or connective tissue, primarily from the leg, shoulder, and some parts of the neck, as well as a firm backfat tissue. To achieve 10 mm granulation, muscle and adipose tissue (75:25) were chopped in a cutter (Seydelman K60, Germany). The chopped meat was placed in a mixer with the remaining filling ingredients: 2.3% table salt, 0.4% saccharose, 0.3% garlic (powder), 0.3% pepper, and 0.8% ground sweet and hot red paprika. After that, the filling was firmly stuffed into natural pig colon casings. The smoking and maturation of sausages took place during the winter months. Temperatures in the smokehouse ranged from 10 to 15 °C, with humidity levels ranging from 75 to 90%. During the first four weeks, Kulen sausages were smoked. The sausages were then moved to the ripening room, which was kept at a temperature of 10 to 12 °C. The ripening was done in a controlled environment in a drying chamber (Maurer, Germany).

The Sremska sausage variants under investigation were made on the same day and in the same way. In a cutter (Seydelman K60, Germany), meat and fat (85:15) were ground to 8 mm. The same amounts of salt, 0.011% $NaNO_2$, 0.3% dextrose, 0.20% garlic, and 0.5% sweet red paprika were used in all Sremska sausage varieties. Pig small intestines with a diameter of 32 mm were filled with the mixture. After stuffing, the sausages were hung on sticks to dry, and the ripening was done in a controlled environment (Maurer, Germany).

Total lipids were extracted using the accelerated solvent extraction method on the Dionex ASE 200 to determine the concentration of fatty acids. Capillary gas chromatography with a flame ionization detector was used to determine fatty acids as methyl esters [9]. According to Maraschiello et al. [10], cholesterol content was determined using an HPLC/PDA on the Waters 2695 Separations Module with a Waters 2996 Photo Diode Array Detector.

The following were calculated using the fatty acid composition data:

1) The relationship between the total main SFAs and the main categories of UFAs is shown by the index of atherogenicity (IA) [11, 12]. The following equation was applied:

$$IA = [(4 \times C14{:}0) + C16{:}0 + C18{:}0] / [\Sigma MUFA + \Sigma PUFA\text{-}n6 + \Sigma PUFA\text{-}n3]$$

2) The thrombogenicity index (IT) indicates the probability of blood clots forming. The link between pro-thrombogenic (saturated) and anti-thrombogenic (MUFAs, PUFAs-n6 and PUFAs-n3) fatty acids [11, 12] is characterized. The following equation was applied:

$$IT = \frac{C14{:}0 + C16{:}0 + C18{:}0}{0.5 \times MUFA + 0.5 \times PUFA\text{-}n6 + 3 \times PUFA\text{-}n3 + PUFA\text{-}n3/PUFA\text{-}n6}$$

For each type of dry fermented sausage, two samples were evaluated. In each sample, each parameter was determined six times. The mean and standard error of descriptive statistics were determined. The data was analysed using single component analysis of variance (ANOVA). Tukey's technique was used to determine the differences between the various types of sausage. Statistica 7.0 was used to perform the calculations (StatSoft Inc.).

3. Results and discussion

Palmitic acid (C16:0) was the most abundant SFA, oleic acid (C18:1 n-9) the most abundant MUFA, and linoleic acid (C18:2 n-6) the most abundant PUFA in all varieties of fermented sausages (Table 1). PUFA levels were significantly higher ($P<0.001$) in Landrace meat Kulen and Sremska sausages than in other kinds. The main source of these differences was increased total n-6 PUFA content ($P<0.001$). Hoz [13]

and Valencia [14] both found lower n-6/n-3 fatty acid ratios (12.05 and 13.86, respectively) in their control groups of dry fermented sausages, compared to our findings. The content of essential PUFA, linoleic acid, in sausage types KM and SL ranged from 6.37% to 14.40% (P<0.001).

Table 1. Fatty acid composition (%), cholesterol content (mg/100g), Index of atherogenicity (IA) and Index of thrombogenicity (IT) (means ± standard error) of different dry fermented sausages

Traits	Dry fermented sausages				
	KM[1]	KL	SM	SL	P[2]
C14:0	1.21±0.04	1.18±0.05	1.18±0.04	1.02±0.07	NS
C16:0	26.28±0.07^{a}	24.77±0.06^{b}	25.88±0.13^{a}	23.99±0.14^{c}	***
C16:1	3.87±0.08^{a}	1.86±0.05^{b}	3.87±0.08^{a}	1.76±0.11^{b}	***
C17:0	0.31±0.02	0.35±0.04	0.29±0.01	0.30±0.02	NS
C18:0	11.25±0.10^{a}	14.12±0.08^{b}	10.88±0.21^{a}	14.19±0.08^{b}	***
C18:1c9	42.73±0.26^{a}	39.47±0.11^{b}	43.41±0.12^{c}	37.74±0.12^{d}	***
C18:1c11	4.38±0.10^{a}	3.26±0.05^{b}	4.55±0.07^{a}	2.91±0.11^{c}	***
C18:2n6	6.37±0.12^{a}	11.66±0.12^{b}	6.58±0.09^{a}	14.40±0.13^{c}	***
C18:3n6	ND	ND	ND	ND	
C18:3n3	0.39±0.03^{ab}	0.35±0.04^{a}	0.46±0.01^{b}	0.44±0.02^{ab}	*
C20:0	0.17±0.01	0.18±0.02	0.17±0.01	0.21±0.02	NS
C20:1	0.85±0.21	0.79±0.06	0.84±0.02	0.72±0.03	NS
C20:2	0.63±0.13^{ab}	0.70±0.04^{ab}	0.54±0.07^{a}	0.91±0.04^{b}	*
C20:3n6	1.33±0.11^{a}	0.67±0.02^{b}	1.11±0.06^{ac}	1.03±0.03^{c}	***
C20:3n3	0.08±0.05^{ab}	0.15±0.02^{a}	0.09±0.04^{ab}	ND^{b}	*
C22:1	0.14±0.03^{a}	0.48±0.03^{b}	0.13±0.02^{a}	0.37±0.02^{c}	***
SFA	39.22±0.14^{a}	40.60±0.21^{b}	38.40±0.22^{c}	39.70±0.15^{a}	***
MUFA	51.97±0.29^{a}	45.86±0.24^{b}	52.80±0.20^{a}	43.50±0.15^{c}	***
PUFA	8.80±0.31^{a}	13.53±0.15^{b}	8.78±0.12^{a}	16.78±0.09^{c}	***
USFA	60.78±0.50^{a}	59.39±0.38^{b}	61.58±0.22^{ac}	60.27±0.20^{abc}	**
MU/PU	5.94±0.19^{a}	3.39±0.02^{b}	6.02±0.09^{a}	2.59±0.01^{c}	***
MU/SF	1.33±0.01^{a}	1.13±0.00^{b}	1.38±0.01^{c}	1.10±0.01^{d}	***
PU/SF	0.22±0.01 ^{a}	0.33±0.00^{a}	0.23±0.00^{a}	0.42±0.00^{c}	***
n-3	0.47±0.04	0.50±0.03	0.55±0.04	0.44±0.02	NS
n-6	7.70±0.20^{a}	12.33±0.11^{b}	7.69±0.12^{a}	15.43±0.11^{c}	***
n-6/n-3	16.96±1.42^{a}	25.21±1.70^{b}	14.38±1.14^{a}	35.86±1.59^{c}	***
Cholest.	50.16±0.11^{a}	61.48±0.26^{b}	59.65±0.26^{c}	64.92±0.12^{d}	***
IA	0.70±0.01^{a}	0.74±0.00^{b}	0.68±0.00^{c}	0.71±0.01^{a}	***
IT	1.24±0.01^{a}	1.31±0.01^{b}	1.19±0.01^{c}	1.27±0.01^{ab}	***

[1]Sausage samples depending on meat pig breeds (Kulen sausage – KM; Sremska sausage – SM / Mangalitsa pork meat, Kulen sausage – KL; Sremska sausage – SL / Swedish Landrace pork meat). Cholest. – cholesterol.

[2]NS – not significant (P≥0.05);*:Statistical significance at the level of P<0.05;**:Statistical significance at the level of P<0.01;***:Statistical significance at the level of P<0.001;

$^{a-e}$Means in the same row with different letters are significantly different (P<0.05).

The levels of MUFA in Mangalitsa pork meat sausages were higher (P<0.001) than in other types. Higher levels of oleic acid, cis-vaccenic acid (C18:1 cis-11), and palmitic acid (C16:1) in these sausages were the main cause of these differences. Individual fatty acids in the SFA fraction showed significant variances, resulting in similar quantities for the total fraction. Sausage type KL had the highest total SFA content, while sausage type SM had the lowest. Stearic acid (C18:0), one of the major SFAs, was found in significantly different amounts in the sausage types (P<0.001). The PUFA/SFA ratios in fermented sausages made of Mangalitsa pork meat were found to be the lowest in our study (0.22 and 0.23 in Kulen and Sremska sausage, respectively).

The cholesterol content of fermented sausages ranged from 50.16 mg/100g (KM) to 64.92 mg/100g (SL), with significant variations (P0.001) amongst the sausage types. The cholesterol level of an Italian style salami ranged from 48 to 57 mg/100g, according to Baggio and Bragagnolo [15]. In a study on fermented sausages in Croatia, Pleadin et al. [16] discovered that the average cholesterol level of

industrially fermented sausages ranged from 58.48 to 105.24 mg/100g, while that of home-made fermented sausages was up to 75.07 mg/100g in their study of fermented sausages from Croatia. Cholesterol levels in the blood are influenced by the ratio of unsaturated to saturated fatty acids, as well as cholesterol consumption from meals. From a nutritional standpoint, the PUFA to SFA ratio, the ratio of "bad" to "good" fatty acids (IA and IT), and the n-6/n-3 fatty acid ratio are all essential indicators of food healthfulness.

If a food's IA and IT are lower, it has a lower atherogenic and thrombogenic potential. In sausage type KL, the IA and IT were the highest, and they differed significantly from the other samples. In sausages made from Mangalitsa pork meat, the IA and IT were lower. Beef has an IA of 0.72, poultry has an IA of 0.50, and pork has an IA of 0.60 [17].

4. Conclusion

Mangalitsa and Landrace meat sausages contain different amounts of cholesterol. Sremska sausage made from Landrace pig meat had the highest cholesterol content of the sausages in this study. Landrace pork sausages had much higher PUFA contents than other kinds of sausages. The main cause of these differences was a higher total content of n-6 PUFAs. The highest levels of MUFA and USFA were detected in Mangalitsa pork sausages. The sausages made from Landrace pig meat had the largest SFA content. Mangalitsa pork meat fermented sausages show better health lipid indices, thrombogenic (IT) and atherogenic (IA), as well as PUFA/SFA ratios.

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Slaughter yield and chemical composition of Siberian sturgeon reared in a recirculating aquaculture system (RAS)

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Slaughter yield and chemical composition of Siberian sturgeon reared in a recirculating aquaculture system (RAS)

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Abstract. Sturgeon fish species are food fish of commercial significance in many countries. Sturgeon farming in Serbia is a relatively new branch of aquaculture, and sturgeon farming has been developing in the last several years. The objective of this study was to examine morpho-physiological and slaughter characteristics and proximate composition of one-year-old sturgeons produced in a recirculating aquaculture system (RAS). Sturgeons were cultured in a RAS system nearby Novi Sad and were fed with commercial feed. Moisture content in fillets ranged from 75.63 to 77.91%, protein content from 18.06 to 18.89% and lipid content from 2.37 to 4.38%. The slaughter performance results are in agreement with those reported by various authors for different strains of sturgeons. Sturgeon species have potential to become an attractive fish in our aquaculture in terms of overall proximate composition and slaughter performance.

1. Introduction

Sturgeon fish species are of increasing interest among consumers mainly due to their sensory properties. Also, sturgeon meat is considered to have beneficial nutritional composition. Furthermore, beneficial effects on health of consumers are also linked to sturgeons. Due to the above mentioned facts, sturgeon is a food fish of commercial significance in many countries. There is also increasing interest among fish farmers in sturgeon species due to the fact that sturgeons are relatively easy to breed, their acceptance of formulated feed is relatively high, their growing performances are satisfactory and their possibility to adapt to intensive rearing systems is relatively high [1]. The development of sturgeon aquaculture is important from the viewpoint that catching them wild is banned, so the production is possible only in aquaculture. In Serbia, rearing sturgeon is a relatively new branch of the aquaculture industry, and sturgeon farming has been developing in the last several years. Russian sturgeons are successfully reared in net cages, tanks and ponds, and they well accept artificial feed. It should be mentioned that the intensive breeding of sturgeon could lead to the spread of infectious diseases, so the proper preventive measures are important [2]. The proximate composition of cultured fish is influenced by several factors such as nutrition, genetics, water quality, health conditions, management practices on farms and

environmental conditions [3]. Today, sturgeon culture systems are mainly based on new technologies and intensive systems such as recirculation systems (RAS) [4], and such culture conditions have enabled increased provision of sturgeon on the international market in the few last decades. The efficiency of production and quality of reared fish are of the greatest importance in commercial aquaculture production. Also, the commercial significance of sturgeon is increasing due to its high meat quality and relatively high market price, and demand for sturgeons is continuously increasing. There are not many literature data regarding quality parameters of sturgeons, especially their proximate composition and morphometric parameters including dressing percentage, but such data are needed to assess the production value of these fish. Having all the above mentioned facts in mind, the objectives of the present study were to determine proximate composition and morpho-physiological properties of sturgeons reared in RAS.

2. Materials and Methods

Cultured male Siberian sturgeon (*Acipenser baerii*) (average weight 301g) were obtained from a local sturgeon RAS farm. The fish were reared in circular RAS tanks and fed with commercial feed which contained 42% protein and 15% lipids. Five male individuals were randomly selected and morpho-physiological and chemical analyses were performed. Exterior measurements were made and slaughter analyses were performed according to methods described by Nikolova et al. [5]. Proximate composition of fish was examined applying standard SRPS ISO methods. Gravimetric methods were utilized to determine the moisture content [6] and total fat [7]; total protein content was determined by the combustion method [8]. Ash content was determined by combustion at 550 ± 25 ºC applying standard method [9]. Energy value was expressed per 100g of sturgeon fillet, and was calculated according to the equation below using conversion factors indicated in the Appendix 13 of the Rulebook on declaration, labelling and advertising of food [10]:

Energy value (kcal/ g) = 4 × carbohydrate content + 4 × protein content + 9 × fat content.

3. Results and Discussion

Proximate composition (Table 1) indicated a varied content of lipids and water in the examined fish. A negative correlation between lipid and water contents in the sturgeon meat was observed (Pearson coefficient of correlation r = 0.92), which is in agreement with the previously reported results [15]. In other studies, the proximate compositions of meat were highly variable in different sturgeon species and hybrids, i.e. Russian sturgeon (*Acipenser gueldenstaedtii*), Siberian sturgeon (*Acipenser baerii*) and hybrid (*Acipenser baerii Br* × *Acipenser medirostris* Ayres) [11, 12, 13]. Furthermore, the proximate composition parameters were highly variable within the one species [14].

Table 1. Proximate composition of Siberian sturgeon (*Acipenser baerii*) fillets produced in a recirculating aquaculture system

Parameter	X	SD	Range	CV
Crude protein (%)	18.54	0.38	18.06-18.89	2.07
Crude fat (%)	3.32	1.09	2.37-4.38	32.78
Moisture (%)	76.63	1.15	75.63-77.91	1.50
Ash (%)	1.45	0.17	1.22-1.58	11.54
Carbohydrates (%)	0.06	0.03	0.02-0.09	49.06
kJ/100 g	438.75	42.19	369-475	9.62
kcal/100 g	104.225	10.2	94-113	9.79

The proximate composition of sturgeon is known to be highly influenced by nutrition [12]. According to results reported by Şener et al. [11], whole body fat content in juvenile Russian sturgeon (*Acipenser gueldenstaedtii*) fed feeds including fish oil, soybean oil and sunflower oil were 4.65%, 4.73% and

5.19%, respectively. Wild sturgeon is classified as a medium-fat fish species, with lipid content between 5-15% [14]. Furthermore, the lipid contents in cultured sturgeons ranged from 5 to 15% and energy content ranged from 116 to 151 calories per 100 g of fresh uncooked fish [16]. Chapman et al. [17] noted the lipid content in Siberian sturgeon could be higher in comparison with other sturgeon species. According to results reported by Ljubojević et al. [15], the moisture content in wild sterlet was 75.38%, protein content was 17.54% and lipid content ranged from 4.8 to 6.1%. Lee et al. [18] reported that moisture content ranged from 77.2 to 77.5%, protein content from 13.1 to 13.8% and lipid content from 4.8 to 6.1% for cultured sterlet. According to results reported by Chapman et al. [17] the proximate composition of edible portions of Russian and Siberian sturgeon ranged from 70 to 76% for moisture, from 17 to 19.6% for protein, from 5 to 10% for lipid and from 1 to 2% for ash.

In this study, a significant correlation was measured between fish weight and protein content (Pearson coefficient of correlation, r = 0.74). Also, Ghomi et al. [19] observed a significant correlation between fish weight and protein content (r = 0.504), which is in accordance with our results. Furthermore, they also did not find any correlation between body fat and fish weight, which was the case in our study. On the other hand, we noted a lower fat content in smaller fish, which is in accordance with the observation by Palmeri et al. [20]. That could be due to the utilization of fat at a faster rate during early growth stages.

3.1 *Morpho-physiological properties and slaughter yield*

Results of morpho-physiological properties and slaughter yield of sturgeons are reported in Table 2. The obtained results were compared with the results of previous studies on the Siberian sturgeon but also on the other sturgeon species [13, 21]. The relative percentages of eviscerated weight (81.02%), gonads (1.79%), liver (0.73%), spleen (0.08%), heart (0.1%), swim bladder (0.49%), head without gills (14.07%), fillets with skin (32.47%) and carcass weight (77.81%) were lower in comparison with results obtained by Nikolova et al. [5] for sturgeons aged six and eight years. That confirmed that age increase leads to increase of the total weight, the total carcass weight, the meat content in the carcasses, and the weight of intestines, liver, heart, gills and head [5]. However, the relative proportion of the separate organs in the study conducted by Nikolova and Bonev [21] did not change with age, with the exception of the gills, which underwent a statistically significant proportionate increase with age. The same authors reported that the differences both in morphometric and morpho-physiological characteristics were insignificant. Furthermore, in the current study, fillet yield in relation to the total weight was 41.73%, while the skinless fillet yield in relation to the whole fish weight yield was 28.18% and fillet yield with skin was 32.47%. Slaughter performance was in agreement with those reported by other authors. According to results obtained by Chapman et al. [17], dressed fillets yields in sturgeon could be different by species. They reported that the skinless fillet yields for Russian sturgeon and Siberian sturgeon were 26% and 32%, respectively. Oliveira et al. [22] reported that fillets yields increased with fish weight. They observed that dressed fillet yields of Gulf of Mexico Sturgeon (*Acipenser oxyrinchus desotoi*) ranged from 19 to 23% of live weight.

Table 2. The slaughter parameters of Siberian sturgeon (*Acipenser baerii*) produced in the RAS

Parameters	X	SD	Range
Total weight, g	301.56	43.99	234.79-347.97
Total length, cm	43.94	2.64	40-47.8
Eviscerated weight, kg	244.33	38.16	184.8-286.56
Total intestines, g	6.66	1.06	4.63-7.64
Gonads, g	5.40	1.08	3.55-6.65
Liver, g	2.22	0.39	1.81-2.86
Spleen, g	0.26	0.06	0.2-0.36
Heart, g	0.30	0.10	0.22-0.49
Swim bladder, g	1.48	0.38	1.16-2.18

Fins and tail, g	12.28	2.63	8.27-14.78
Head without gills, g	42.42	5.60	36.66-45.8
Gills, g	5.57	0.83	4.56-6.32
Fillet with skin, g	97.94	17.98	68.75-109.8
Carcass weight (Total weight without intestines and whole head), g	234.66	36.94	177.74-275.43
Slaughter value 1 (Eviscerated weight/Total weight)*100, %	80.89	1.23	78.71-82.35
Slaughter value 2 (Carcass weight/Total weight)*100, %	77.67	1.12	75.70-79.15

The investigation of morpho-physiological parameters is very important for fish reared in relatively small amounts of water, such is the case with RAS. Both genetic and environmental factors determine body growth as well as slaughter and morpho-physiological characteristics of fish. These characteristics are very important if fish are grown for meat. Also, morpho-physiological and slaughter characteristics could be useful tools to evaluate physiological status, health status and well-being status of fish. Nikolova and Bonev [21] reported that liver size is connected to physiological condition of the fish, the heart weight is influenced by swimming activity and spleen weight is dependent on the nutrition factors.

4. Conclusion

In conclusion, sturgeon species have potential in Serbia to become an attractive fish in terms of overall proximate composition and dressing percentage. From a nutritional point of view, sturgeon fillet composition was characterized by a good content of crude protein and other nutrients. The slaughter performance was similar to those reported by other authors for different strains of sturgeon. Balanced nutrition is one of the most important factors for obtaining optimal proximate composition of sturgeon meat from the nutritional standpoint. The possibilities for utilising the currently relatively little used sturgeon species for high quality food and for introducing it as a new species in aquaculture production are big. Reliable analytical data regarding sturgeon quality is required. All reported results could be a useful tool in order to improve sturgeon production in different facilities in our country.

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Health status and microbial quality of common carp reared in a pond fed with treated wastewater from a slaughterhouse

Health status and microbial quality of common carp reared in a pond fed with treated wastewater from a slaughterhouse

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Abstract. Wastewater from slaughterhouses in many countries is still discharged into rivers, without having been adequately treated. Such wastewater contains plenty of organic matter which is an ideal source of nutrients for fish, but also for the development of microorganisms. Thus, usage of wastewater in aquaculture could become a health risk for humans, fish due to the introduction of microorganisms into the aquatic environment. In the available literature, there is insufficient data on health and meat safety regarding common carp reared in purified wastewater. The aim of this study was to assess the health and meat safety of common carp cultivated in a fishpond supplemented with slaughterhouse wastewater that was subjected to tertiary treatment. The number of parasites was not significant and not a single parasitic disease was found in this study, but the number of parasite species detected was as expected and typical for carp production. No spring viraemia of carp or koi herpesvirus disease was found. The carp cultivated were in good health and completely safe for human consumption in terms of the presence of microbial contaminants. The safe use of wastewater for fish rearing should be encouraged, but proper treatment of wastewater must be applied before its use.

1. Introduction

The meat industry is characterized by high water consumption and is undoubtedly a significant source of organic pollution in the environment. The fact that slaughterhouse wastewater is still discharged into natural water bodies without adequate purification is a significant concern from the ecological viewpoint. This practice could be a significant hazard for the environment and consequently for human health. Some authors noted that wastewater could be used as a source of water and nutrients in fish production [1,2,3]. Pelić [4] reported that purified slaughterhouse wastewater could be used in common carp production as a novel approach to aid sustainable aquaculture development. Also, it is useful from the viewpoint of resolving the problem of slaughterhouse wastewater.

The use of fish meat in human diets is highly recommended. Fish is a valuable source of essential amino acids, protein, essential fatty acids and fats [5]. Fish meat is a very valuable source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Since these fatty acids can only be

synthesized by aquatic organisms, consumption of fish meat is the only way humans can intake EPA and DHA [6].

However, consumers are increasingly directing their attention towards safety requirements associated with fish consumption due to the presence of different environmental contaminants, microorganisms and parasites, especially if fish are reared in wastewater-fed ponds or integrated production systems [7]. The potential public health risks associated with consumption of fish reared in wastewater include bacterial and parasitic infections, like diarrhoea and skin infections. Untreated wastewater can contain different harmful substances with negative effects on human health and the environment, such as parasites, pathogenic microorganisms, heavy metals, pesticides, antibiotics and hormones [8]. At the same time, the proper use of treated wastewater in fish production has important environmental and economic significance, due to recycling nutrients and reuse of water.

There are both ecological and financial motives for water purification which intertwine and complement each other. The use of available technology to clean the organic load from slaughterhouse wastewater, making it suitable for fish farming, is an ecological solution for water re-use. On the other hand, an integrated system carries some risks, mostly related to the safety of fish meat produced in this way.

The aim of this research was to examine the effect of using appropriately treated slaughterhouse wastewater on the health of carp and safety of carp meat for human consumption.

2. Common carp rearing in pond fed with treated wastewater from slaughterhouse

The research was carried out in several stages. A wastewater purification system was built on the property of a slaughterhouse in Pećinci, Serbia. The efficiency of the wastewater treatment plant was examined by chemical analyses of water at different purification stages. After this, a fishpond was built on the same property. It was mostly fed with water from the slaughterhouse wastewater purification system, but with some added well water. The purified water was first fed to a pre-fishery pond where it was aerated, after which the water moved into a fishpond where part of nutrients from the purifiers was used for carp nutrition. Carp fingerlings in good health were stocked in the fishpond for growth in optimal ambient conditions. After the fishpond, the water was then used to irrigate the soil surrounding the slaughterhouse. Compared with no treatment, the entire system increases the quality of purified wastewater, which reaches the limit concentrations set before it inflows into a natural, recipient water body. The health condition of fish was monitored during the production cycle by diagnostic examination of the causative agents of viral, bacterial and parasitic aetiology. Additionally, cultivated common carp were collected from the fishpond in spring and autumn and assessed for microbial quality.

3. The effect of slaughterhouse wastewater subjected to tertiary treatment on common carp health

The health condition of fish was controlled during the breeding season at least twice a month, having in mind that the results can be beneficially used only if any eventual therapy is administered on time. Monitoring the health of fish primarily involved examination of the body surface (presence of visible changes or injuries on the skin), then examination of the gills (colour change, presence of changes in the gills, appearance of necrosis) and examination of the internal organs. No viral diseases, including spring viraemia of carp and koi herpesvirosis, were detected by clinical examination or laboratory diagnostics. Only sporadic occurrence of erythrodermatitis was detected during the study, but this condition did not cause major health problems or losses, since the usual measures were applied. Zaibel et al. [9] also revealed that wastewater that had undergone tertiary treatment did not affect fish's growth, immune function or disease resistance.

In the current study, external parasites were recorded in small numbers that were characteristic for carp production, and no localised damages on the fish were observed. The following parasites were recorded: *Myxosporidia* spp., *Lernaea cyprinacea*, *Dactylogyrus* spp. and *Ichthyophthirius multifiliis*. The results obtained were in accordance with the results obtained by Novakov et al. [10]. Carp infection with *Lernaea cyprinacea* in spring was significantly higher than in other seasons, as others have stated [11].

Also, infection with *Dactylogyrus* spp. was recorded in all seasons. The current results, although parasite infestation was not significant, support the hypothesis that infestations of parasites including protozoa (*Ichthyophthirius multifiliis*), Monogenea (*Dactylogyrus* spp.) and Copepoda (*Lernaea cyprinacea*) are widespread and cause losses in fish farms in Serbia [10].

4. The potential public health risks associated with using treated slaughterhouse wastewater in fish production

The potential public health risk is the main factor that constrains use of wastewater in fish production. Danso et al. [12] noted that many studies confirmed that there is no evidence of consumer health risks from the consumption of fish cultivated in wastewater-fed fishponds. Primary and secondary treated waste effluents were successfully used to grow Nile tilapia that were safe for human consumption [13]. Furthermore, [14] reported that most of the muscle samples of fish experimentally exposed to *E. coli*, *Aeromonas*, enterococci and faecal coliforms were not contaminated. The same authors did not observe greater contamination under conditions of fish stress, i.e., high organic load, a water temperature of 37°C or low levels of dissolved oxygen. Lan et al. [2] reported there was no significant difference in the number of presumptive thermotolerant coliforms (in the muscle tissue and gut content) between fish farmed in wastewater-fed fishponds and non-wastewater-fed fishponds. Moreover, studies conducted in Egypt and India showed that fish farmed in fishponds supplemented with treated wastewater had lower levels of microorganisms than fish obtained from surface waters [15,16].

5. Microbial quality of common carp reared in a pond fed with treated slaughterhouse wastewater

The counts of all the examined microorganisms in the carp were permissible and did not exceed prescribed hygiene norms. There was no significant difference in the bacteriological quality of fish collected in spring and in autumn. The number of bacteria (total bacterial count, *Enterobacteriaceae*, coliforms and *Escherichia coli*) were highest in digestive tract content, followed by skin and were lowest in fillets. Sulphite-reducing clostridia, *Salmonella, Staphylococcus aureus* and *Listeria* spp. were not detected in the examined fish samples.

Dang and Dalsgaard [17] also reported that the level of faecal contamination was low in fillets of rohu, grass carp and sliver carp obtained in household-based integrated systems where fish farming was integrated with pig farming and horticulture. On the other hand, high levels of *E. coli* were found in the digestive tract of their examined fish. High levels of *E. coli* were also reported in the digestive tracts of fish but low levels in the fillets of fish from fishponds fed with urban wastewater in some Asian countries [18]. Additionally, Lan et al. [2] reported that fish reared in fishponds fertilized with urban wastewater contained very low levels of thermotolerant coliforms in muscle tissue, but high levels of coliforms were present on their skin and digestive tract contents. Proper handling of fish and the prevention of faecal cross-contamination during degutting and preparing fish for consumption at the market or in the home are the main critical points to control the food safety of fish flesh produced from wastewater-fed fishponds.

6. The presence of zoonotic parasites in fish reared in a pond fed with treated slaughterhouse wastewater

In the current study, zoonotic parasites were not detected in the carp. Fishbone zoonotic parasites are food safety hazards associated with usage of wastewater in fish production. According to the available literature data from Serbia, trematodes do not cause a health problem in cultivated carp. In contrast, in Asian countries, the occurrence of zoonotic trematodes in carp is a significant public health risk [19]. In integrated systems, pig manure can be a reservoir for trematodes, and eggs could be introduced into fishponds via infected pig waste [17]. The methods for preventing trematode transmission are the use of commercial feed in pig nutrition and proper heat treatment of the fish before consumption. Phan et al. [20] investigated the risk of fishborne zoonotic trematode infection in fish reared in wastewater-fed ponds in peri-urban areas of northern Viet Nam. The overall prevalence was 5% (6.5% in spring and

2% in autumn). The metacercariae were zoonotic intestinal trematodes of the family *Heterophyidae*. The intensity of infection was relatively low. It should be highlighted that the prevalence was low in comparison to previous findings of zoonotic trematodes in non-wastewater fish in Viet Nam [21]. Also, Hop et al. [22] reported that the prevalence of trematodes in fish reared in wastewater-fed fishponds in Viet Nam was low in comparison with previous findings of trematodes in fish from conventional fishponds. However, the fish reared in wastewater-fed fishponds are certainly at risk of infection with trematode parasites. The main risk for humans is consumption of raw or improperly prepared fish. As the consumption of raw fish meat is not widespread in Serbia, it can be concluded that carp meat produced in the pond that is partially filled with treated wastewater from the slaughterhouse is safe for human consumption from the aspect of the presence of zoonotic parasites.

7. Preventive measures

It is necessary to continuously work on reducing the prevalence of the parasites commonly present in fishponds. This is possible by improving the fish rearing conditions and implementing prophylactic measures such as drying of objects, freezing, mechanical cleaning and disinfection with lime [10]. Visual inspection of fish for parasites before being placed on the market is obligatory. This procedure is the best preventive measure that minimizes the risks associated with consumption of fish farmed in wastewater-fed ponds. Additionally, microbiological analyses of fish are very important. Proper handling of fish in the home and heat treatment are the best preventive measures against microbial and parasitic contaminants in fish. Adequate treatment of wastewater is the main prerequisite for its use in aquaculture.

8. Consumer acceptance of fish cultivated in wastewater

Consumer acceptance of fish reared in wastewater is a very important issue. The main reasons for avoiding consumption of fish from wastewater, except food safety concerns, are consumer behaviour and cultural habits. In Egypt, consumers did not accept fish produced in treated sewage waters despite the fact that the fish were safe for consumption [23]. Research conducted by Suzette et al. [24] in Ghana showed that the factors that influenced consumers' attitudes towards fish in general included food safety (63%), freshness of the fish (51%), taste (44%), packaging (41%) price, size and species of fish. Furthermore, the authors reported that the factors that affected consumers' attitudes to fish produced in treated wastewater were proximity of consumers to the treatment plant, price, consumer's religion and age, and whether or not they consume the fish species. It is interesting that the source of the fish did not significantly affect the preference of the consumer to consume fish cultured in the treated wastewater [24]. Gebrezgabher et al. [25] reported that surveyed consumers generally accept fish reared in treated wastewater if the fish price is suitably low. Danso et al. [26] reported that consumers in Viet Nam want to know if wastewater is used in fish production and if fish is certified by a relevant government agency. The authors highlighted that there is a need for government to provide adequate food safety control and quality control.

Consumers' acceptance of fish from treated wastewater could be increased by their education on the process of wastewater treatment and development of food safety guidelines. Proper treatment of wastewater before its usage in aquaculture production is a prerequisite for consumer acceptance of fish reared in wastewater.

9. Conclusions

The use of purified slaughterhouse wastewater in carp production had no adverse effects on fish health and resulted in production of carp characterized by adequate meat quality that corresponded with that of carp reared in conventional production systems. The results of this research and their comparison with earlier research on carp health strongly suggest the use of properly purified slaughterhouse wastewater results in production of carp without harmful effects. The use of treated slaughterhouse wastewater in fish production is a novel approach to the sustainability of the meat industry and environment protection. The application of this concept at slaughterhouses in Serbia is crucial from the

aspect of environment protection, having in mind the requirements and standards aimed at minimizing environment pollution imposed by the EU. In that respect, such requirements have to be fulfilled, and Serbian legislation on environment protection must be harmonized with the EU regulations.

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Influence of CLA addition in non-ruminant diets on lipid index values

Influence of CLA addition in non-ruminant diets on lipid index values

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Abstract: In monogastric animals, tissue fatty acid profile directly reflects the fatty acid profile present in the animal's diet. Inadequate ratio of fatty acids in food can lead to negative effects on human health. Conjugated linoleic acid (CLA) is a group of isomers of linoleic acid (C18:2), and its most interesting role is in the prevention of tumors, atherosclerosis and diabetes. CLA is found in ruminant meat and milk, and since pigs and poultry do not have the ability to synthesize CLA, it is possible to add them to animal feed with biotechnological solutions. The scientific public imposes modern parameters for determining the nutritional value of fatty acids, in which the AI – index of atherogenicity, TI – index of thrombogenicity and H/H □ hypocholesterolemic/hypercholesterolemic ratio are distinguished. The aim of this study was to determine the effect CLA addition to the diet of non-ruminants on the lipid indices of certain categories of meat, from the aspect of consumer health needs. A significant influence of the correction of feed's fatty acid composition on the lipid indices in food of animal origin was determined.

1. Introduction

The basic task of production animal feeding is to achieve the highest possible production of quality meat with minimal feed consumption and the lowest possible production costs. Animal nutrition has gone through several stages in its development, starting from complete empiricism to the modern concept of nutrition, which is based on very precise research of metabolic and biochemical changes at the cellular level. The goal of fattening pigs and broilers is to increase the body weight of the animal to obtain a larger amount of meat and fat by using mostly plant nutrients, with a smaller amount of nutrients of animal origin. With the development of scientific disciplines in the field of nutrition, as well as consumer awareness, the imperative in animal husbandry has become not only the quantity of food produced, but also the quality of food produced.

Fats are a heterogeneous group of compounds of different structures that are insoluble in water and soluble in organic solvents (ether and chloroform). The role of fat in human and animal nutrition is primarily energetic. Oxidation of one gram of fat yields 37 kJ, but in the case of carbohydrates, the yield is 17 kJ [2]. The raw materials for the synthesis of unsaturated fatty acids in the body are essential fatty acids that are ingested through feed. Essential fatty acids serve as the building units of many hormones, especially prostaglandins, leukotrienes, thromboxanes and others, but they are also a very important component of cell membranes. Fat requirements in monogastric animals are considered to be relatively small, up to 2%. Lack of essential fatty acids can lead to severe disorders. Up to 6% fat in the mixture accelerates the utilization of feed.

The chemical composition of food of animal origin, especially certain ingredients such as fatty acids, has attracted the attention of experts for years because of their impact on human health [4]. There are many examples in the literature where certain practices in nutrition and breeding increase the content of n-3 unsaturated and other desirable fatty acids in meat, milk and eggs. If a certain animal nutrition strategy is adopted, the results can be seen in a short period of time. Importantly, the change in the ratio of n-6/n-3 unsaturated fatty acids acid in the human diet has become a cause for concern, as this ratio has changed in favor of n-6 unsaturated fatty acids due to lifestyle and diet in which fish and vegetables are declining [15]. The fat content and fatty acid composition of meat has changed from that in grazing animals to that in intensively kept and fed animals in modern technological conditions. The n-6/n-3 ratio in the body is one of the main parameters for determining the nutritional value of fat, given the effects it has on the body.

The taste of meat is mostly determined by the fatty acid composition of intramuscular fat. Also, the taste of semi-shelf stable (chilled) and shelf-stable meat products largely depends on the composition of fat depots. The amount of fat in the carcass is mostly determined by genetic predispositions and the composition of feed and the fatty acid composition is determined by the fatty acids in the diets of monogastric animals. This is explained by the ability of pigs and broilers to absorb a large percentage of fat from feed in unchanged form [8]. This effect depends on the amount and duration fats are consumed. In fattening, by using dietary supplements, more nutritionally valuable food can be produced if the source of fatty acids is taken into account. Conjugated linoleic acid (CLA) is a term used for a group of isomers of linoleic acid (C18: 2), which has been proven to improve the quality of fat, so its biological role is assured. CLA have always been part of the human diet, and they are found in ruminant meat and milk [3], but not in products from monogastric animals. Primarily, CLA has the role of a micronutrient, and its most interesting role is in the prevention of tumors, atherosclerosis and diabetes [10].

These facts have led to new challenges for experts who care about animal nutrition, and thus indirectly about human health. With biotechnological solutions, it is possible to add CLA to feed for non-ruminants, since pigs and poultry are not able to synthesize this group of fatty acids. The quantitative ratio of fatty acids as biological components in the diet plays an important role in maintaining human health. As a consequence, modern parameters for determining the nutritional value of fatty acids are the index of atherogenicity (AI), index of thrombogenicity (TI) and hypocholesterolemic/hypercholesterolemic ratio (H/H). The AI and TI were developed by Ulbricht and Southgate in 1991 [17]. H/H was first proposed by Santos-Silva et al. in 2002 [14]. The H/H ratio could serve to protect consumers from hypercholesterolemia, but has some limitations. Similar to the AI and TI, the H/H ratio might include more kinds of fatty acids such as other molecular species of monounsaturated fatty acids (MUFA), and different weights can be assigned to different molecular fatty acid species [6]. Proper calculation of the AI and TI determines the potential for cardiovascular diseases in humans who consume meat. The aim of this study was to determine the effect of CLA addition in the diet of pigs and broilers on these lipid indices in meats from the animals.

2. Materials and Methods

Broilers of Cobb 500 provenance (60) with an initial average body weight of 40 g were used. Broilers were divided into two experimental groups (Group C – control group and Group E – experimental group) of 30 individuals each and fed with complete feed mixture for broilers of standard raw material and chemical composition (Table 1). Three mixtures were used, complete mixture for fattening broilers I (starter), complete mixture for fattening broilers II (grower) and complete mixture for fattening broilers III (finisher), which completely met the needs of broilers [11]. The groups differed, so for the experimental group, 2% CLA Lutalin ® from BASF was added to the diets at all stages of fattening. Total CLA content in the

complete mixture for broilers in group E after the addition of the preparation was 4.43 ± 0.15%. CLA was not detected in the complete mixture for broilers in group C.

Pigs (40) were from the mother of a crossbreed of Yorkshire and Landrace and the father of a Duroc, and had initial body weight of 60 kg. Pigs were divided into two experimental groups (C group – control group and E group – experimental group) of 20 individuals each and fed with a complete mixture for feeding pigs of standard raw material and chemical composition (Table 1). The complete mixture for fattening of pigs (finisher) completely met the needs of pigs [12]. The groups differed, so 2% CLA Lutalin ® from BASF was added to the diet of the experimental group. The total CLA content in complete mixture for the pigs in group E was 5.12 ± 0.03% (individually). CLA was not detected in the complete mixture for pigs in group C.

The Lutalin® preparation used, manufactured by BASF, is an oil with an energy value of 9 kcal/g, produced by chemical isomerization from sunflower oil in the form of CLA methyl esters. Lutalin® contains CLA *trans*-10,*cis*-12 and *trans*-9,*cis*-10 isomers in a 1:1 ratio. At the end of fattening, six individuals from each group of animals were sacrificed in both experiments and samples of breast and drumstick meat were taken for analysis in broilers, while muscle tissue and smoked pork neck (after processing) was examined in pigs.

Chemical analyses to determine protein, moisture, cellulose, fat, and ash of the feed were conducted according to AOAC methods [1].

Table 1. Raw material and chemical composition of complete mixtures for broilers in fattening (group C and group E), (%)

Raw material composition of the mixture						
Component	**Groups**					
	Starter C	**Starter E**	**Grower C**	**Grower E**	**Finisher C**	**Finisher E**
Corn	50.85	48.85	44.15	42.15	44.95	42.95
Wheat	-	-	10.00	10.00	15.00	15.00
Full fat soya	15.00	15.00	17.00	17.00	20.00	20.00
Soybean meal	12.40	12.40	1.00	1.00	1.00	1.00
Soybean cake	17.00	17.00	23.30	23.30	14.70	14.70
Monocalcium phosphate	1.20	1.20	1.00	1.00	0.90	0.90
Chalk	1.60	1.60	1.60	1.60	1.60	1.60
Salt	0.35	0.35	0.35	0.35	0.35	0.35
Premix	1.00	1.00	1.00	1.00	1.00	1.00
Lysine	0.20	0.20	0.20	0.20	0.10	0.10
Methionine	0.20	0.20	0.20	0.20	0.20	0.20
Adsorbent	0.20	0.20	0.20	0.20	0.20	0.20
CLA	-	2	-	2	-	2
Chemical composition of the mixture						
Mixture	**Groups**	**Protein**	**Moisture**	**Lipid**	**Ash**	**Cellulose**
		X ± SD				
Complete mixture for	**C**	24.98±0.57	8.04±0.24	6.09±0.37	5.45±0.14	2.04±0.05
	E	24.97±0.47	8.06±0.27	6.96±0.35	5.50±0.15	2.04±0.04

broilers I (starter)						
Complete mixture for broilers II (grower)	**C**	22.17±0.21	9.38±0.09	7.03±0.26	4.88±0.13	2.16±0.04
	E	22.11±0.47	9.38±0.10	7.09±0.29	4.92±0.12	2.16±0.05
Complete mixture for broilers III (finisher)	**C**	20.91±0.87	9.98±0.07	5.44±0.11	4.76±0.21	2.38±0.26
	E	20.78±0.80	10.00±0.06	5.46±0.06	4.72±0.22	2.57±0.24

Table 2. Raw material and chemical composition of complete mixture for fattening pigs (group C and group E), (%)

Raw material composition of the mixture						
Komponenta		**Finisher C**		**Finisher E**		
Corn		48		46		
Barley		28		28		
Soybean meal		16		16		
Wheat bran		5		5		
Troumix 210		3		3		
Lutalin CLA		0		2		
Total		100		100		
Chemical composition of the mixture						
Mixture	**Groups**	**Protein**	**Moisture**	**Lipid**	**Ash**	**Cellulose**
		X ± SD				
Complete mixture for pig nutrition III (finisher)	C	15.22	12.28	2.96	2.68	4.17
	E	15.22	12.28	2.96	2.68	4.17

After sacrificing animals, the fatty acid composition of breasts and thighs with drumsticks of broilers and muscle tissue and smoked pork neck of pigs (n = 6) was determined and on the basis of the fatty acid composition, lipid indices (AI, TI, H/H) were calculated.

The fatty acids in meat and meat products were determined according to Milanković et al [9]. The fatty acid content is expressed as a percentage (%) of the total fatty acids identified.

The calculations of lipid indices were according to the following formulae:

$AI = [(C12:0) + (4 \times C14:0) + (C16:0)] / [\Sigma n6 + \Sigma n3 + \Sigma MUFA]$

$TI = [(C14:0) + (C16:0) + (C18:0)] / [0.5 \times + \Sigma MUFA) + (0.5 \times \Sigma n6) + (3 \times \Sigma n3) + (\Sigma n3 / \Sigma n6)]$

$H/H = [(C18:1 + C18:2 + C18:3 + C20:3 + C20:4 + C20:5 + C22:4 + C22:5 + C22:6) / (C14:0 + C16:0)]$

Statistical data processing was done in GraphPad Prism software version 7.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). The results are presented graphically as intermediate values using Microsoft Office Excel 2010. Two–way ANOVA with Tukey's multiple comparison test was performed to compare lipid indices among examined groups of broilers and pigs. Statistical significance is shown at $P<0.05$.

3. Results and Discussion

Table 3 shows the values of AI, TI and H/H of breast and drumsticks with thighs of control and experimental groups of broilers.

Table 3. Lipid indices of control and experimental group of breasts and drumsticks with thigh of broilers (n = 6)

	Experimental group				*P*-value		
	Breast		Drumstick with thigh				
	Control group	Experimental group	Control group	Experimental group	Diet (row factor)	Meat (column factor)	Interaction (RxC)
AI	0.387^{a}	0.467^{c}	0.303^{b}	0.473^{c}	*	*	*
TI	2.732	2.637	2.875	2.673	ns	ns	ns
H/H	2.675^{a}	2.300^{b}	2.750^{a}	2.247^{ac}	*	ns	*

Legend: Within a row, means with a different superscript letter significantly differ (^{a,b,c,d} – $P < 0.05$); ns = no significance ($P > 0.05$); * ($P < 0.05$).

From the presented results, the AI of drumstick with thigh of the control group broilers was significantly lower than the AI of the control group breasts ($P <0.05$). The AI of the breast and drumstick with thighs of the experimental group of broilers was significantly higher than the AI of the breast and drumstick with thighs of the control broilers ($P <0.05$). No significant difference was found between the AIs of the breast and the drumstick with thigh in the experimental group of broilers. The diet had a significant ($P <0.05$) effect on AIs, and a significant difference was observed between different types of meat.

No significant differences were found between the TIs of the breast and drumstick with thigh of the control and experimental groups of broilers, as well as their mutual interactions.

Broilers fed with added CLA had a statistically significantly lower H/H indices in both breast meat and drumsticks with thighs compared to the H/H indices of these two types of meat of the broiler control group ($P <0.05$). A significant interaction between diet and meat type was found ($P <0.05$).

Table 4 shows the AI, TI and H/H of muscle tissue and smoked pork neck of the control and experimental groups of pigs.

Table 4. Lipid indices of control and experimental group of muscle tissue and smoked pork neck of pigs (n = 6)

	Experimental group				*P*-value		
	Muscle		Smoked pork neck				
	Control group	Experimental group	Control group	Experimental group	Diet (row factor)	Meat (column factor)	Interaction (RxC)
AI	0.552[a]	0.895[c]	0.573[b]	0.853[d]	*	ns	*
TI	1.465[a]	2.195[c]	1.530[b]	2.048[d]	*	*	*
H/H	0.593[a]	0.350[c]	1.962[b]	1.370[d]	*	*	*

Legend: Within a row, means with a different superscript letter significantly differ ([a,b,c,d] - $P < 0.05$); ns = no significance ($P > 0.05$); * ($P < 0.05$).

From the presented results, the AIs of muscle tissue and smoked pork neck of the experimental group of pigs was significantly higher than the AIs of muscle tissue and smoked pork neck of the control group of pigs (P <0.05). The AI of muscle tissue of the experimental group of pigs was significantly higher than the AI of smoked pork neck of the experimental group of pigs (P <0.05), while the AI of muscle tissue of the control group of pigs was significantly lower than the AI of smoked pork neck of the control group of pigs (P <0.05). A statistically significant interaction (P <0.05) was found between the type of meat and the diet of pigs.

The TIs of muscle tissue and smoked pork neck of the control group of pigs were significantly lower than the TIs of the examined types of meat of the experimental group of pigs (P <0.05). Significant differences were found between the TIs of both muscle tissue and smoked pork neck of the control and experimental groups of pigs (P <0.05). The interaction between diet and meat type on TI was statistically significant (P <0.05).

The experimental group of pigs had significantly lower H/H indices of muscle tissue and smoked pork neck than the H/H indices of the examined types of meat of the control group of pigs (P <0.05). A statistically significant interaction of the H/H index (P <0.05) was found between the compared parameters.

Among the risk factors for the development of chronic non-infectious diseases (cardiovascular and cerebral diseases, arterial hypertension, malignant neoplasms, diabetes, obesity, biliary tract calculosis, osteoporosis, dental caries), nutrition is of great importance. Today, the scientific community knows that the chemical and fatty acid composition of meat depends on the composition of animal feed, which has an indirect impact on human health. The negative attitude towards meat consumption has a number of different causes. One of the reasons is the debatable nutritional value of meat if we take into account the values of lipid indices (AI, TI and H/H) which have a direct impact on the risk of disease in humans. Lower AI and TI indicate more nutritionally valuable food, so they help prevent cardiovascular diseases related to fat intake [17]. A higher H/H index indicates a higher nutritional value of fat. The addition of CLA to animal feed, as one of the strategies in changing the fatty acid ratio of meat, indirectly enables the management of AI, TI and H/H indices.

In studies with broilers fed with mixtures with different CLA concentrations (0.2%, 3%), Du and Ahn found that the average content of total saturated fatty acids increased significantly as the CLA concentration in broiler feed mixtures increased, while the average content of total monounsaturated and polyunsaturated

fatty acids decreased [7], which has negative consequences on the AI and TI. Sirri et al. observed that in broilers fed diets with different concentrations of CLA (0; 2%; 4%) the content of n-3 fatty acids in the meat of broiler drumstick with thigh decreased as the concentration of CLA in the feed mixture increased [16], which is not in accordance with the our results for TI in broilers.

According to Cech et al., statistically significant differences in the n-3/n-6 ratio as well as in the content of n-3 and n-6 fatty acids occurred in pigs fed with 2% CLA, which ultimately meant a decrease in the TI index, and so there was a positive impact on disease prevention in humans [5]. The results so far confirm that the use of CLA in the diet of pigs affects the fatty acid composition of meat [13], i.e., it increases the saturated fatty acid content in intramuscular fat, and reduces monounsaturated fatty acids. These data are in compliance with the results of this study in pigs, because through the AI, TI and H/H achieved, the interaction of CLA in animal diet in both types of meat was proven.

4. Conclusion

The addition of 2% CLA, correcting the fatty acid composition of animal feed, significantly affects the AI, TI and H/H indices of pig meat and the AI and H/H indices of broiler meat, while the TI of broiler meat is not affected.

Acknowledgment

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Effects of regular control of food colours content in meat products in Serbia

Effects of regular control of food colours content in meat products in Serbia

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Abstract. Research of added food colours presence in meat products was carried out for a period of almost six years, as a continuance of previous study on market in Serbia. Improved method of high performance liquid chromatography was applied for identification and quantification od added colours in meat products. The colours were determined in variety of meat products, smoked meat and bacon, fermented sausages and heat-treated dry sausages, boiled sausages, cooked sausages, canned meat and meat meals, meat semiproducts and mechanically separated meat and, as a separated category, sea fish pastes, both from domestic market and from import. Over the 1400 products were analysed. The research results showed great improvement both in content of colours and labelling of products of meat products in Serbia.

1. Introduction

Colour is, in general, the most important sensory attribute of food and might sometimes have dramatic impact on the expectations, as well as on the subsequent taste/flavour experiences of consumers. Better understanding of the consumer's sensory expectations caused by the food colour, lead to better understanding of the ways in which what we see can attune our perception of flavour, and, as a consequence, alter our food behaviours [1].

The use of food additives, and therefore food colours in meat products, is regulated in Serbia as well as in EU [2-5]. Like as in other categories of food, colours affect the visual experience of the products, give them a characteristic appearance and thus directly affect the acceptability of meat products by consumers [6,7]. Recent studies show that the appearance of a meat product is the first and, most often, crucial sensory factor that influences the consumer's decision to choose a particular product, and one of the most significant contributions to the appearance is its colour. Taste, smell, texture and other sensory factors are of secondary significance for choice and more important to the decision whether the consumer will buy the same product again. Product presentation, which is greatly contributed by the appearance of the product in addition to the appearance of the packaging, can often be of more influence than the imperfection of other sensory qualities of the product.

However, the use of food colours in meat products has its good and bad sides. On the one hand, they overcome the difficulties associated with manufacturing technology and achieve uniformity of product appearance [8], and on the other hand, their use can mask intentional changes in product composition and use of inferior raw materials [9]. Also, some of the colours used in meat products can cause adverse health reactions in children and some population groups [10]. Unauthorised use of colours for adulteration affects the nutritional properties of the product and directly deceive consumers [11,12]. Adverse health effects of colour use are one of the reasons why the European Food Safety Authority

(EFSA) periodically evaluates food safety risks and issues scientific opinions on the use of certain colours in food and suggests values for their acceptable daily intake (ADI) [13].

From 2012 to 2014, the Institute of Hygiene and Meat Technology organized a study of the red food colours presence in meat products that were in stores in Serbia. Laboratory of the Institute for these purposes, developed a method of high-performance liquid chromatographic (HPLC) method for the simultaneous qualitative and quantitative determination of the added colour in meat products [14].

Results of that study showed that most products on the domestic market had a legal irregularity, whether it was a misdeclaration, addition of food colours to products in which their use are not allowed, or addition of forbidden colours. The results indicated that adequate control was not being carried out in Serbia and that it was necessary to constantly monitor the use of food colours in meat products on the market [14]. In accordance with this conclusion and new legal regulations for the safety of the additives use and labelling of meat products [3,11], as well as special requirements of the meat industry, continuous control of the presence and content of colours in meat products has been performed since then. The original liquid chromatographic method was improved both in the efficiency of sample preparation and in number of analytes. This review presents the results of the application of the improved HPLC method for colour control in meat products in the period from 2016 to the first half of 2021.

2. Materials and methods

2.1. Reagents

Colour standards purity ≥98% (Tartrazine, E 102, Sunset yellow FCF, E 110, Carminic acid, E 120, Azorubine, E 122, Amaranth, E 123, Ponceau 4R, E 124, Erythrosine, E 127, Red 2G, E 128, Allura Red AC, E 129, Patent Blue V, E 131, Indigo carmine, E 132, Brilliant Blue FCF, E 133, Green S, E 142 and Brilliant Black BN, E 151), were purchased from MERCK (Darmstadt, Germany).

Other chemicals and preparation of reagent solutions, buffers and mobile phase were the same as previously described [14].

2.2. Meat products

Meat products are obtained as part of regular control of food quality and safety, from domestic market, and, directly, from producers and importers. The research included over a thousand samples from the vast variety of meat products. For the presentation purpose, products of similar properties and production technologies were associated in larger groups, to avoid scarcity of samples in some groups which is, on the other hand, important for reliability of statistical analysis results. The samples were grouped in following categories: smoked meat and bacon, fermented sausages and heat-treated dry sausages, boiled sausages, cooked sausages, canned meat and meat meals, meat semiproducts and mechanically separated meat and, as a separated category, sea fish pastes.

2.3. Preparation of samples for HPLC

Homogenize part of the sample for determination. Weigh 5 g of the sample to the nearest 1 mg. If sample contain more than 10 % of fat, remove the fat with 2-3 portion of light petroleum. Extract colours from sample with ethanol-water solution. Place in the ultrasonic bath for 30 minutes. Add Carrez I and Carrez II solutions, stir on a vortex mixer to homogenize the mixture and centrifuge at 3000 rpm. If needed, repeat the extraction until the supernatant is colourless. Combine the supernatants. Transfer to a centrifuge cuvette and centrifuge for 10 minutes at 3000 rpm. Transfer the supernatant to a 10 ml measuring flask and fill it to the mark with deionised water. Filter the prepared samples through 0.45 μm pore size membrane filters into autosampler vials.

2.4. Chromatographic determination

Condition for HPLC determination were the same as previously described [14].

3. Results and discussion

The initial chromatographic method described in previous study [14] has been greatly improved in two ways. First, number of analytes has been increased from initial 7 red food colours to 14 colours. Second, preparation of samples has been optimised for extraction and rapid determination from large number of diverse samples, not just meat and meat products, with minor modifications of the preparation procedure.

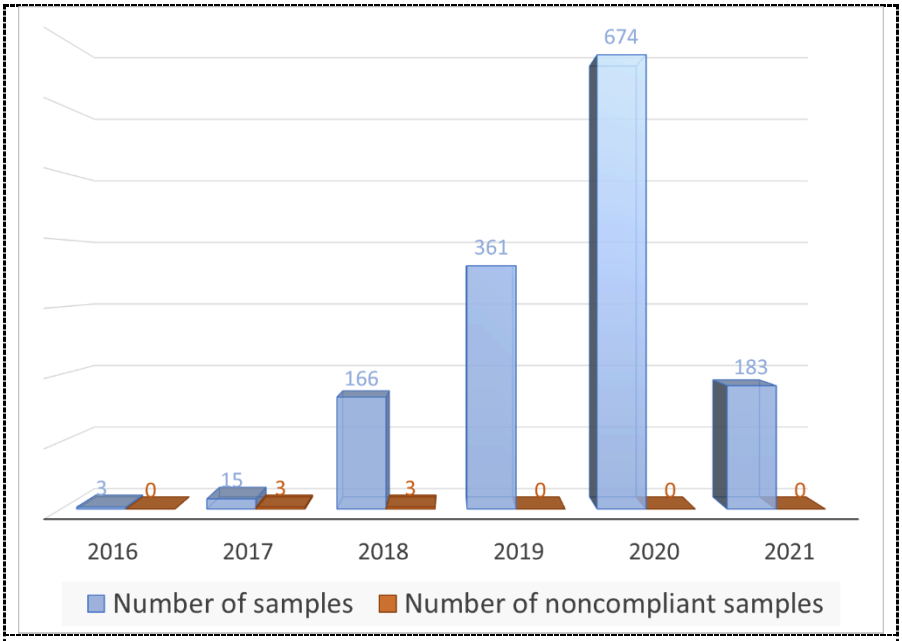

Figure 1. Number of samples per year.

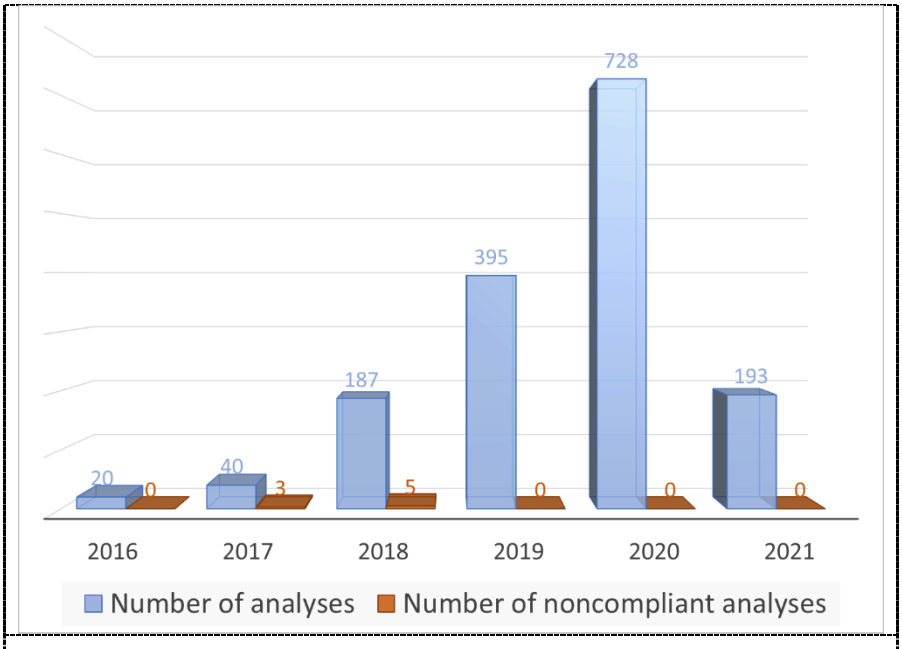

Figure 2. Number of analyses per year.

The optimized method was applied to determine the presence and content of these colours in meat products, as well as to check the safety of products and their compliance with legislation. The research

included the period from the beginning of 2016 to the first half of 2021. In the mentioned period, 1402 samples were examined and 1563 analyses were performed. The distribution of the number of samples and analyses by years, as well as the number of non-compliant samples and analyses are shown in Figures 1 and 2.

It is evident that number of analysed samples has been increased in the period of observation, as well as number of analyses, but number of noncompliant samples decreased, and in last two and half year not a single case of irregularity considering food colours content in meat product has been recorded.

As it previously noted, the samples were grouped in following categories: smoked meat and bacon, fermented sausages and heat-treated dry sausages, boiled sausages, cooked sausages, canned meat and meat meals, meat semiproducts and mechanically separated meat and, as a separated category, sea fish pastes. Distribution of samples and analyses by categories are rendered in Figure 3.

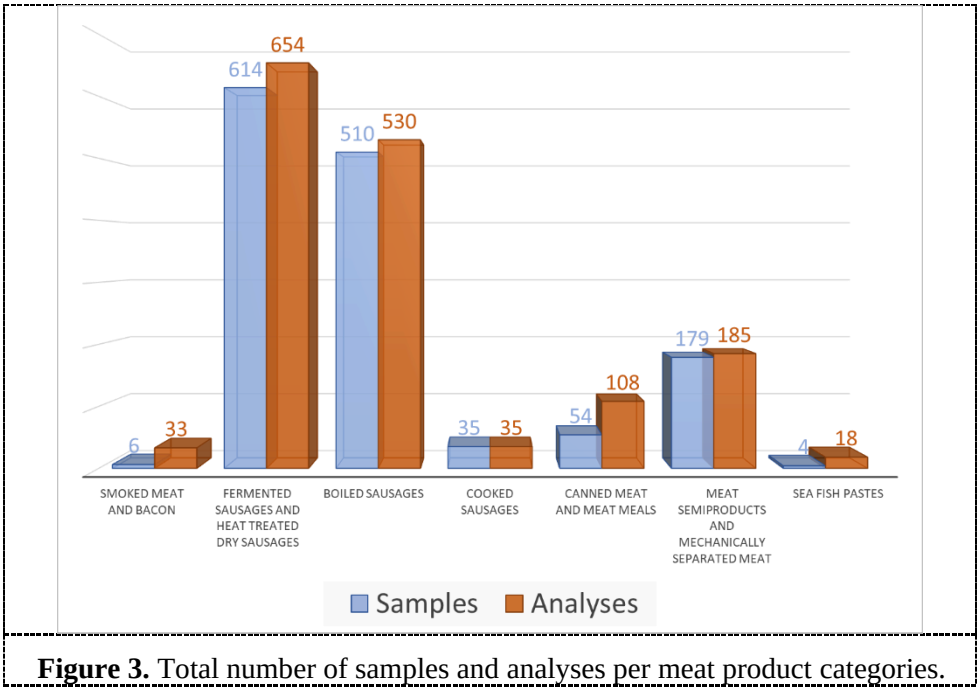

Figure 3. Total number of samples and analyses per meat product categories.

Apparently, fermented and boiled sausages are the two categories with greatest number of analysed samples, namely, over the 80 % of total samples and over the 75 % of total analyses. This is a consequence of regular safety control of these categories of meat products, and comprehensibly regulated added colours content in them [3,4,5]. It is surprising that a relatively small samples number of cooked sausages has been analysed in the research period, considering that the use of colours in them is also regulated [3,4,5] as in the two mentioned groups of products.

3.1. Compliance with legislation

The study published in 2015 concluded, based on the results of testing 74 meat products from three categories over a period of two and a half years, that close to 10% of products contained unpermitted added colours. In regard to product labelling, in slightly more than half (52.63%) of the products in which colour was not declared presence of added colour was confirmed. Added colour was labelled only in 23% of the total number of analysed products [14].

Current study, as it can be seen, was more comprehensive, including longer period of observation and largely greater number of samples. Number of analysed samples has been increased from year to year, as a consequence of the adoption of new legislation related to the safety of the use of food additives

and food labelling [3,11]and as well as the need of the meat industry to control raw materials and retail chains to control safety of meat products of their brands.

It is evident that despite the huge increase in the number of examined samples, the share of non-compliant analyses is declining. While a couple of sporadic cases were recorded in 2017 and 2018, there were no such occurrences in the later period. In 2017, there were three cases of adding cochineal, E 120, to canned meat, and in 2018, one case of unpermitted use of E 120 and two cases of Allura Red AC, E 129, and E 120 in the same samples.

The results of the added colours content in analysed meat products considering food labelling are shown in Table 1. Comparing the presented results with the results of the previous study, a great improvement of the conformity of the product declaration is noticed. While previously more than half of the tested samples were mislabelled, now that percentage is far lower and about one fifth of the samples with confirmed colour content, and less than 9% of the total samples. Nevertheless, although the results are promising, they show that it is necessary to continue with the control and influence the producers and distributors of meat products to direct additional attention to the declaration of products.

Table 1. Share of samples with labelled and unlabelled colour in the total number of analysed samples with the presence of colour confirmed

Samples with added colour	Number	%
Total	602	100.00
Declared colour	482	80.07
Nondeclared colour	120	19.93

3.2. Self-control of raw materials at the request of the manufacturer

Some producers in the meat industry introduced the practice of periodically checking the input raw materials for meat products because there was a reasonable suspicion that dyes were added to some of the raw materials. Such raw materials contaminated the products, although the manufacturer did not add colour in his original recipe or used some of the colours that are allowed in *quantum satis* quantities. For the purposes of raw material control, the described method was used to determine the added colours in meat, mechanically separated chicken and pork meat.

3.3. Product authenticity and branding

The production of traditional meat products often completely prohibits the use of added colours, or the use is strictly limited and regulated. Authentic products are highly demanded and appreciated by consumers and must meet strict legal criteria. A large number of traditional products are exported, and the ban on exports due to security reasons caused by the unpermitted use of food colours causes great damage to both industry and the state.

On the other hand, many large retail chains require the meat industry to deliver products that are proven safe, without added colours or other additives. Also, in case of adding allowed colours, a valid certificate is required, including confirmation that their quantity is within the allowed limits. The use of HPLC method for simultaneous determination of food colours in meat products enables control and reliable confirmation of the colours content in products obtained in the traditional way or produced for the needs of trade brands or, for example, to obtain the label of authentic "Serbian quality".

4. Conclusion

Continuous determination of the content of food additives ensures the protection of consumer safety. Also, these analyses confirm that the food is prepared in accordance with good manufacturing practice and confirm that it is suitable for distribution through the sales network. This implies the application of adequate and reliable analytical techniques for qualitative and quantitative determination of the content of added additives in food. Food colours are additives that can be added in accordance with legal

regulations and must be clearly declared on the product. These same principles have been applied to the use of food colours in meat products.

The results of the research conducted with the aim of re-evaluating the situation on the Serbian market six years after the initial study performed in the period from 2012 to 2015 show that the state of the use of food colours in meat products according to legislative has changed significantly. While in previous study [14], far more irregularities were noticed on a smaller number and in several categories of samples, now it can be said that the situation is completely under control. Except for a few sporadic cases of unpermitted use of colours in previous years, not a single case has been reported in recent years.

A great improvement of the compliance in the product labelling is noticed. Previously in more than half of the tested samples were observed lack of added colour labelling or a non-present colour is declared, now that percentage is far lower and about 20 % of the samples with confirmed colour content, and less than 9 % of the total samples. These results show that it is necessary to continue with the control and additional attention need to be paid to the declaration of colours in meat products.

Regular control of the colour content in meat products has achieved that today we have a more auspicious situation and safer products on the domestic market, that our traditional and other meat products are easier to export and to acquire nutritional declarations and prestigious quality labels. In this way, better consumer protection is provided, as well as better sales of products of the domestic meat industry.

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Trichinellosis among the human population in Vojvodina

Trichinellosis among the human population in Vojvodina

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Abstract. The origin of the parasites of *Trichinella* species goes back to very ancient times – as far as the Paleozoic era, and the organism has been present in the territory of today's Europe for millions of years. The first epidemic in the region of Serbia was reported in Zemun in 1923. Trichinellosis is the most important parasitic food borne disease in our country. In Vojvodina alone, 58 epidemic outbreaks were reported in the period 2015-2019 with 853 affected patients. The average incidence was 3.5 diseased individuals per 100 000 population. High morbidity and hospitalization rates and the occurrence of lethal outcomes qualifies trichinellosis as belonging to the category of relatively severe human diseases in Serbia. However, there are promising data indicating that implementation of relevant control measures and improvement of the awareness of the population have led to a statistically significant decrease in the number of diseased persons with trichinellosis in both Vojvodina and the entire country.

1. Introduction

The first trichinellosis epidemic in Zemun, Serbia, was confirmed in 1923, more than 70 years after the first identification of *Trichinella* species as a parasitic agent in London in 1835 and introduction of pork meat inspection in Germany in 1866. Several years before the epidemics in Zemun (in 1918), the infection of pigs imported from Serbia has been confirmed in slaughterhouses in Berlin [1]. Several decades then passed from the first identification until recognition of the importance of this zoonotic parasite and its inclusion into the food control protocols. Meat inspection for trichinellosis in Serbia started as late as 1950, and mandatory meat examination at industrial slaughterhouses was introduced in 1952. Legislation on the mandatory pig meat inspection at slaughter for private domestic consumption dates back to the 1970s.

The history of trichinellosis control in Serbia shows a close correlation with the development of agriculture, industry, and society as a whole. At the end of the 1980s, a period characterized by peak levels of industrial pig farming and the minimum share of domestic pig breeding in the total swine production. *Trichinella* was found in only 90 animals out of the 1.2 million pigs slaughtered at slaughterhouses [1]. The later period of intensive financial crisis was characterized by the collapse of big production companies and the increase of household pig production during the 1990s, resulting in massive epidemics with sometimes lethal outcomes.

The *Law on Protection of the Population from Infectious Diseases* [2] established rules for the control of trichinellosis in humans. Since 1966, trichinellosis has been categorized as a mandatory reported disease in Vojvodina. Ever since, the occurrence of the disease in humans has officially been recorded. Massive epidemics have occurred during the 1980s and 1990s (907 diseased individuals were reported in 1985) [3].

In this paper, we present the major epidemiological features of trichinellosis in Vojvodina as well as the potential causes of epidemics. During epidemic outbreaks in Vojvodina, the collection and processing of the data is performed in active collaboration between the Institute of Public Health of

Vojvodina and the Scientific Veterinary Institute Novi Sad. The data pertaining to the entire territory of Serbia were obtained from the official web sites of the Institute of Public Health of Serbia "Milan Jovanović Batut".

2. Epidemiological characteristics of trichinellosis in Vojvodina

2.1. Incidence

The average annual trichinellosis incidence in Serbia during the past 14 years was 1.5 (Table 1). The incidence rate in Vojvodina was somewhat higher as compared with the rest of the country; however, the difference was not statistically significant. The highest incidence was recorded in Vojvodina in 2005. An obvious decreasing tendency in the country-level number of diseased humans (Table 1) has been established, predominantly resulting from an increased percentage of industrially grown pork in human diets. Compared with other diseases caused by parasitic foodborne pathogens (echinococcosis and toxoplasmosis), trichinellosis has by far the highest incidence among the human population [4].

Table1. Incidence of trichinellosis in Serbia (per 100 000 population)

Year	Serbia	Central Serbia	Vojvodina
2005	4.5	1.1	13.6
2006	2.5	1.7	4.9
2007	2.4	2.2	2.8
2008	1.2	0.7	2.8
2009	0.7	0.1	2.2
2010	1.5	1.9	0.5
2011	1.7	1.1	3.5
2012	0.6	0.7	0.6
2013	1.3	0.5	3.6
2014	1.2	0.9	1.9
2015	1.1	0.5	2.8
2016	2.7	2.7	2.7
2017	0.2	0.0	0.3
2018	0.2	0.2	0.1
2019	0.4	0.2	0.9
Average	1.5	1.2	3.5
SD[a]	1.2	0.8	3.3
Minimum	0.2	0	0.1
Maximum	4.5	2.7	13.6
CV[b]	0.8	0.8	1.1
Sc[c]	-55	-39	-52
Confidence factor	99.7%	97.1%	99.5%
Incidence trend	Decreasing	Decreasing	Decreasing

[a-] standard deviation, [b-]coefficient of variation, [c-]Mann-Kendall statistic

2.2. Outbreaks

During the past 14 years, on average, four epidemic outbreaks were reported in Vojvodina annually (Table 2). The majority of patients (83.2%) were reported during the epidemics. Commonly, such epidemics occur within a single family or amongst members of two or several related families. A correlation between the number of diseased patients and the number of epidemic outbreaks could not be established – in 2007, 55 diseased patients were reported in 10 epidemic outbreaks, whereas 103 diseased individuals were reported in only one epidemic outbreak in 2005.

T. spiralis is the most frequently identified species in domestic and wild pigs in Vojvodina. It is the only *Trichinella* species detected during autochthonous epidemics in Vojvodina [5]. *T. britovi* was

recently identified as a causative agent in a massive epidemic transmitted from the region of central Serbia to Vojvodina [6]. In neighboring countries, *T. spiralis* is also a dominant species, but epidemics associated with T. *britovi* and *T. pseudospiralis* have also been reported [7].

In Vojvodina, trichinellosis shows highly seasonal prevalence trends ($p<0.0049$) with case numbers peaking in the period from December to February (63.6%) [8]. Similar tendencies were found in other parts of Serbia [9]. The seasonal character of the disease is associated with the seasonal domestic slaughter of pigs by the end November and production of traditional meat products. Similar traditions and, hence, seasonal patterns are observed in neighboring Bulgaria and Romania, but also in China and Argentina [7, 10, 11, 12, 13].

Table2. Outbreaks of trichinellosis in Vojvodina, 2005-2019

Year	Number of patients	Number of outbreaks	Number of patients affected in outbreaks	Percentage (%) of patients affected in outbreaks
2005	277	8	277	100
2006	98	6	80	81.6
2007	57	10	55	96.5
2008	55	4	51	92.7
2009	44	4	43	97.7
2010	10	2	8	80.0
2011	69	1	67	97.7
2012	9	1	4	44.4
2013	69	8	49	71.0
2014	36	1	34	94.4
2015	53	6	42	98.1
2016	51	4	48	94.1
2017	6	1	6	100
2018	2	0	0	0.0
2019	17	2	17	100
SUM	853	58	781	
Average	56.9	3.9	52.1	83.2
SD[a]	67.0	3.1	66.8	27.5
Minimum	2	0	0	0
Maximum	277	10	277	100

[a-] standard deviation

2.3. The structure of cases

Even though the majority of patients were males (60.2%), gender-related differences were not statistically significant ($p = 0.37$) [8]. In the majority of animals, parasitic diseases occur more commonly in male individuals, which is predominantly due to their behavior (aggression) and consumption of larger amounts of food. Testosterone has an immunosuppressive effect, thus increasing the susceptibility of male individuals to parasites, whereas progesterone shows antiparasitic effects. Exceptionally, male animals show higher resistance towards *Plasmodium berghei, Trypanosoma cruzi* and *Strongyloides* spp., yet not to *Trichinella* spp. Female sex hormones enhance immune response and lead to higher antibody levels and stronger adaptive immunity. Administration of stilbestrol (synthetic estrogen) to male rats results in significantly lower number of intramuscular larvae [14].

The vast majority of patients from Vojvodina are adults over 20 years of age (83.2%). Age-specific incidence varies from 9.5 (age 0-9) to 44.5 (age 40-49) per 100 000 population. It was confirmed the incidence of trichinellosis in children under 9 years of age is significantly lower than that in older age groups ($p = 0.03$-0.04) [8]. Considering the underdeveloped immune system in children, a more severe clinical manifestation of the disease would be expected in this population; however, trichinellosis disproves this hypothesis. The disease shows a milder clinical picture and a shorter course in children than in adults [15]. This is probably due to the fact that children consume lesser amounts of meat, especially traditional meat products that have a strong and salty taste. Moreover, the manifestation of allergic reactions in children is weaker as compared to other age categories [16].

2.4. Morbidity and mortality

The high infectivity of *Trichinella* for the human population is reflected by the high morbidity rate recorded in Vojvodina (12.0-100%). The morbidity is the ratio of number of diseased to the number of exposed individuals [8]. Hospitalization rates vary across Serbia according to regions and periods of data processing, ranging between 13.5 and 72.7%. A significantly higher hospitalization rates was established in Vojvodina (41.6± 31.1%) as compared to the city of Belgrade (13.5%) [8,9]. These differences are more likely associated with hospitalization criteria rather than with the severity of the clinical picture.

During the past 14 years, three lethal outcomes of trichinellosis cases were reported in Serbia and all of them in the same year (2005) [3]. The primary cause of death in two cases was acute myocarditis and endocarditis, whereas the third case was an immunocompromised person who had already been hospitalized for another reason. In 2005, trichinellosis mortality rates (number of lethal outcomes to total population ratio) were 0.02 and 0.10 per 100 000 in Serbia and Vojvodina, respectively. The trichinellosis lethality rate (ratio of the number of deaths to number of diseased patients) was 0.8% in Serbia and 0.7% in Vojvodina. The mortality rate in Serbia (0.4%) was higher than the average mortality rate in 55 countries, where trichinellosis occurs autochthonously (0.2%) [17]. Long-term health effects among diseased individuals were reported only in infections associated with *T. spiralis* and *T. murrelli* [18].

Morbidity and hospitalization rates and the occurrence of lethal outcomes indicates that trichinellosis has to be considered relatively severe human disease in Serbia. The severity of the clinical picture is also associated with the fact that infections are most frequently caused by T. *spiralis* which is the most pathogenic of all *Trichinella* species for humans.

2.5. Incriminated food

The food that is considered as the most common cause of epidemics in Vojvodina, as well as in Romania and Bulgaria, is meat products originating from pigs from extensive farming. In Vojvodina, traditional meat products are associated with 54.9% of epidemic outbreaks as the only source of infection, and with an additional 23.5% of outbreaks as a mixed source with meat. Consumption of raw sausages for roasting originating from backyard pigs was identified as the cause of 5.9% of epidemics [8]. In spite of the variations between traditional meat products in the region (fermented sausages, products for grilling, dry meat products, charcuterie) their common feature was that they were produced in the household and underwent poor thermal processing [8, 13]. Traditional meat products considered a source of infection in Vojvodina include fermented and dry sausages, ham and raw sausages for roasting.

2.6. The sources of epidemic

In spite of the mandatory meat inspection in Serbia, the main underlying cause of the occurrence of massive epidemics in Vojvodina (103 diseased in 2005 and 48 cases in 2006) is the consumption of traditional meat products purchased from illegal production of uncontrolled meat. In 2011, the epidemics with 67 diseased individuals was related to an error during meat inspection (an unauthorized and untrained person performed the examination). In Vojvodina, the consumption of non-inspected pork meat is considered a major source of epidemic outbreaks, whether the meat originates directly from

backyard pigs (54.9%) or from illegal production of traditional products (33.3%). Errors during meat examination were identified as the cause of 11.8% epidemic outbreaks [8].

Major reasons for errors in meat examination included the following [6, 8]:

a) The meat was not examined,
b) The meat examination is performed by unqualified person,
c) An inadequate method was applied, e.g., examination of wild boar meat by compression method,
d) An inadequate sample was used, e.g., examination of intercostals muscle by compression method,
e) Examination was performed using an inadequate amount of the sample e.g., less than 10 g diaphragm from wild boar or less than 2 g diaphragm from domestic pig.

Errors in meat examination can occur and always result from non-adherence to standard procedures. In order to reduce the number of diseased individuals, it is essential to improve the knowledge and awareness of consumers and producers, and apply strict measures to prevent illegal trade of traditional meat products. Comprehensive control of all legal entities authorized for meat inspection should be included into the protocols and measures for disease control.

In spite of significant improvements of the awareness of the population and pig producers and development of the meat processing industry, the current epidemiological situation in Serbia strongly suggests that trichinellosis still remains one of the most important parasitic zoonoses. Such an ingloriously high rank is due to the severity of clinical trichinellosis illness and occurrence of lethal outcomes, as well as to the spread of human disease and animal infections across the entire country. Proper farming management is the cornerstone of infection control in animals. In Western European countries, more than 90% of pigs originate from intensive farming. Thus, human infections associated with consuming pork meat from domestic pigs are minimal. In Serbia, less than 5% of the total number of pigs is from intensive production, whereas the majority originates from backyard breeding or small- or medium-scale family farms. Backyard breeding is characterized by complete lack of relevant biosafety measures, while family farms apply such measures at highly limited levels.

3. Conclusions

Epidemic trichinellosis outbreaks in Serbia are largely influenced by illegal trading of traditional meat products. Considering that traditional products are highly valuable foods, their production must be maintained, yet under strict control. Modern Serbian legislation prescribes a range of relevant measures for quality control of traditional products in craft workshops. However, according to our research, the highest risk is associated with illegal domestic production and trade and consequent epidemics, which could affect and endanger consumers numbering in the hundreds.

Establishing continuous trichinellosis control relies on knowledge and awareness about proper disposal of infected animal carcasses and, importantly, on implementing appropriate biosafety measures in farming practices. The knowledge and experience in adequate meat examination as well as controlling of all legal entities authorized for meat examination are of vital importance.

The range of measures to be taken cannot ensure complete eradication of the infection in game animals. However, human infection could be eradicated, and Serbia could take a position as a country with developed, intensive pig production, preserved production of a veriety of safe, traditional products, and healthy consumers.

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Green Economy and Meat Processing–Future Prospects

Green Economy and Meat Processing – Future Prospects

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Abstract. This paper provides a brief overview of the possible strategies for reducing hydrocarbon emissions from the meat industry according to the Green Deal program of the EU in the next decades. An overview of emerging technologies (**h**igh-pressure processing (HPP), **s**hock wave technology (SW), **o**hmic heating (OH) **and p**ulsed electric field (PEF), cultured meat) that should reduce gas emissions is given, as well as methodologies that can be applied (labelling, sustainable cooking, product lifecycle management (PLM) and product data management (PDM) applications). Noticeably, most novel strategies draw the conclusion that we should go for lower consumption of meat, especially beef, and change habits to eat and prepare foods in energy and environmentally friendly ways, as well as apply the so-called "green" food declaration in the future. Transforming into a climate-friendly economy, protecting biodiversity, and reorienting the agri-food industry growth can contribute to creating greater resilience of society.

1.Introduction

Climate change has significant effects on food systems [1]. There is no single and generally accepted definition of the concept of a green economy, but it is certainly the result of efforts to make the economy more environmentally responsible and at the same time create a balanced and positive impact on the economy, society and the environment. Consumers are generally interested in how food production affects the state of the environment but also expect food quality to be preserved, safe and affordable [2]. Climate change should be at the heart of the European Union's economic strategy: "transforming into a climate-friendly economy, protecting biodiversity, and reorienting the agri-food industry growth" and can contribute to creating greater resilience of society. According to that program (the Green Deal), the European Union would have to legally commit itself to achieving "climate neutrality" by 2050 [3]. The main goal of this strategy is to support industry to innovate and to become global leaders in the green economy.

Many sources, including scientific publications, suggest various methods to produce and consume food economically to reduce gas emissions, but the majority of them claim that global reduction in meat consumption is required in the future [4]. Consuming smaller amounts of meat or eating more chicken, eggs or pork is a good way to individually reduce emissions, but it is unrealistic to expect that farming to be discontinued in order to quickly eliminate this contribution [5]. However, the rise in the availability of alternative protein sources, coupled with the associated health, environmental and economic benefits from eating less meat, is bolstering a plant-based food system [6]. A significant share of gas emissions in the food sector stems from losses in distribution chains or excessive food wasting by consumers (24%). Within this contribution, almost 15% belongs to food degradation because of inadequate food storage and handling (inadequate storage temperatures, food spoilage during the transport and mistakes in production processes). The remaining 9% is due to returns in retail because of food past it´s expiration date. Finally, it was estimated food waste is responsible for 6% of global greenhouse gas emissions [7]. In the European Union, a slight decline in meat consumption is predicted by about 1.1 kg on average annually by 2030. This is primarily because of

changes in food consumption habits and higher amount of chicken consumption. There is also an increase in awareness of the impact of food production on global climate change among the world population as well as the consumption of organic and environmentally certified food. Also, this decline in consumption occurs in the period when efforts are being made to introduce innovative production technologies, modernization of production processes, with the aim of environmentally efficient production. COVID 19 pandemic is projected to affect beef consumption in the EU by 2030, it could drop from 10.6 kg to 9.7 kg per capita [8].

2.Strategies to lower the footprint of meat processing

According to World Resources Institute (WRI), the world has to close a gap of 56% between the amount of food available today and that required by 2050. The world is projected to hold nearly 10 billion people by 2050. The same source says that consumption of milk and meat—foods that rely heavily on pasture for their production—is likely to grow by 68%. These rates of growth exceed those that prevailed from 1962 to 2010. Strategies to secure a sustainable food future should incorporate analyses, research and business measures. The WRI program includes methods to reduce food production`s impact on the environment (such as climate smart agriculture, climate friendly diets, reducing food loss and waste, green labelling) [9]. Clear communication of food to consumers in the food chain is essential. The open statement that certain food is produced in an environmentally friendly way in the food trade is very suggestive for the consumer. They decide which food to buy, so it is up to the producer to describe product transparently to the customer through an improved strategy of it´s labeling [10]. The EU estimates that more than 80% of a product's environmental impact is determined in the product conception phase. Product lifecycle management (PLM) refers to the management of data and processes used in the design, engineering, manufacturing, sales and service of a product across its entire lifecycle and across the supply chain. According to Maarit [11], Green PLM can be summarized as: "product conception processes that help to minimize the product's impact on the environment throughout its entire lifecycle." Hence application of PLM and also product data management (PDM) applications from vendors uniquely dedicated to food can contribute to future strategies to lower gas emissions from the food industry. There is also one more strategy, that of encouraging more sustainable cooking. The conversation about sustainable cooking clearly needs to be louder. This policy would make a contribution to a sustainable food environment in the future [12].

3.New technologies in meat processing and climate change

The meat industry has undergone significant changes recently by developing and introducing new technologies in the whole food chain (precision livestock farming, involving sensors and robotics in slaughterhouses and cutting departments and improvement of fermentation processes in the production) [13]. Application of emerging meat-processing technologies (high-pressure processing (HPP), shock wave technology (SW), ohmic heating (OH) and pulsed electric field (PEF)) to replace conventional energy-intensive processes has potential to reduce energy consumption and production costs, and improve the sustainability of the meat sector. These technologies are indicated to be more environmentally friendly. The design of HPP, PEF, OH and SW equipment should be advanced to achieve more environmentally friendly and energy efficient options for meat processing [14]. On August 5, 2013, the first hamburger grown from stem cells in a laboratory, and not in a cow, was served in London. This event was a milestone in the development of the scientific and technological capability to produce factory-grown, or cultured, meat [15]. Economically observed, meat from the laboratory is still an experimental technology under development. Although it is assumed that the new breeding technology will greatly help to preserve the environment, it is not possible to predict the real environmental and social implications. Cultivated, also known as artificial, *in vitro* meat, is considered as the product which is obtained from domestic animal cells. The cultivation process begins by taking cells of interest from a donor animal without injuring them. In the next step, under controlled

conditions with the addition of nutrients and growth factors, the culture will proliferate and increase in overall mass [16].

4.Conclusion

The green economy is an important part of the economy today, nationally and globally, and we now need to work on it in a more sustainable, comprehensive way. Influenced by new emission reduction strategies, the meat industry must also find ways to produce food in an environmentally friendly manner. Future design of emerging technologies and equipment included in modern meat processing should be advanced to achieve more environmentally friendly and energy efficient options. The future production and sale of ″green″ food products including meat becomes more profitable in domestic and international market. There is also growth in the number of consumers who prefer purchasing products that meet high standards of environmental protection. Understanding consumer needs and attitudes towards green products is good starting point in sustainability planning for green food producers. Consumer self-identification with characteristics related to the friendly attitude towards the environment is driving force of buying green products.

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Determination of patulin in apple juice by liquid chromatography-electrospray tandem mass spectrometry

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Determination of patulin in apple juice by liquid chromatography-electrospray tandem mass spectrometry

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Abstract. Patulin is a mycotoxin produced by several fungi, (*Penicillium*, *Aspergillus*, *Byssochlamys)*. The main sources of patulin intake in human diet are apples, apple juice and apple nectar, and for this reason, apple based foods are monitored for the presence of this mycotoxin. Commission Regulation EC No 1881/2006 lays down maximum residue limits (MRLs) of 50 μg/kg in apple juice and cider, 25 μg/kg in solid apple products, and 10 μg/kg in products for infants and young children. In Serbia, maximum permitted amounts of patulin in fruit juices, reconstituted concentrated fruit juices and fruit nectars, as well as in solid apple products, including apple compote and apple puree, intended for direct human consumption are prescribed in the Regulation on maximum concentrations of certain contaminants in foodstuffs. This paper presents the LC-MS/MS method for quantitative determination of patulin in apple juice. Criteria for method validation were taken from Commission Decision 2002/657/EC. Linearity was confirmed in the concentration ranges of 0-100 μg/kg, with the limit of detection (LoD) of 9.85 μg/kg. The performance of the method was successfully verified by participating in a proficiency study.

1.Introduction

Patulin**,** with the formula shown in Figure 1, is a secondary metabolic product of moulds such as *Penicillium*, *Aspergillus* and *Byssochlamys* species [1]. *Penicillium and Byssochlamys* are the most studied species, while *Penicillium expansum* is known as the major producer of patulin. Patulin is a common contaminant of fruit and vegetable based products, most notably apples, and it is particularly associated with apples exhibiting “brown rot” or other rotting characteristics. Fruits that are damaged or improperly stored are susceptible to the growth of patulin producing moulds [2, 3]. Both patulin and the moulds which produce it, especially *Byssochlamys* strains, are heat stable, so the normal pasteurization treatment is not sufficient to decompose it [4]. Several studies have shown toxic, mutagenic, carcinogenic and teratogenic properties of patulin [5]. Fliege and Metzler extensively studied patulin’s chemical and biochemical properties, emphasizing its electrophilic properties [6, 7]. Patulin exerts toxicity through covalent binding to the sulfhydryl groups on proteins and glutathione [6, 7]. *In vivo* toxicity assessment shows damage to vital organs and systems including the liver, kidney, intestinal tissues and immune system [8, 9].

Patulin occurrence in the food commodities poses a serious threat and necessitates effective methods to remove it from food products such as removal of decayed and trimming of mouldy portions of rotten fruits [10]. It also creates a demand to improve handling and food processing techniques. Because apple juice is such a popular beverage and the possibility for life-long exposure exists, the permissible levels of patulin are regulated. Commission Regulation EC No 1881/2006 lays down maximum residue limits (MRLs) of 50 µg/kg in apple juice and cider, 25 µg/kg in solid apple

products and 10 μg/kg in products for infants and young children [11]. In Serbia, maximum permitted amounts of patulin in fruit juices (50 μg/kg), reconstituted concentrated fruit juices and fruit nectars (50μg/kg), as well as in solid apple products, including apple compote and apple puree (25 μg/kg), intended for direct human consumption are prescribed in Article 2, Annex 1 of the Regulation on maximum concentrations of certain contaminants in food [12].

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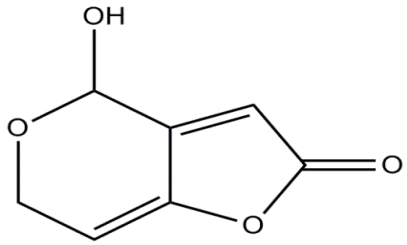

Figure 1. Structural formula of patulin

Since patulin is a regulated compound and has negative impact on food safety, it is necessary to develop the method for its reliable quantitative determination for the purpose of official controls. Therefore, the aim of this work was the development of a sensitive, simple and rapid method for the determination of patulin in apple juice by reverse phase liquid chromatography tandem mass spectrometry.

2.Materials and Methods

Patulin (CAS No. 149-29-1) analytical standard was purchased from Toronto Research Chemical (Toronto, Ontario, Canada). Water, methanol, ammonium acetate, ethyl acetate were all HPLC grade and purchased from Sigma-Aldrich (St. Louis, USA). Nylon filters, pore size 0.22μm were purchased from AMTAST (Lakeland, Florida, USA). Stock solution of patulin, c = 1.00 mg/mL was prepared in methanol, and working solution, c = 10.0 ng/μL, was prepared by diluting stock solution with methanol. All solutions were stored at -20ºC.

Mass spectrometric analysis of patulin was carried out on Shimadzu mass spectrometer LCMS-8040 coupled to a Shimadzu UHPLC instrument (Shimadzu, Europa, Duisburg, Germany). The instrument was controlled by LabSolution software. Sparation was carried out using Kinetex 50 x 2.1 mm 2.6μ C-18 100Å analytical column with UltraGuard cartridge (Phenomenex, Torens, CA, USA). The oven temperature was set to 40ºC. The mobile phase consisted of 40mM ammonium acetate in water (mobile phase A) and 40mM ammonium acetate in methanol (mobile phase B) flowing at a rate of 0.30 mL/min.in gradient mode. Electropray ionization (ESI) was used in negative mode, with the following parameters: probe voltage 4kV, temperatures of block heat (BH), desolvation line (DL) and interface were 400 ºC, 250 ºC and 350 ºC rsepectively, nebulizing and drying gas flow were 3 and 15 L/min respectively. Argon was used as the collision gas. The precursor and product ions and collision energies for patulin are presented in Table 1.

Table 1. Mass spectrometry parameters for patulin

Compound	Precursor ion (m/z)	Product ions (m/z)	Collision energies (eV)	Ionization mode

Patulin	153,10	109,15 81,10	9 12	ES-

Apple juice sample (10 g) was weighed into polypropylene jars with caps. Ethyl acetate 20 mL was added, the jars were shaken and placed in an ultrasonic bath for 15 min. The extracted sample was centrifuged at 4000 rpm for 5 min (ProfiLab GmbH, Berlin, Germany) and supernatant transferred to clean flask and evaporated in stream of nitrogen at 50 ºC. Dry residue was dissolved in 1mL of methanol and filtered through nylon 0.22μm syringe filter into a HPLC vial. Quantification was carried out using matrix extracted calibration curves at four levels. With every analysis batch, blank apple juice was fortified at four different levels with working standard solution and submitted to the full extraction procedure.

Validation was performed in accordance to the Commission Decision 2002/657/EC [13]. The linearity of the method was evaluated on three different days over the range 0-100 μg/kg, and each calibration curve was constructed with five concentration levels (including zero) and was fitted to a linear equation. The linearity of curves measured as average regression coefficient (R^2) was 0.9962, which was satisfactory. The acceptance criteria were that the average regression coefficient (R^2) should be greater than 0.996. Other validation parameters (decision limit CCα, detection capability CCβ, accuracy, repeatability, reproducibility, measurement uncertainty) were determined based on the procedure given in the software ResVal for the validation of the analytical methods made in EURL RIKILT, Wageningen, The Netherlands. A total of four experiments were performed for four days. Blank sample was divided into identical sub-samples and fortified at different concentration levels. Twenty-one fortified samples, seven for each validation level at 25, 50 and 75 μg/kg, plus seven blank samples and five calibration level samples at 0, 10, 25, 50 and 100 μg/kg (ppb) were analysed on each day for three days. On fourth day, to determine ruggednes, two small changes – variables of the method (amount of ethyl acetate and time in ultrasonic bath) were varied.
After validation, the method was used in routine laboratory work and the performance of the method was verified by participating in proficiency testing organized by Progetto Trieste, Italy.

3. Results and Discussion

In this work we presented a simple, sensitive method for determination of patulin in apple juice. The extraction method is based on simple liquid extraction with ethyl acetate with high recovery rate. A good chromatographic separation was achieved with a reversed phase (C-18) column and in gradient elution using 40mM ammonium acetate in water (mobile phase A) and 40mM ammonium acetate in methanol (mobile phase B) at flow rate of 0.30 mL/min. We monitored molecules after proton loss $[M-H]^-$ of 153.1 m/z and two fragments of 81.10 and 109.15 m/z respectively, as presented in Table 1. The most intense fragment, 109.15 m/z, was used for quantification. The ratio of abundance of these two fragments was used to conclusively identify patulin.

Typical chromatograms of blank and apple juice fortified with patulin are shown in Figure 2.

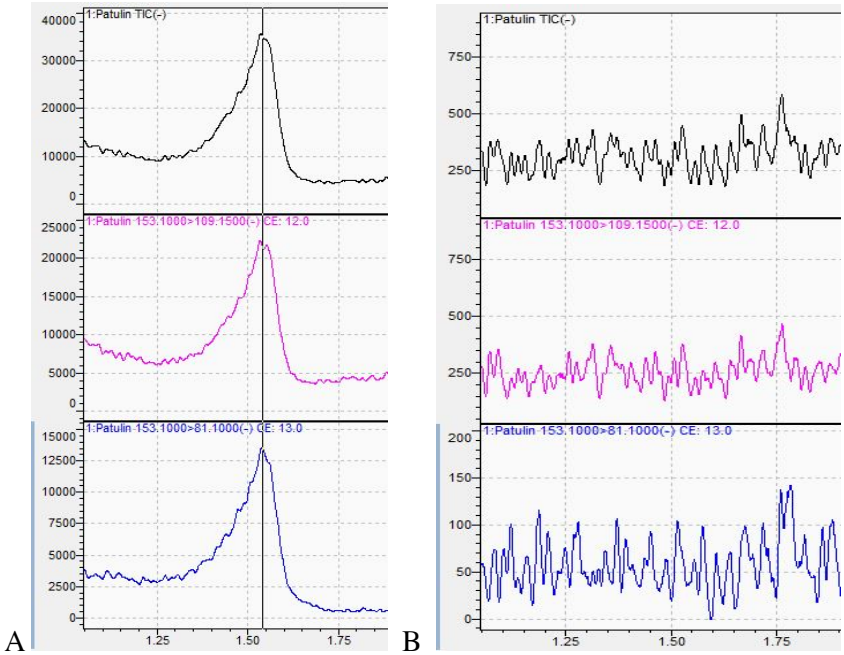

Figure 2. Chromatograms of blank apple juice fortified with patulin at level of 10 μg/kg (A) and blank apple juice (B)

As can be seen no interference from the matrix, that might disturb the signal, was observed at the retention time of patulin, so method is considered very specific. The limits of detection and quantification were 9.85 μg/kg and 19.7 μg/kg respectively, showing that the developed method had sufficient sensitivity to detect patulin at the regulatory level (50 μg/kg). The decision limit CCα and the detection capability CCβ were 54.77 μg/kg and 59.55 μg/kg respectively. The other validation parameters are shown in Table 2. The results of accuracy, repeatability and within laboratory reproducibility expressed as relative standard deviation (RSD) were satisfactory. Extended measurement uncertainty was 11.1%.

Table 2. Validation parameters

Compound	Fortified level (μg/kg)	Accuracy (%)	RSD repeatability (%)	RSD reproducibility (%)
Patulin	25	101	6.7	7.0
	50	97.8	5.8	6.0
	75	100.3	3.0	3.1

The performance of the method was verified by participating in proficiency testing organized by Progetto Trieste, Italy in 2020, PT code VF2054. A total of 17 laboratories participated in this study, and sample of apple juice was analysed. Z-score value obtained by our laboratory was 0.72.

4. Conclusion

The presented method for determination of patulin in apple juice is simple, rapid and has sufficient sensitivity to detect patulin at the regulatory level. After validation and successful participation in the proficiency testing, the method is suitable for routine laboratory work and efficient food control in accordance with European legislation. Patulin is heat stable, so the pasteurization process is not sufficient to decompose it, meaning apple juices are the major concern associated with patulin contamination. The need to use high-quality raw materials, that is, healthy apples without mouldly and rotten portions, as well as good hygiene practices and control measures, should also be emphasized.

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Restrictive nutrition and compensatory growth of broilers: Impact on growth production results and carcass characteristics

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Restrictive nutrition and compensatory growth of broilers: Impact on growth production results and carcass characteristics

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Abstract. Increase in growth rate through genetic selection and improved nutrition in broiler chickens has been associated with high body fat deposition. This is particularly evident under *ad libitum* feeding that is normally practiced. Excessive fat deposition in the body of broilers is a common problem for poultry producers and consumers. Studies have shown that feed restriction could decrease fat content and increase protein deposition in carcasses, thus resulting in their improved composition. In addition to determining the optimal time to start and the duration of the restrictive nutrition, the success of the given programs largely depends on the intensity of the restriction, as well as the type of applied technique. Considering that in a large number of studies, the application of restrictive programs resulted in a reduction of body fat in broilers, but at the same time the desired body weight was not achieved at the slaughter (market) age, these programs should not be *a priori* rejected as ineffective. It is necessary to determine whether the market price of the obtained product (poultry meat) with its reduced fat content justifies the lower body weight of broilers achieved at the end of the fattening period.

1. Introduction

Exceptional progress made in the field of animal nutrition during the past few decades, along with constant (sharp) genetic selection aimed at increasing body weight of broilers and improving feed efficiency, have resulted in reaching the biological limit for achieving animal production results in this segment of poultry production. In the last 50 years, body mass growth rates in broiler chickens have increased by more than 300% [1]. This dramatic increase in broiler growth intensity was manifested primarily during the first four weeks of fattening [2]. Unfortunately, these improvements in production results have led to a number of problems: reduced animal resistance, increased susceptibility to stressors, high incidence of metabolic diseases (the most common are sudden death syndrome, ascites and foot problems), and increased body fat storage [3, 4, 5]. Although a large number of nutritional techniques have been proposed to overcome these problems, the concept of limitation, i.e. slowing down the growth of broilers during the early period of fattening, by applying a restrictive diet and the subsequent manifestation of compensatory growth, has found the widest application in practice.

2. Compensatory growth

The growth of an animal implies primarily an increase in the amount of structural body tissues muscles, bones and organs, and is characterized by the deposition of proteins and minerals in the body of the individual. Genetic potential largely determines the growth intensity, body structure and definite body weight of an animal, and feed is the substrate from which the body should be built [6]. The growth intensity

of an animal is estimated on the basis of the percentage ratio of the achieved gain in a unit of time in relation to its initial body weight or size. Under optimal conditions of nutrition and environment, animals show undisturbed growth in accordance with their (genetically) predetermined growth curve. Under unfavorable conditions for growth (such as insufficient / inadequate nutrition, disease, poor housing conditions, heat stress and many other factors), the animal deviates from its original growth path. However, animals retain the endogenous growth impulse persistently and for a long time, so that when favorable conditions are provided again, they often show compensatory growth (in the literature it is also referred to as "catch-up" growth). Compensatory growth is most often defined as: "The rate of growth exceeding that normally observed in the same breed of chicken at the same age" [7] or as: "A physiological process whereby an organism accelerates its growth after a period of restricted development, usually due to reduced feed intake, in order to reach the weight of animals whose growth was never reduced" [8].

As a consequence of compensatory growth, the individual manages to make up for the previous growth retardation and reaches (even exceeds) the body weight of animals that grew under optimal conditions. The described phenomenon is especially interesting for livestock production, and since the diet during the winter period is often insufficient (lack of quality bulk feed), the original experiments were focused on sheep and cattle as experimental models [9]. Soon after, compensatory growth found its application in poultry production, first in the rearing of laying hens, and then in the fattening of broilers. Numerous programs have been developed that intentionally cause growth restriction at a certain age, after which the animals manifest accelerated growth. In addition to improving the growth intensity, the application of the given programs has other advantages, such as improved feed conversion and reduced deposition of adipose tissue in the body of animals, while many trials have confirmed a positive impact in controlling the occurrence of metabolic disorders.

Two hypotheses about the mechanism of compensatory growth are most often cited in the literature. The first is the "central control" hypothesis, which suggests that the body has a "pre-set point" for body size that corresponds to a particular age and that this control is located in the central nervous system. After a period of malnutrition, the body tries to reach a size that is appropriate for a given age in the shortest possible time. The second is the "peripheral control hypothesis", which suggests that body size is controlled by tissues in which the number of cells, or more precisely the DNA, determines the extent of growth after a period of malnutrition or disease [10].

A large number of studies have been conducted on the phenomenon of compensatory growth in broilers, and the results obtained are inconsistent and often contradictory. The reasons for these discrepancies should be sought primarily in the different design of applied programs (method of growth restriction, start of application, duration, restriction level, type of diet provided after restriction), differences in the duration of fattening (earlier programs included broiler fattening up to 56 or 49 days, while in modern broiler hybrids, fattening ends up to 42 days or earlier), different responses of sex of the tested individuals (males have a greater ability to show compensatory growth compared to females), differences in the tested hybrid (fast-growing broiler hybrids show less compensatory growth compared to slow-growing hybrids) and others. Having in mind all the above factors, it is clear that compensatory growth cannot be manifested after the application of any broiler growth restriction program, or more precisely, it cannot be expected the animals will reach the same final body status as those that grow under optimal growth conditions. Therefore, some authors [11] suggested that the term compensatory growth should be replaced by the term accelerated growth, regardless of whether it resulted in the desired body weight of broilers at slaughter age being achieved, while the period of *ad libitum* feeding, which is applied after a period of limited growth (caused by limited feed intake), should be named the recovery period. The phenomenon of compensatory growth in broiler chickens remains complex because the physiological, nutritional, metabolic, and endocrine aspects involved are still not well understood [12].

3. Restrictive feed intake

The most widely accepted technique for limiting broiler growth is a controlled (predefined) reduction in feed intake during the early period of fattening. This control is achieved by applying various programs of quantitative and qualitative restriction of nutrient intake.

In practice, the most common are quantitative restriction programs, which involve limitation of the amount of feed delivered to broilers over a certain period of time. The programs can be realized through:

a) Restrictions of time provided for broilers to access the feed.

Feed is given only for a certain period of time within 24 hours or the "skip-a-day" feeding system is used, i.e. one day of feed and one day without feed.

b) Changing the light regime.

The lighting period during which the animals consume feed is shortened, i.e. the period of darkness during which the animals rest is extended, all within 24 hours.

c) The use of chemicals. Restriction of feed intake by chemicals has no major practical significance and is mainly used in experimental studies. The use of 1.5% and 3% glycolic acid in the broiler diet (in the period from days 7 to 14 of fattening) reduces feed consumption by 22% and 50%, respectively [13]. Similar effects can be achieved with the use of the opioid antagonists, naloxone and naltrexone, at a dose of 2.5 to 10 mg/kg of body weight [14].

d) Changing the form of feed that animals consume.

Complete feed mixtures for broilers are mainly produced in three different forms: mash, pellets and crumbled pellets. Manufacturers of modern broiler hybrids recommend the use of crumbled pellets during the starter, crumbled or whole pellets during the grower and whole pellets during the finisher phase of fattening. The process of pelleting reduces the volume of feed mixtures, their disintegration and dustiness, provides easier manipulation, prevents decomposition, and since feed is heated with steam during preparation, it is better utilized in the animal's body. In the literature, pellets are usually referred to as "energy more dense" in relation to mash, which practically means that by reducing the volume (by compressor pressure and passing through the matrix), a greater presence of nutrients (energy) is achieved in the same bite. Therefore, individuals spend more time consuming mash diet and expend more energy on this process. Simple replacement of pellets with a mash form of diet results in slowing down the growth of broilers during a given feeding period [15, 3, 6].

e) Reducing the amount of feed provided for individual during 24 hours, without limiting time for accessing the feed.

Reducing the amount of feed during the early period of fattening is an economically viable option for the farmer, considering that feed mixtures intended for the initial phase of fattening (starter mixtures) have a significantly higher commercial price compared to other mixtures (grower and finisher mixtures), and any reduction in consumption of these feed mixtures, if compensatory growth has been achieved, increases the economy of such production. However, the implementation of this program requires the provision of sufficient feeding space to prevent competition between individuals and their uneven growth, and an additional problem is the proper dosing of drugs and coccidiostats [16). The most common procedure for reducing the amount of consumed feed in practice is to provide an amount of feed that meets only the maintenance energy requirements of broilers. In this case, it is necessary to apply the specific mathematical formula developed by Plavnik and Hurwitz [17] (which precisely determines the necessary parameters):

$$EI \text{ (kcal ME / day)} = 1.5 \times BW^{0.667}$$

Where: EI (Energy Intake) = daily energy intake of feed, expressed in kcal ME (kilocalories of metabolic energy), $BW^{0.667}$ = metabolic body weight of the animal at the beginning of the restrictive diet (body weight of the individual expressed in grams, per exponent 0.667).

If the EI values from the previous formula are expressed in KJ values instead of Kcal, then the specified formula can be represented as follows:

$$EI\ (KJ\ ME / day) = 6.2 \times BW^{0.667}$$

The change in the presented formula was made in accordance with the established ratio between KJ and Kcal which is: 1kcal = 4.184 kJ, so that 1.5 (kcal from the previous formula) x 4.184 = 6.2 (KJ).

After determining the EI value from the formula, the required daily amount of feed is determined from the EI value and the energy quotient value of the feed mixture the broilers are fed in a given period (usually starter or grower). By applying the equation, the broilers (during the early period of fattening) are provided with energy requirements for maintenance (35-40 Kcal ME per day per bird) and daily feed intake at the level of 35% in relation to the *ad libitum* diet can be accomplished [18].

Unlike quantitative restriction, qualitative restriction of nutrient intake implies the provision of feed with a composition (raw material and/or chemical) that deviates from the usual. This technique is realized by:

a) Formulating meal with low (below recommended values) energy levels
b) Formulating meal with low (below recommended values) protein levels
c) Diluting meal that was originally formulated in the usual way (dilution is most often achieved by introducing poorly digestible ingredients, such as rice or oat hulls, into the structure of the meal)

A practical problem with the application of qualitative restriction is the appearance of wet litter due to higher content of crude fiber in the meal (diluted diet), as well as increased feed consumption (individuals increase consumption of diluted feed to meet their energy requirements), which increases the price per energy unit of feed [3, 16].

4. The success of the feed restriction program

The extent of compensatory growth can be quantified by the compensatory index, which can be calculated as the ratio of the difference between weight variation at the end of restricted and compensatory growth periods, respectively, relative to the variation at the end of the restricted growth alone. A value of 100% indicates full recovery [8]. According to Wilson and Osbourn, [19] there appear to be six main factors (acting together) governing an animal's ability to recover weight and the final conformation and composition:

(a) The nature of undernutrition.
(b) The severity of undernutrition.
(c) The duration of undernutrition.
(d) The stage of development at the commencement of undernutrition.
(e) The relative rate of maturity of the species.
(f) The pattern of re-alimentation.

Regardless the type of applied technique, by increasing the intensity (severity) and/or duration of the restriction, the individual's ability to recover and exhibit compensatory growth reduces [19). Considering the optimal length of the restrictive feeding program, McMurtry et al. [20] suggested that for male broilers, the given period should be limited to a maximum of seven days, and for females to a maximum of five days. Jones and Farrell [21] preferred a short duration of restriction, with a period of four days being considered the best choice, because it allows individuals to fully recover and exhibit compensatory growth. It is difficult

to determine the optimal age of the individuals to start applying the restrictive program, having in mind the different duration of the fattening period, the type of applied program and the desired results. The first week of broilers fattening is considered particularly sensitive given the high intensity of metabolic processes, insufficient development of the digestive tract and limited physical ability to apply the restriction to individuals [4]. In flocks with an even sex ratio, it is recommended to start a restrictive program at the age of 5-7 days. If only males are used, then this program can be successfully applied at the age of 3-11 days, while in females a decrease in body weight can be expected if program is applied at a later age (9-11 days of age). Therefore, it is recommended for females to start the restriction at the age of 5 days [17]. There are no clear explanations for these gender differences, but they have been attributed to male hormones responsible for the manifestation of accelerated growth [19, 17].

The restrictive program does not end with the transition to a recovery period, i.e. *ad libitum* nutrition, but requires consideration of the special (additional) requirements of broilers during this period. In most studies, after the cessation of the restriction, during the *ad libitum* feeding period, the broilers received feed common to a given fattening period. However, Fjeld et al. [22] indicated that protein content in broiler diet could be a limiting factor during the recovery period. More precisely, Plavnik and Hurwitz [23] indicated increased broiler requirements for essential amino acids during the 7-day period after cessation of restriction and the need to supplement their diets so that compensatory growth can be fully manifested. After switching to *ad libitum* nutrition, broilers greedily ingest feed (the first 24-36 hours after the restriction period), which can lead to suffocation and death. Therefore, the return of individuals to the *ad libitum* diet should be carried out gradually and carefully [16].

5. Influence of restrictive nutrition on feed conversion (feed efficiency)

Accelerated growth of broilers, after a period of restrictive feeding, is achieved thanks to energy and other nutrients from feed, which are mostly directed and used for productive purposes (for growth), and less to meet the requirements for maintenance (non-productive purposes). Lower maintenance (life support) requirements result in smaller body size, lower body weight, and slower metabolism of animals that have been subjected to restrictive program [24].

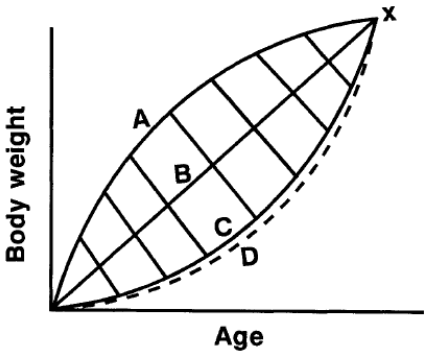

Figure 1. Different broiler growth curves (slow and fast growing lines) [25]. Lines A, B and C represent three potential growth curves of broilers reaching approximately 2 kg body weight (x) at 42 days of age.

Figure 1 gives a schematic representation of the different broiler growth curves for birds weighing 2 kg at 42 days of age, described by Leeson and Summers [25]. When broilers grow at a steady rate (intensity), their growth corresponds to line B and represents perhaps a biological ideal in terms of minimizing stress, i.e. continuous, stable growth, with no major periods of slow or rapid growth. In practice, however, few

animals grow with such statistical precision. Birds with growth shown through lines A and C, also reach a weight of 2 kg at 42 days, but the routes they follow are quite different. Bird A has faster initial growth and then slower growth as it approaches slaughter weight x. Bird C initially has slower growth, which then accelerates towards slaughter weight x. Slow-growing lines of broilers (bird C) grows more slowly initially, but faster later, while the fast-growing lines (bird A) behave in the opposite fashion [26]. Bird C will achieve better (lower) feed conversion compared to bird A, because they will have lower requirements for life support, since at any age, before reaching point X, they have a lower body weight and need less nutrients to maintain life. It should be noted that smaller individuals still have (relatively) higher life support requirements than large ones, if the values are observed in relation to body weight; however, in absolute quantities (expressed in absolute values of grams of feed or KJ), they are certainly smaller too [10]. In other words, lower life support needs throughout the fattening period result in directing more ingested feed for growth, i.e. for productive purposes, and thus, improved feed efficiency, i.e. better feed conversion.

Scientists [27, 19] found that after restrictive nutrition, broilers achieve higher relative feed consumption (relative to body weight) compared to individuals who received feed *ad libitum*. In this way, broilers also consume a larger amount of energy through feed, which is necessary for achieving compensatory growth. Increased feed intake can be explained by the adaptation of the broiler digestive tract to restrictive dietary conditions, i.e. relative (in relation to body weight) increase in the weight of the digestive organs, especially the stomach, glandular and muscular stomach, pancreas and liver during and after the restriction period. In addition to the previously described factors, numerous studies have found that individuals subjected to a restrictive diet exhibit a certain degree of metabolic adaptation to given conditions, which is reflected in lower metabolic heat production, as a consequence of slowing metabolism. Lower metabolic heat production in the individual's body (since it leads to reduced energy consumption for unproductive purposes) has a positive effect on feed conversion. The mentioned metabolic adaptation continues even after the period of restriction, i.e. after the transition to the *ad libitum* diet, but it does not last long. Calorimetric measurements found no differences between broilers (in metabolic heat production) five days after the end of the restriction period [27]. Although the mechanisms responsible for these processes have not been fully elucidated, the decreased metabolic heat production during the restriction period is associated with an established decrease in serum triiodothyronine (T3) concentration [28] and lower sympathetic nervous system activity in birds (29). It should be noted, however, that low circulating T3 during energy restriction could be the consequence rather than the cause of low basal metabolism [8].

6. Influence of feed restriction on body fat storage

Increase in growth rate through genetic selection and improved nutrition in broiler chickens has been associated with high body fat deposition. This is particularly evident under *ad libitum* feeding that is normally practiced [30, 9]. The results of numerous studies have indicated an association between high fat content (and intake) in the human diet with the incidence of cardiovascular disease and cancer. Therefore, in recent years, consumer preference for chicken meat in the human diet has increased, and chicken meat products with additionally reduced fat levels would have an advantage when consumers are choosing meat products. The high deposition of fat in the body of broilers, in addition to being undesirable to consumers, also constitutes an economic loss for producers. Namely, by increasing the share of fat in the structure of growth, its energy value increases, and thus, the amount of consumed feed that is necessary for its realization increases as well [10, 6]. Field observations on the influence of obesity in individuals at an early age on the development of obesity in adulthood have initiated numerous studies on metabolic programming in poultry, i.e. the possibility of managing the processes responsible for the development of broiler obesity (hyperplasia and hypertrophy of adipocytes). Metabolic programming can be defined as a physiological process whereby early adaptation to a nutritional stress permanently changes the physiology and metabolism of the organism and continues to be expressed even in the absence of the stress that initiates it [31]. Studies have shown that

feed restriction could decrease fat content and increase protein deposition in carcasses, thus resulting in improved carcass composition [32, 33].

Slowing down the growth of broilers is achieved by applying a restrictive diet, mainly during the second week of age, with the aim of changing (reducing) the hyperplastic growth of adipocytes, which at this age is of the greatest importance (participation) in adipose tissue growth. In this way, adipocyte proliferation is reduced and/or delayed, thus reducing the incidence of obesity in the broilers of market age [17]. In other words, the reduction of abdominal fat in response to restrictive feed intake in the early period of broiler fattening is the result of a reduced number of fat cells in their older age [34, 35, 36]. Also, during this restriction period, individuals mobilize body fat faster to provide the necessary energy supply [31]. It has been shown that early feed restriction results in lower hepatic acetyl-CoA carboxylase activity, a rate-limiting enzyme for fatty acid synthesis. This can limit hepatic triglyceride synthesis, causing lower serum triglyceride concentration, and therefore, it partly contributes to reduce fat accumulation [37]. It is necessary, however, to take into account the fact that after a period of restrictive nutrition, when individuals re-feed *ad libitum*, adipocytes retain the possibility of hyperplasia and this process continues, so the described technique can have negative effects on carcass quality if not applied at the right time. With a restrictive diet, the process of adipocyte hyperplasia is certainly delayed, and given the short period of broiler fattening, it is necessary to determine the optimal time (but not too early) to start a restrictive diet, in order to achieve the planned improvements. Also interesting are the observations of scientists who found that during the period of restrictive diet, the activity of enzymes responsible for lipogenesis in the liver decreases (in broilers 50% of fat synthesis takes place in the liver) [38], but after switching to *ad libitum* nutrition, their activity grows significantly. Only two weeks after the cessation of the restrictive diet, the level these enzymes decline again and reach even lower values compared to those observed in individuals in which no restrictive diet program was applied [10, 36]. The results presented indicate the importance of proper application, and above all, the importance of determining the optimal time to start a restrictive diet (not too late), which in recent studies, is usually conducted at the end of the first week (5-9 days) or at the beginning of the second week of fattening (7-11 days). In the described way, excessive fat deposition is reduced, and individuals are given enough time to show compensatory growth and reach the same body weight as those who were on the *ad libitum* diet from the beginning of fattening. In previous research, restrictive feeding was practiced during older age (mainly during the second and third weeks), as well as longer duration of the program, but at that time broiler fattening lasted much longer (up to 56 days), which had its physiological and nutritional justification.

In addition to determining the optimal time to start and the duration of the restrictive diet, the success of the given programs largely depends on the intensity of the restriction, as well as the type of applied technique. By increasing the intensity and duration of the restriction, the ability of the animal to recover decreases, and thus its ability to undergo compensatory growth lessens too. If the restrictive feed intake is too mild (insufficiently intensive) or its application is short-lasting, the animals will show compensatory growth, but the level of body fat will be increased. Otherwise (if the restrictive intake is too intense and lasts a long time), the animals will not be able to show compensatory growth, and the impact of the applied program on the level of body fat will not be consistent. Therefore, in order to succeed in implementing a restrictive program, it is necessary to find an optimal balance between the intensity and duration of limited feed intake. The influence of the intensity of the restriction on the success of the applied program can be summarized through three possible cases [21]:

1. If an animal loses a certain percentage of its body weight during a restriction, recovery will be slow and body fat levels will be reduced.
2. If the animal gains weight during the specified period, the recovery will be complete, but the body fat level may increase.

3. If the animal maintains a constant body weight during the restrictive period, the recovery will generally be complete and the fat level reduced.

In order to maintain a constant/unchanged body weight during the restriction phase (as in the above case 3), it is necessary to provide only life maintenance requirements through diet. Although it has been shown in previous examples that applying the formula EI (KJ ME / day) = 6.2 x $BW^{0.667}$ provides broilers with only maintenance requirements, Jones and Farrell [21] found that broilers subjected to a given diet nevertheless gain a certain percentage of body weight (which corresponds to the previously mentioned case 2). Only when they applied a more intensive restriction program (3.1 kJ x $BW^{0.667}$ per day) were maintenance requirements alone achieved, and the body weight of broilers was unchanged (without increase and without weight loss) [21]. By applying the given restriction program for 4 days (from the 7^{th} to the 11^{th} day of fattening), broilers achieved compensatory growth, and the level of body fat was reduced. By shortening the implementation of the given program to 3 days, the mentioned improvements were absent. These results illustrate very well the connection between the intensity and length of the restriction period and the possibility of achieving the desired results in practice.

In addition to the above, restriction of feed intake in broilers can be achieved by other techniques, such as the use of a diet with reduced protein content. However, after switching to an *ad libitum* diet, broilers show accelerated growth (which is not always compensatory), but this type of program, in most cases, results in an increase in body fat [35, 39]. Therefore, the application of such programs has been largely abandoned in practice. Research has also shown the frequency of feeding can also affect fat metabolism. Rare, but abundant meals encourage the development of obesity because excess of feed intake (energy and other nutrients) is deposited in the form of body fat. Yu et al. [7] found the application of a restrictive broiler diet, which included only the provision of maintenance requirements for animals in the period from 8 to 14 days of fattening, had the greatest effect on reducing body fat when applied daily, while the effects were significantly less pronounced when the same program was applied using the skip-a-day technique.

Considering that in a large number of studies, the application of restrictive programs resulted in a reduction of body fat in broilers, but at the same time the desired body weight was not achieved at the slaughter (market) age, these programs should not be *a priori* rejected as ineffective. Namely, it is necessary to consider their economic profitability, i.e. to determine whether the market price of the poultry meat with its reduced fat content justifies the lower body weight of broilers achieved at the end of the fattening period.

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Intensive genetic selection and meat quality concerns in the modern broiler industry

Intensive genetic selection and meat quality concerns in the modern broiler industry

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Abstract. The genetic selection toward bigger broilers provoked the development of muscular myopathies and abnormalities. Since the affected meat is downgraded and often inadequate for further processing, economic losses to the broiler industry are inevitable. In addition, not only the nutritional value of the meat has been decreased, but also sensory properties and technological traits seem to concern consumers more. This can have a very negative attitude toward poultry meat as consumers connect these traits with poor animal welfare. To avoid these problems in the future, new studies must be focused on identifying markers in live birds for newly developed myopathies. Also, better strategies for determining genetic factors, nutritional and slaughter conditions, and hence meat quality, are a complex concept, and all factors together have an impact on parameters of meat quality.

1. Introduction

Of all animal products over the last three decades, the highest absolute and relative growth was made in the poultry meat industry. This not only demonstrates the significant role that poultry meat has as a low-priced protein source for a growing global population, but also the efficient production strategies. Between 1961 and 2019, world meat production rose from 9 to 132 million tonnes, and today poultry meat accounts for about 39% of global meat production. What makes poultry meat so popular among other types of meat is the fact that there are no religious taboos in the consumption of poultry meat, the carcasses are small-sized, broiler meat can be consumed as a whole bird and in parts, and there is no need for refrigeration in contrast to pork and beef meat. This also explains why poultry meat has, apart from egg consumption, become the main protein source for a growing population in many less-developed and threshold countries. Because of this rapid increase in the consumption and demand for poultry products, scientists along with producers have made an effort in developing new methods in the formulation of poultry diets, farming systems and techniques of genetic selection.

2. Factors affecting meat quality

The most significant concern of the modern broiler industry is how to produce high-quality meat in an economically and environmentally sustainable manner. The main goals for poultry productivity are to

achieve high meat yield, good meat quality and high feed conversion efficiency, and this is quite a challenge, particularly because altering one factor will affect the others as well.

Meat quality is determined by complex interactions between the animal's genotype and its environment (nutrition, rearing methods and slaughter conditions). The first level in improving meat quality is taking advantage of the diversity of breeds or strains to exploit valuable animal characteristics. The second level denotes within-breed genetic variability in order to improve meat quality through genetic selection. This intensive selection toward fast-growing and high-yielding broilers resulted in having modern broiler lines that reach processing by 6 weeks of age with a breast muscle that is almost 90% larger than the broilers produced in the 1950s [1]. This tremendous growth of *m. pectoralis major* has led to changes at the morphology and molecular levels that have a negative impact on meat quality [2, 3]. As the number of muscle fibres is determined at the time of hatching, continued selection for post-hatch muscle growth will only impact increasing muscle fibre sizes, not the number of fibres [4]. Increasing fibre size leads to poor vascularisation of muscle fibres, as the distance between capillary support and muscles is increased [3] with consequences of muscle hypoxia, difficulty in delivering essential nutrients and removing waste products of muscle metabolism, such as lactic acid, which necessarily accumulate in the muscles [5]. These alterations are thought to be notably responsible for the development of known myopathies and muscle defects, such as: deep pectoral myopathy, focal myopathy, white striping, pale, soft and exudative meat and wooden breast **[**5].

3. Intensive genetic selection and modern broilers concerns

Using genetic selection techniques for increasing body weight and growth rate of broilers that have bigger breast muscles and lowering feed conversion has led to noticeable changes from commercial broilers in the 1950s to the broilers in 1991 and 2001. From these studies, it is clear that the implementing genetic selection in the poultry industry has made 85 to 90% improvement in the birds, and created broilers as we see them today [6, 7]**.** Unfortunately, with intensive genetic selection that is developing at tremendous rates, modern broilers are more prone to health issues associated with fertility **[**8, 9], diseases [10, 11, 12] and consequently, with meat quality as well [13, 14, 15]. Recently, the biggest concern to the broiler industry is the occurrence of myopathies that are affecting not only the welfare of animals but also the nutritive value and consumer acceptability of products. Two myopathies, wooden breast and white striping, are found globally [16, 17, 18]. Even though these myopathies do not concern meat safety but, rather, meat quality, they cause huge economic loss for all producers in the modern poultry industry around the world. According to Tijare et al. [19**],** the occurrence of wooden breast in flocks was up to 96.1%. Economic loss to the US poultry industry with the incidence of severe wooden breast is estimated to be from $US 200 million up to $US1 billion per year [15, 20]. In order to reduce or even eradicate these myopathies, we must develop novel methods of selection and make changes to the environment.

4. Meat quality modification using genetic selection

Meat quality parameters are easy for researchers to measure, but defining specific factors that contribute to meat quality is quite a challenge. One of the underlying determining factors of meat quality is genetic input. Breeders are now well informed and educated, taking the role of creators of modern broiler lines, selecting desired performance traits (fast-growth, high-weight breast meat, efficient feed conversion, resistance to diseases, low incidence of myopathies, and others). Decisions regarding which meat quality traits to include in broiler breeding schemes are dependent on their level of heritability and genetic correlation with other economic traits. **The** heritability of several traits is well documented by Mir et al. [21], in relation to declining postmortem pH (0.35-0.49), lightness or meat colour (0.5-0.75) and drip loss (0.55-0.64). In recent years, problems with selection programs in broilers are more evident. The desire to control meat colour and pH is having a negative impact on meat quality traits, as those traits are being neglected through each generation of breeding. In genotypes selected for high growth and muscle yield, a slight variation towards higher ultimate pH (pH 5.9 vs. 5.8 on average) has been detected [22, 23, 24, 25], probably due to lower glycogen content in breast muscle. Also, the quality of meat has

been affected by significant positive and negative correlations between the cross-sectional area of muscle fibres and ultimate pH or glycolytic potential in broiler pectoral muscle [26]. This could explain why muscles with increased fibre sizes (and therefore ultimate pH) have lower values for meat lightness, drip and cooking loss, and toughness in broiler breast meat [24, 25, 26, 27].

Based on one study [28], the heritability of wooden breast in broilers is relatively low, and it is hypothesized that non-genetic factors contribute to alterations observed in meat with this myopathy [29]. Nowadays, several companies are searching for new markers that could be used in selection to improve meat quality [30]**.**

5. Efforts to preserve genetic variability in the broiler industry

Even though technology, especially genomics, is evolving very fast, for most developing countries, these developments are still costly and out of reach. Major breeding companies will try to implement genomic knowledge to get lines of broilers that are resistant to many threatening diseases and that are in good condition. If they succeed, it will threaten to eradicate many local indigenous breeds. According to FAO **[**31**]**, the regions with the most specialized poultry industry were also regions with the greatest proportion of their breeds categorized as at risk – Europe and the Caucasus, and North America (49% and 79% of poultry breeds respectively). Moreover, these regions also registered the highest number of breed extinctions.

Hence, genomic science must be applied in the conservation of local poultry populations as well, and to point out the importance of preservation of genetic variability among animals. New breeding schemes have improved the productivity of indigenous chickens using cross-breeding. With this strategy, it is possible to provide greater productivity, but it can have a negative effect on losing or diluting morphological features and instincts for broodiness in birds. Therefore, the balance between human requirements and aspirations with the rules of nature must be our priority.

6. Conclusion

The high absolute and relative growth rates in the poultry industry are a result of the success in breeding, which produced bird strains with excellent feed conversion and good health available. Over the last 10 years, with the development of different molecular studies, the interest of scientists in the field of genetic control of meat quality has not decreased. Many new challenges have appeared regarding a better perception of the origin of metabolic defects (related to the postmortem pH drop) or structural defects (white striping and wooden breast). Efficacy of selection depends on identifying quick and nondestructive methods which apply biological or genetic markers of poultry meat quality that will help in further genetic selection programs. Of course, the main goal is increasing favourable attributes of meat quality parameters in the selected lines and concurrently avoiding the incidence of meat quality defects. However, we must not forget that chickens produced for food are living beings, which have been suffering in silence for too long, surviving in unsuitably crowded conditions, and being overweight and unable to move. We have reached a point where meaningful reforms must be implemented. One of the solutions is moving back to breeding slower-growing birds, and there are a lot of possibilities that can be further investigated so that productivity and welfare come to balance again.

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The role of marbling as an intrinsic characteristic at the point of meat purchase–the Taguchi approach

The role of marbling as an intrinsic characteristic at the point of meat purchase – the Taguchi approach

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Abstract. Meat quality is considered a complex concept depending on many characteristics that could be intrinsic or extrinsic. At the same time, intrinsic and extrinsic quality cues affect consumers' purchasing decisions. The importance of each quality cue was analysed and discussed in previous literature. Thus, colour and level of marbling of fresh meat were defined as key quality cues at the point of meat purchase. These characteristics are mostly related to pork and beef. The aim of this study was to identify quality characteristics that most closely match the consumer's preferences and at the same time could be related to quality losses. For that purpose, this paper gives a novel approach of the potential application of Taguchi loss function associated with quality characteristics and related losses for colour and level of marbling. This application can be implemented by providing a quality characteristic's proper target values and limits, which would make the meat production process more consistent.

1. Introduction

The concept of meat quality from a consumer perspective is defined in terms of the moment of quality evaluation. Quality expectation is formed by consumers at the purchase point, while experienced meat quality arises after preparation and at the moment of consumption. In addition, experienced quality is based on confirmation or rejection of the expectations [1]. Different moments of quality evaluation are related to different quality cues observed by consumers. These quality cues are in previous literature defined as intrinsic and extrinsic [2]. Typical intrinsic quality cues are based on the physical appearance of product like colour, fat content, texture, amount of drip, level of marbling etc. [1, 3-8]. Other types of intrinsic quality cues are experience and credence intrinsic characteristics [9]. Experience intrinsic quality characteristics comprise attributes observed at point of consumption such as flavour, leanness, juiciness, tenderness, taste, freshness etc. Credence intrinsic quality characteristics consist of credence attributes that are related to health and process benefits that could satisfy consumer's moral and ethical needs. Examples of credence intrinsic quality cues are healthiness of product and nutritional value [4, 10-13]. On the other hand, extrinsic quality cues are defined as product-related attributes for which consumers form first impressions at the point of purchase. Some extrinsic quality cues for meat are brand, price, packaging, place

of purchase, origin of the animal, breed, animal welfare, feeding system, environmental impact of the livestock production, and certification labels [5, 9, 11, 12, 14, 15].

However, consumers differently evaluate the importance of intrinsic quality cues for different types of meat (beef, pork, chicken and game meat). Basic intrinsic attributes of beef are meat colour, fat content and cut [1], but indispensable characteristics are fat marbling, amount of drip, texture, freshness, juiciness, tenderness, flavour, taste etc. When it comes to pork, the most important intrinsic quality cues are freshness, colour, smell, appearance, cut, fat distribution and meat-bone-fat ratio [16]. On the other hand, purchase of chicken meat in Serbia depends on three main attributes: colour, freshness and fat content [17, 18]. Finally, the most important intrinsic attributes of game meat examined by European consumers were texture, appearance and colour [19]. It is obvious that colour is recognized as one of the main intrinsic characteristics in every type of meat.

Meat colour is defined as a very important visible characteristic of considerable influence on consumers at the point of meat purchase [20]. Through consumers' perceived quality examination, it was shown that consumers preferred pinkish and light-coloured beef over darker beef [3, 6, 7]. Thus, it was confirmed that light red colour is an indicator for youthful and high quality meat. Since the first impression that consumers have of any meat product is colour, it is important to know how to measure and control this quality cue. Although many factors could influence meat colour and meat colour evaluation [21], there are several common instrumental colour analyses that have been used extensively for meat and meat products. Beside examples of using Minolta and Hunter branded colorimeters, many studies presented how to use computer vision system (CVS) for meat colour analysis, and use of this is increasing [22, 23].

When it comes to determining the quality of meat, marbling score is the dominant parameter. Definition of marbling score was provided in study of Shiranita et al., [24], as "measure of the distribution density of fat in the rib-eye region". It has been reported that a certain degree of marbling could affect juiciness, tenderness, palatability, and flavour of meat [25, 26]. In addition, marbling could directly affect a consumer's consumption decisions. Consequently, marbling as an influencing factor in the meat purchase decisions, has to be maintained and controlled. Many countries have developed methods and standards for evaluating the marbling degree of meat [25]. As is known, standard methods were used for visual appraisal of meat marbling. Although visual appraisal has been used by the industry for a long time, it is of subjective nature depending on experience and intuition of inspectors [24]. Therefore, the application of instrumental measurement techniques for automatizing the visual inspection of meat has been attracting considerable attention.

Nevertheless, imaging techniques, particularly CVS, have been extensively studied and considered as the most suitable technique for the objective grading of meat marbling [25]. Furthermore, the CVS method seemed to be suitable for evaluating the colour of different types of meat such as beef, pork, and chicken [27, 28] as well as meat products [22, 29]. Besides that, as a non-destructive and objective method, this system includes the possibility of analysing the surface of complex food matrixes such as processed meat products. The process of image analysis starts once the pictures of fresh meat or meat products are captured using appropriate computer software for image analyses of colour or marbling [30].

The objective of this paper is to give an overview of the potentials of using the Taguchi approach in defining quality function loss associated with marbling of pork and beef.

2. Taguchi method

Over the last decades, the relationship between the economics of production and quality has been subject of many studies and managerial topics. This relationship considers various approaches from reducing setup costs (and time) to improving process quality. Realizing that there are different types of losses and consistently striving to achieve a particular target of product quality, Taguchi established a new approach [31]. Its main aim was to avoid the concept of zero defects, which could encourage managers to set wider tolerances than necessary. The Taguchi method is a specific statistical technique for implementing robust processes in product design and improvement of product quality at a relatively low cost [32]. According to Taguchi, quality robustness stems from consistency in production. With that consistency, it is much easier to detect the noise (non-controllable) factors causing the quality variations. Thus, mentioned consistency is achievable with optimal process design – the best process settings to optimize multiple quality characteristics [32].

2.1. Taguchi loss function (TLF)

Main inputs for Taguchi loss function are measured values of particular quality characteristics and determined target values. Quality loss for a single product is proportional to the square of the deviation of the quality characteristic from its target value. This loss or cost was characterized by Taguchi as a quadratic function:

$$L(x) = k \cdot (x - \mu)^2 \quad (1)$$

where L (x) is the loss per unit, k is Taguchi's loss parameter – a constant that depends on loss type, x is the actual value of the quality characteristic and μ is the target value. While k constant depends on loss value and type, structure of TLF depends on quality characteristic type [33]. Thus, there are different types of losses (related to manufacturer or consumer's dissatisfaction) and three different types of TLFs in terms of characteristic type. The first one is "the nominal value, the best value" which has curves with two-sided functions (Figures 1 and 2).

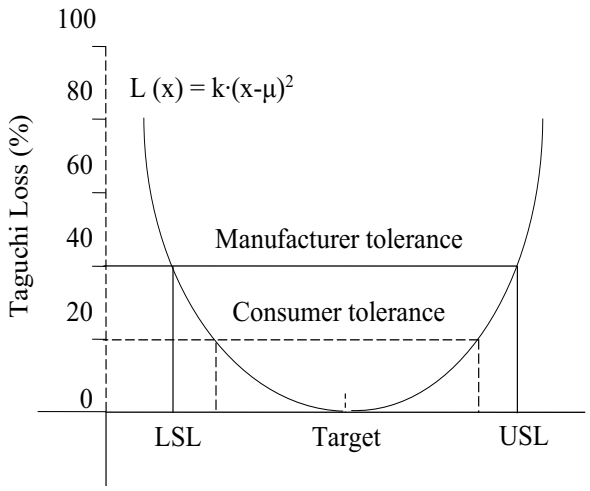

Figure 1. The two-sided equal specification Taguchi loss function (adopted from [46]).

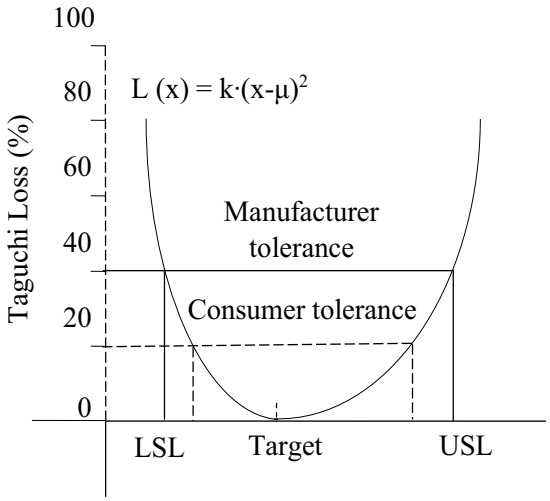

Figure 2. The two-sided with specification preference Taguchi loss function (adopted from [46]).

In two-sided functions, variation is allowed in both directions from the target value. In Figure 1, both lower and upper limits have equal distance from the target value. It means that characteristics measurements equally vary, towards lower and upper limits. On the other hand, Figure 2 shows that for some characteristics, less variation is allowed in this case, towards the low specification limit (or it could be vice versa).

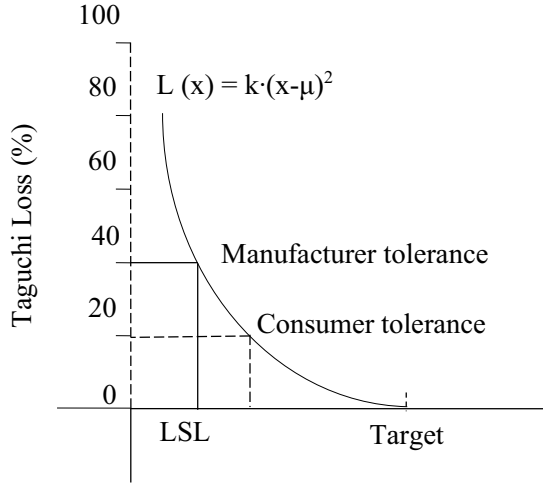

Figure 3. The 'higher is better' Taguchi loss function (adopted from [46]).

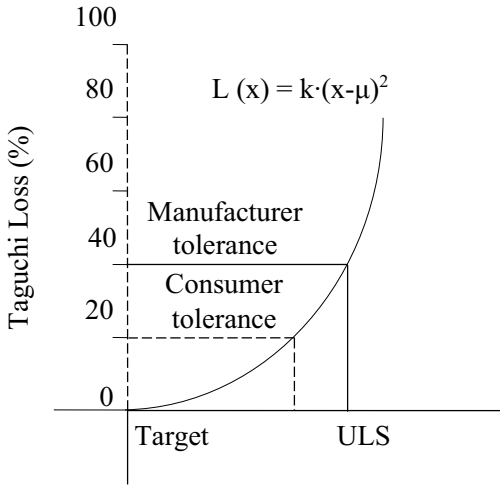

Figure 4. The 'smaller is better' Taguchi loss function (adopted from [46]).

A second TLF type is related to characteristics in which higher values are preferable (Figure 3). In that case, zero loss is at the target value and each deviation form target value towards the lower specification limit will result in loss. Finally, the third type of TLF is used for characteristics for which smaller values are desirable (Figure 4).

2.2. Literature review

When it comes to the food sector, the Taguchi approach in terms of loss function and orthogonal array (OA) has not been widely used. Up to now, the most common areas for Taguchi approach applications were: the managing and controlling the equipment, facilities, machines, electronic circuit technology, metal industry, computer science etc. According to previous literature about the Taguchi approach, its application in the food sector was barely in focus. In this paper, Table 3 gives an overview of the latest publications associated with food production, specific food related processes and food products. Application of Taguchi approach was much more common in studies of food process optimization than in designing food product quality.

Table 1. Compilation of papers in the food sector in which the Taguchi approach has been used

Implementation areas	Description of design	Result	Ref.
Food related process	Application of TLFs in process of filling bottles.	Process optimization and reducing costs.	[33]
Food product	Parameter design experiment was conducted in order to develop a caramel formulation in relation to the effect of the external noise factor – temperature.	Optimal caramel product formulation.	[34]

Food related process	Application of the Taguchi method for tea-beverage customers segmentation into 5 clusters.	Effective market segmentation.	[35]
Food related process	Parameter design was applied using TLF based on four attributes - quality, on-time delivery, price and service.	Development of method for supplier evaluation and selection.	[36]
Food production	Taguchi orthogonal array (OA) method was used. Six factors in biscuit production line.	Investigation of factors that mostly affecting food processing system.	[37]
Food production	Taguchi method was used to optimize the manufacturing process of bread production.	Optimal setting of parameter.	[38]
Food production	Taguchi OA method was used to optimize the process factors for developing ready to eat peanut chutney.	Optimized treatment.	[39]
Food related process	OA was applied to design experiments.	Optimal parameter design for increasing the yield of caffeine removal.	[40]
Food related process	Taguchi technique was used to optimize postharvest handling process.	Optimum conditions for handling and storing eggplant.	[41]
Food product	OA was applied to optimize the baking parameters (product formulation).	The optimal formula for producing a djulis sourdough bread.	[42]
Food related process	Taguchi method was used to optimize microwave frying process and its parameters.	The optimum condition for frying.	[43]
Food related process	TLF were used for evaluating quality loss.	Development of supplier evaluation and selection process.	[44]
Food product	OA was applied to determine the optimal production process and composition of candied carrot.	Best combination of parameters.	[45]

As can be seen in Table 1, the Taguchi approach has been used in various food sectors, but no application to fresh meat quality has been reported.

3. Taguchi approach in measuring marbling

Potential application of the Taguchi approach in evaluating fresh meat quality losses associated with marbling is presented in the flowchart (Figure 5). The details of these steps are briefly described in the following sections.

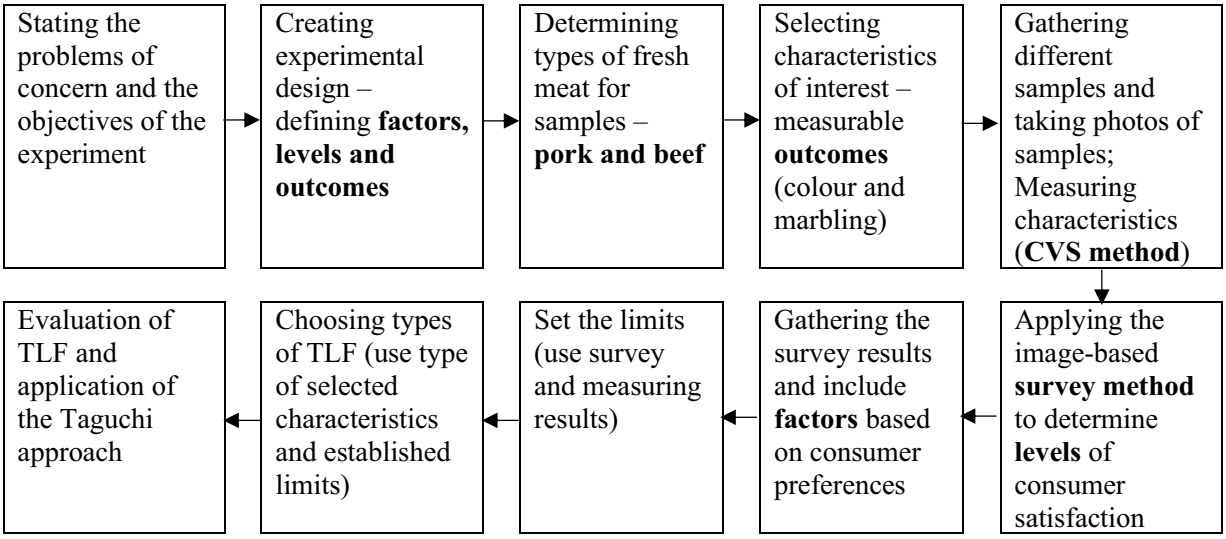

Figure 5. Flowchart of the potential application of the Taguchi method in evaluating fresh meat quality losses.

3.1. Stating the problems of concern and the objectives of the experiment

The experimental design of Taguchi-based approach is conducted by stating the problems of concern that in our case refer to inadequate marbling as quality loss occurrence in meat production and tendency for consumers' needs to be better understood. Hence, the main objective of this paper is to identify and calculate losses in fresh meat production that are related to consumers' dissatisfaction. Using TLF it is possible to evaluate every loss due to product manufactured with quality characteristics that deviate from the target value.

3.2. Creating experimental design

The experimental design consists of planning the experiment (determining types of fresh meat for samples and selecting the quality characteristics and measurement systems) and performing the experiment that considered steps from gathering different samples to the end of the flowchart (Figure 5). Gathering different samples, in this flowchart, means choosing samples with different levels of marbling. Thus, after samples are photographed, the image-based survey would show different levels of marbling of pork and beef meat to consumers. In this part of the experiment, consumers should be asked to select their preferred chop from presented images, considering marbling. Additionally, the consumers would be asked to select their maximum and minimum acceptable levels of marbling. Measuring quality characteristics would start by using CVS [22], after the pictures of samples are captured.

3.3. Potential application of Taguchi loss function

Based on consumers' responses to the survey and previous measurements, limits for marbling could be set. After quality characteristic limits are set, types of TLFs could be chosen. Defined inputs for application of TLFs are marbling measurements. Methodology for evaluation of Taguchi losses would be modified according to the work of Kethley et al. [46]. Such methodology suggested conversion of the raw performance measurements into the common Taguchi unit of measure, the percentage of loss for particular characteristic. Figure 6 shows a generic TFL.

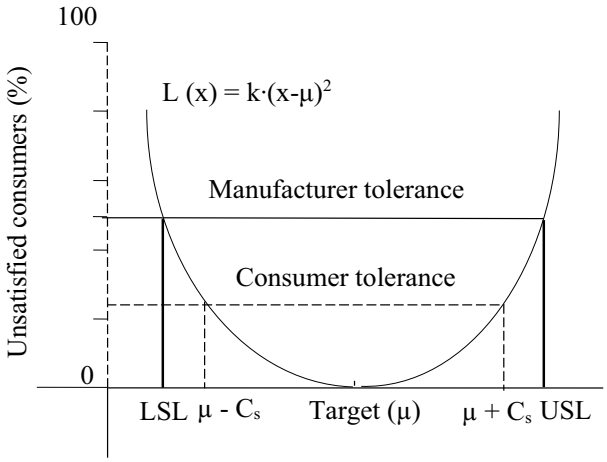

Figure 6. Generic TFL.
LSL – lower specification limit (determined as minimal marbling share); USL – upper specification limit (determined as maximal marbling share); Cs – level of customer satisfaction.

Let x be the level of marbling of the fresh meat and μ the consumers' preferable level of marbling. L(x) denotes the loss due to the difference between x and μ. When $x < \mu$, the level of marbling is undesirably smaller than the target value, and when $x > \mu$, the level of marbling is undesirably bigger than the target value. If the process goes in this direction of deviation, towards functional ($\mu - C_s$ and/or $\mu + C_s$) limits, we consider it as consumers' tolerance (at 50% of unsatisfied consumers). Any possible value of x (characteristic measurement) within scope from the target to the LSL or from the target to the USL represents consumers' dissatisfaction.

4. Conclusion

As the Taguchi approach is designed to identify quality characteristic levels that most closely match the consumers' preferences and at the same time provide relation to quality losses, its validation should be under consideration in future research. Therefore, a combination of instrumental techniques, consumers' preferences and Taguchi loss function analysis can provide realistic data to develop a product with optimal levels and limits of key quality characteristics. This study can be used as the basis for designing applications of Taguchi loss function in other meat production areas, such as production of fermented sausages, dry meat, hamburgers, cooked hams and other meat products.

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Artificial neural network prediction of microbiological quality of beef minced meat processed for fast-food meals

Artificial neural network prediction of microbiological quality of beef minced meat processed for fast-food meals

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Abstract. In this study, the microbiological quality of 72 minced beef meat samples collected during six months from a local butcher was defined after laboratory analysis and developing advanced mathematical models. This new simultaneous approach provided adequate precision for the prediction of the microbiological profile of minced beef meat as one of the easily spoiled products with a short shelf life. For the first time, an artificial network model was developed to predict the microbiological profile of beef minced meat in a fast-food restaurant according to meat and storage temperatures, butcher identification, and work shift. A concurrent statistical study of practical analysis and the developing mathematical models provided adequate precision for the prediction of the microbiological profile of minced beef meat. The developed ANN provided a good prediction of the microbiological profile of beef minced meat with an overall R^2 of 0.867 during the training cycle.

1. Introduction

The artificial neural network model (ANN) is a widely accepted mathematical tool that regularly provides an empirical solution to the problems from a set of experimental data, and is capable of handling complex systems with nonlinearities and interactions between decision variables [1-3]. Predictive ANN modelling is often use in meat science for analysing different characteristics of meat, e.g. temperature and moisture content [4], bacterial growth in modified atmosphere packed cooked meat products [5], rheological features [5], sensory measurements [6], heat capacity prediction [7], slaughter weight in meat-type quails [8], *etc*.

Meat is the most popular type of food in fast-food restaurants around the world [9], as well as an inseparable part of traditional dishes (e.g., moussaka, sarma), and meat products (e.g., sausages, ćevapčići, burgers) [10]. One of the main characteristic of minced meat is water activity of approximately 99%, as well as satisfactorily nutrients which can support microbial growth [11]. Many chemical and physical factors can influence meat quality, such as storage temperature, moisture, oxygen availability, packaging, microbiological contamination, *etc*. [12]. The microorganisms are commonly present on the meat surface, but also can be distributed throughout comminuted products during the entire production process, especially during the mincing and mixing process used to produce burgers and other minced meat-based products [13].

In order to estimate the microbiological profile of minced beef meat from a local specialist beef butcher shop, the predictive capabilities of the ANN were tested as a function of meat and storage temperatures, butcher identification, and the work shift. This study aims to determine and predict the microbiological profile of minced meat used in a local fast-food restaurant in Novi Sad, Serbia. For this purpose, 72 minced meat samples were purchased during six months from a local butcher after the mincing and mixing process, and the results were used for mathematical modelling and ANN prediction of the microbiological profile of minced-meat products in the mentioned restaurant.

2. Materials and methods

2.1. *Sampling*

For this study, minced beef originated from a local butcher in Novi Pazar, Serbia. Approximately, one hundred grams of minced meat was obtained from each processed meat batch immediately after transport to the fast food restaurant in Novi Sad, Serbia and placed in a sterilized sampling box. Samples were collected for six months (January-June 2019, every Monday, Wednesday, and Friday) for each batch.

2.2. *Microbiological analysis*

The microbiological profile of minced meat samples was examined following standard methods, presented in Table 1. The selected microbiological analysis was chosen based on the Guide to Microbiological Criteria for Food [13]. All analyses were done in triplicate and the results were compared to the allowable values for every type of food. Briefly, all results are presented as logarithm-transformed colony count in one gram of sample (log CFU/g), except for *Salmonella* spp., where the absence of the bacterium required. According to the Serbian Rulebook [15], the obtained results can classify meat as satisfactory (result is below m-value), acceptable (result is between m- and M-value), or unsatisfactory (result is above M-value). The m- and M-value define limit values, minimum and maximum for each analysis separately, and depend on the type of food.

Table 1. ISO methods for microbiological analysis

Microbiological analysis of minced meat		**Allowable limit values (m- and M-values) according to the Guide to microbiological criteria for food (log CFU/g)**
Microorganism/ group of microorganisms	**Method**	
Aerobic and mesophilic bacteria	ISO 4833-1:2013 [16]	m=5 M=6
Escherichia coli	ISO 16649-2:2018 [17]	m=2 M=3
Salmonella spp.	ISO 6579-1:2017 [18]	nd*
Listeria monocytogenes	ISO 11290-1:2017 [19]	nd
Staphylococcus aureus	ISO 6888-1:2018 [20]	m=2 M=3
Lactic acid bacteria (LAB)	ISO 7889:2003 [21]	**

*nd- not detected in 25 grams of samples; ** the Guide to Microbiological Criteria for Food [13] does not indicate value for this parameter

2.3. *Artificial neural network (ANN) predictive modelling*

A multi-layer perceptron model (MLP) with three layers (input, hidden, and output) was used as a concept for ANN modelling, while input and output data were normalized before calculation, to enable improvement of the ANN model's performance. Furthermore, the experimentally obtained data were

randomly separated into training, cross-validation, and testing data (with 70%, 15%, and 15% of experimental data, respectively), and a series of different topologies were used, in which the number of hidden neurons varied from 5 to 20. The training process was run 100,000 times with random initial values of weights and biases. The Broyden-Fletcher-Goldfarb-Shanno (BFGS) algorithm was used for the solution of the unconstrained nonlinear optimization in the ANN modelling. Successful ANN training was achieved when learning and cross-validation curves approached zero [22].

3. Results and discussion

The developed optimal ANN model showed a good generalization capability for the experimental data obtained, and it can be used to predict the accurate output for a broad range of the input parameters. According to ANN performance, the optimal number of neurons in the hidden layer for aerobic and mesophilic bacteria, *Escherichia coli*, lactic acid bacteria (LAB), and *Staphylococcus aureus* prediction was equal to 10 (network MLP 9-10-4), whereby high values of R^2 (overall R^2 was 0.867 during training period) and low values of SOS were obtained (Table 2). When striving to implement mathematical modelling in the field of predictive microbiology, the artificial neural network (ANN) model is considered the most suitable predictive tool [23].

Table 2. ANN summary of observed results

Network name	**Performance**			**Error**		
	Train.	**Test.**	**Valid.**	**Train.**	**Test.**	**Valid.**
MLP 9-10-4	0.867	0.832	0.854	0.484	0.490	0.897
	Training algorithm		**Error function**	**Hidden activation**		**Output activation**
	BFGS 6447		SOS	Logistic		Identity

The predicted values were very close to the desired values in most cases, in terms of R2 value, for ANN models. The ANN model predicted experimental aerobic and mesophilic bacteria, *E. coli*, LAB, and *S. aureus* reasonably well for a broad range of the process variables as seen in Figure 3, where the experimentally measured and ANN model predicted values are presented. The accuracy of the ANN model could be visually assessed by the dispersion of points from the diagonal line in the graphics presented in Figure 1.

Table 3 presents the elements of matrix W_1 and vector B_1 (presented in the bias row), as well as the elements of matrix W_2 and vector B_2 (bias) for the hidden layer.

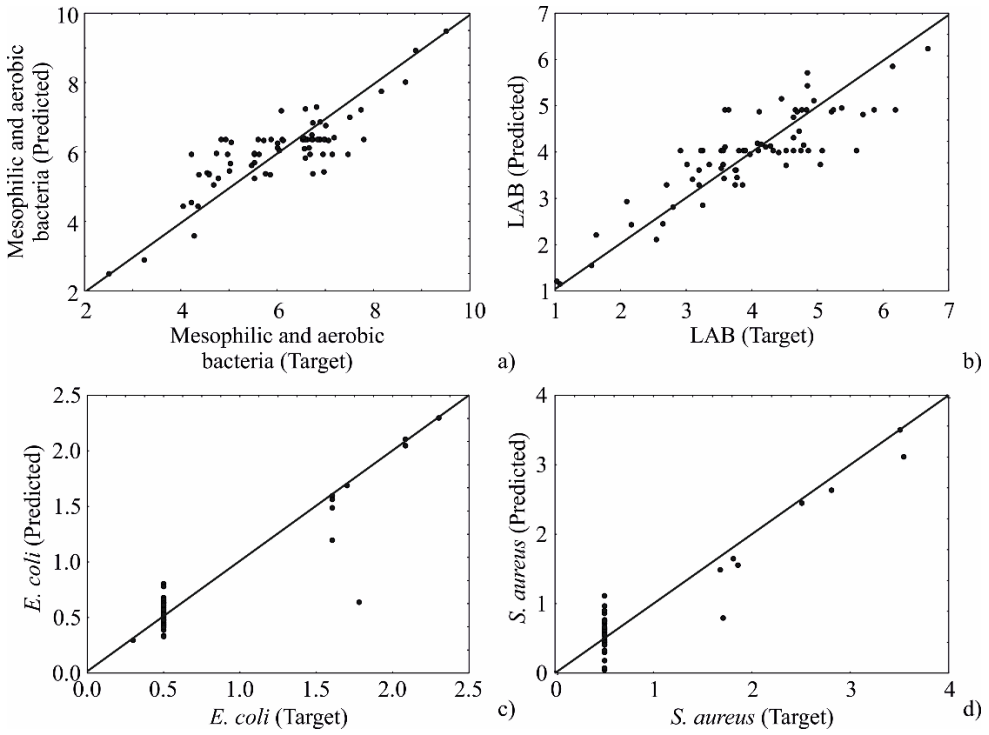

Figure 1. Experimentally gained and the ANN predicted values of the microbiological profile of minced meat

Table 3. Elements of matrix W1 and vector B1 (presented in the bias row) as well as W_2 and vector B_2 (presented in the bias column)

Independent variables	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Store temperature	3.70	0.37	-147.96	-17.66	-82.56	-15.34	231.07	29.54	28.94	-14.48
Meat temperature	99.23	142.56	58.25	20.10	65.60	27.46	-94.96	105.16	151.15	25.96
Butcher (A)	-65.60	-105.46	-37.80	-7.51	-20.39	-14.53	35.69	-78.06	61.40	-121.10
Butcher (B)	107.49	303.75	29.99	2.40	20.45	2.62	-80.11	115.68	41.76	52.01
Butcher (C)	41.19	-63.16	59.55	3.81	19.07	4.05	34.42	-64.03	-101.17	53.46
Butcher (D)	-65.26	-103.04	1.93	2.01	14.19	7.24	-114.51	98.64	-34.24	56.38
Day (Friday)	6.55	6.67	52.01	7.05	73.63	7.72	44.36	78.00	93.39	73.42
Day (Monday)	4.87	10.37	25.22	-1.54	-2.84	0.28	-37.29	58.36	-73.23	66.34
Day (Wednesday)	6.45	15.05	-23.48	-4.91	-37.44	-8.57	-131.65	-64.04	-52.44	-98.96
Bias	17.80	32.05	53.77	0.68	33.38	-0.58	-124.66	72.23	-32.28	40.75
Output variables	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Bias**
Aerobic and mesophilic bacteria	65.57	-65.39	-5.27	-0.52	5.61	5.91	-3.40	-0.25	0.08	-5.50	0.30
LAB*	3.41	-3.35	-38.85	0.96	38.44	15.05	-27.28	0.49	-0.15	-16.01	0.56
E. coli	55.53	-55.56	71.22	0.43	-71.60	2.03	50.36	0.16	-0.10	-2.21	0.09
S. aureus	20.40	-20.35	-13.56	-0.59	13.24	-33.94	-9.39	0.40	0.04	34.45	-0.06

*LAB - lactic acid bacteria

The goodness of fit between experimental measurements and calculated results is presented in Table 4. The mathematical model had an insignificant lack of fit tests, which means that all the models represented the data adequately. A high R^2 is indicative that the variation was accounted for and that the data fitted adequately to the proposed model.

Table 4. The goodness of fit tests for the microbiological profile prediction of beef minced meat

	χ^2	RMSE	MBE	MPE	r^2	Skew	Kurt	SD	Var.
Aerobic and mesophilic bacteria	1.612	1.176	0.000	26.823	0.592	-0.125	-0.274	0.738	0.545
LAB	1.047	0.947	0.000	31.823	0.731	0.144	0.085	0.595	0.354
E. coli	0.087	0.2737	0.000	36.413	0.819	4.195	27.617	0.171	0.029
S. aureus	0.153	0.362	0.000	67.756	0.880	0.933	3.896	0.228	0.052

*LAB - lactic acid bacteria

4. Conclusions

A simultaneous statistical study comparing practical analyses with the developing mathematical models showed the models provided adequate precision for the prediction of the microbiological profile of minced beef meat as one of the easily spoiled products with a short shelf life due to a fast decrease of quality parameters and microbial growth. Furthermore, the developed ANN provided good prediction of the microbiological profile of beef minced meat with an overall R^2 of 0.867 during the training cycle.

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Optimization of pasteurization of meat products using pasteurization values (p-values)

Optimization of pasteurization of meat products using pasteurization values (p-values)

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Abstract. This study aimed to examine the effect of shortening the effective heat treatment time by 15 minutes for finely and coarsely chopped sausages and canned meat in pieces packed in polyamide casings with diameters of 75 and 90 mm. Product safety was ensured in accordance with legislation and with the producer food safety system. Optimization led to a decrease in p-values for finely chopped sausages (diameter 90: 148.8 → 97.64 minutes; diameter 75: 111.2 → 58.4 min), coarsely chopped cooked sausages (diameter 90: 115.5 → 79.1 min; diameter 75: 245.3 → 106.6 min) and for canned meat in pieces (diameter 90: 249.7 → 95.91; diameter 75: 213.9 → 48.42 min). The use of p-value in optimization confirms the pasteurization level and ensures the safety of the product in the defined storage conditions. It is also possible to compare the levels of heat treatment for different products that are differently packaged.

1. Introduction

Thermal processing is still one of the most effective methods for inactivating undesirable microorganisms in foods. Heat is used to inactivate pathogens and in aid in development of typical flavours, aromas, texture, and colour of a boiled (cooked) food [1]. Pasteurized products include all those meat products which during production were exposed to the preservative effect of temperature below the boiling point of water, whereby a temperature of at least 70° C is achieved in the geothermal centre, in accordance with the Regulations [2], while the required time duration at the defined temperature in the geothermal centre is not stated in the Regulations.

Pasteurized meat products belong to easily perishable foods, with physicochemical properties (water activity (a_w), pH) that enable the growth of microorganisms. Destruction of microorganisms during heat treatment depends on temperature and heating time, and the peak of destruction depends on the natural resistance, number, and phase of reproduction of microorganisms, as well as on the properties of the substrate [3]. Therefore, in production, it is necessary to provide the conditions for ensuring a cold chain so that the product retains the quality properties it possesses for its defined shelf life. To ensure the safety and quality of pasteurized products within defined shelf life, they need to be under cold chain conditions (conditions of storage, transport, and trade at temperatures from 0° to 4°C).

The optimization of the heat treatment process described herein was aimed at a complete understanding of the pasteurization process while ensuring the safety and quality of meat products within their defined shelf life. In this way, the consumption of energy required for the realization of heat treatment of pasteurization levels is reduced, while the aroma of the product is intensified.

Pasteurization destroys vegetative forms of bacteria, while spores survive. The lethality of the pasteurization process is that necessary to destroy *Enterococcus faecium* according to the 5D concept, which is > 40 [2]. The D-value (decimal reduction time) is the time necessary to reduce by 90% a microbial population present in a well-defined medium, and it is indicative of the thermal resistance of a microorganism at a constant temperature [4]. Knowledge of the D-values for a particular micro-organism makes it possible to design thermal processes that target that specific organism [5]

The limit for pasteurization value (hereafter, p-value) is calculated based on the time for a one decimal reduction of the stated microorganism, relative to the average level of contamination (10^6 cfu/g) multiplied by product weight, and is expressed in minutes.

This research aims to optimize the pasteurization process by applying p-values, which improve the overall process control, reduce costs, and improve the sensory properties of the product, which is less exposed to the effects of elevated temperature.

2. Materials and Methods

Systematic monitoring of the pasteurization process was performed in real-time over one year in a meat industry production plant during regular production. Heat treatment was validated for finely chopped sausages (diameters 90 and 75 mm), coarsely chopped sausages (diameters 90 and 75 mm) and canned meat in pieces (diameters 90 and 75 mm). Validation of a minimum pasteurization time was done for each specific food-thermal process, by inserting a thermocouple into the “coldest” spot of the food, and ensuring that this point is submitted to the minimum pasteurization value required [1]. Validation was realized during regular production in Eller brand heat chambers (single door chamber that can accommodate 4 trolleys) before and after shortening the effective pasteurization time by 15 minutes. Accurate and precise recording of the temperature in the geothermal centres was realized using a thermocouple, ELLAB, model E Val Pro, with six compensating cables. The thermocouple is a simple, widely used component for measuring temperature [6]. Special attention was paid to the placement and fixation of probes in the geothermal centre while ensuring the defined positions during pasteurization. The thermocouple allowed us to monitor the temperature in real-time during pasteurization. The time frame was monitored within the Ellab E Val Pro thermocouple software, in minutes at which the mass of the product was at specific temperatures during heat treatment (55°C, 70°C, 72°C and 74°C were recorded).

The temperature of 55°C is important because it is considered at that level, heat treatment of pasteurization level is initiated, in accordance with the method of determining the p-value according to Vukovic and Nitsch [7]. The temperature of 70°C is defined as the limit to be reached during pasteurization in a geothermal centre [2]. Temperatures of 72°C and 74°C are generally defined in the HACCP documentation of food business operators as critical limits for the assessment of pasteurization for meat products.

The time frame for maintaining the temperature in the geothermal centre for these temperatures (55°C, 70°C, 72°C, and 74°C) was determined. Then, based on the mass of the product and the heat treatment graph, the p-value was calculated.

The p-value is determined from the lethal effects measured at the cold point of the product during the heat treatment. The pasteurization process is validated by monitoring F_{70} values in relation to the thermoresistant microorganism *Enterococcus faecium*, integrating temperatures of over 55°C at the geothermal point of meat products [3]. Using D-values of a heat resistant *Enterococcus faecium* E-20 strain as a basis, parameters for pasteurization that effectively eliminate vegetative, non-spore forming

bacteria can be developed [8]. One of the ways to obtain p-values, which was applied in this paper, is the computational method, which was developed by Vukovic and Nitsch in 2004 [7]. The method is based on Gaussian integration.

Immediately after the end of the active phase of pasteurization, after reaching the HACCP defined values in the geothermal centre (72°C and 74°C for meat products with or without mechanically separated meat), temperature measurement continued during the cooling phases.

All validated meat products were stuffed into polyamide casings in two diameters, 90 and 75 mm, and during heat treatment were exposed to the preservative heat in controlled conditions of the chamber in one programmed cooking step at 80°C medium temperature.

The list of products that were optimized by applying p-values, and the defined pasteurization programs before and after shortening the heating times are given in Table 1.
Optimized pasteurization time meant a shortening of the effective pasteurization time by 15 minutes.

Cooling was performed with water (temperature 13-14°C) by showering the meat products on the pasteurization trolleys. The cooling dynamics consisted of two steps of active intensive showering for 4 minutes, followed by a 1.5-minute break. After that, the cooling process was repeated by showering until the temperature in the geothermal centre of the product dropped to the value of 55°C at the hottest control point. After that, the pasteurization process was finished, and the product was further sent for cooling to the circulating air cooling room.

The validation process was performed regularly for one year, using six probes placed diagonally in the trolleys, in the geothermal centre of products differently positioned. Since the optimization of the heat treatment process must have the imperative of ensuring food safety, the lowest results obtained in the validation procedure of the regular and optimized regime were monitored and considered.

3. Results and Discussion

The results of monitoring the time frames at which the product was at set temperatures during defined pasteurization time, and the calculated p-values are presented in Table 1.

Table 1. Validation of pasteurization during defined time

Meat product	Diameter (mm)	Mass of the product (kg)	The time frame for the product during heat treatment at defined limits (min)				p-value (min)
			55°C	70°C	72°C	74°C	
Finely chopped sausages	90	2,060	104	48	37	25	148.8
	75	1,706	72	33	26	19	111.2
Coarsely chopped sausages	90	2,114	95	38	27	18	115
	75	1,698	98	49	43	34	245.3
Canned meat in pieces	90	2,056	101	56	48	37	249.7
	75	1,723	90	48	41	32	213.9

The results of monitoring the time frames at which the product was at set temperatures during optimized pasteurization time, and the calculated p-values are presented in Table 2.

Table 2. Validation of pasteurization during optimized time

Meat product	Diameter (mm)	Mass of the product (kg)	The time frame for the product during heat treatment at defined limits (min)				p value (min)
			55°C	70°C	72°C	74°C	
Finely chopped sausages	90	2,072	93	37	25	13	97.64
	75	1,712	62	28	21	6	58.4
Coarsely chopped sausages	90	2,102	80	30	19	12	79.1
	75	1,709	84	35	28	18	106.6
Canned meat in pieces	90	2,061	97	38	25	10	95.91
	75	1,763	56	22	15	7	48.42

It was noticed that the products, after the interruption of the pasteurization process, suffer an additional temperature increase of 2-4°C in the geothermal centre, due to the additional effect of the generated heat, and then begin to cool.

The optimization of finely chopped cooked sausages indicates that it is possible to reduce the pasteurization time by 15 minutes while ensuring food safety. For this food, p-values decreased from the regular 148.8 minutes (diameter 90 mm) to the optimized 97.64 minutes, and from the regular 111.2 minutes (diameter 75 mm) to the optimized 58.4 minutes. The obtained p-values ensure the safety of the products obtained in this way because they are in accordance with the legal regulations [2].

The optimization of coarsely chopped cooked sausages indicates that it is possible to reduce the pasteurization time by 15 minutes while ensuring food safety. For this food, p-values decreased from the regular 115 minutes (diameter 90 mm) to the optimized 79.1 minutes, and from the regular 245.3 minutes (diameter 75 mm) to the optimized 106.6 minutes. The obtained p-values ensure the safety of the products obtained in this way because they are in accordance with the legal regulations [2]. The results of the regular pasteurization process, especially with a diameter of 75 mm, indicate the too intense thermal process that needlessly increases production costs through energy consumption while reducing the nutritional value of the product.

The optimization of canned meat in pieces indicates that it is possible to reduce the pasteurization time by 15 minutes while ensuring food safety. For this food, p-values decrease from the regular 249.7 minutes (diameter 90 mm) to the optimized 95.91 minutes, and from the regular 213.9 minutes (diameter 75 mm) to the optimized 48.42 minutes. The obtained p-values ensure the safety of the products obtained in this way because they are in accordance with the legal regulations [2]. The results of the regular pasteurization process, for both diameters (90 and 75 mm), indicate the too intense thermal process that needlessly increases production costs through energy consumption, while reducing the nutritional value of the product. In order to meet the expectations of the demanding consumers and become competitive on the market, it is important that during the development of optimized products, special attention is paid to its sensory properties [9].

The obtained validation results indicate that the safety of the products was ensured, but that the pasteurization process itself was uneven and that it varied significantly from product to product, without taking into account the size and shape of the packaging.

The results show that an optimized pasteurization regime using p-values can make a significant contribution to producing products with better nutritional value through milder pasteurization regimes

and without compromising product safety. By shortening the pasteurization time, the conditions are created to increase productivity.

For validation of the pasteurization, in the heating chamber, it is necessary to correctly place the top of the control probe in the geothermal centre of the product. Workers in charge must have the appropriate skills to repeat this procedure during each cooking, to obtain accurate results.

During the commercialization of an optimized pasteurization regime, shelf life studies for the optimized product is required, while special attention should be paid to the provision of the cold chain after production, in retail.

4. Conclusion

Each optimization process for pasteurization-level heat treatment must be adapted to the production facility and specific production conditions. Since this is a level of heat treatment that does not destroy all microorganisms, those that cause spoilage as well as pathogens, it is necessary to properly cool meat products, and then to provide and secure the cold chain in transport and retail. It is also necessary to re-perform the risk analysis within the food safety assurance system for the optimized product.

The use of p-value in optimization confirms the pasteurization level and ensures the safety of the product in the defined storage conditions. It is also possible to compare the levels of heat treatment for different products that are differently packaged. This provides more complete insights into the production process, reduces unnecessary costs, contributes to a better taste of products of higher nutritional value, and increases productivity by creating space for more production batches.

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Tracking brucellosis–a re-emerging disease

Tracking brucellosis – a re-emerging disease

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Abstract. Brucellosis caused by members of the genus *Brucella* is of major concern for animal and public health and is recognized as a re-emerging zoonotic disease. Brucellosis causes flu-like symptoms like fever, sweats, weakness, pain in muscles, joint and back, with some symptoms persisting for longer time periods. Infections occur through consumption of unpasteurized dairy products or undercooked meat, inhalation, and contact with animals. Human-to-human transmission is rare. Surveillance of this disease in animals and humans and prevention of infection risks factors are the most effective strategies to prevent brucellosis. With the progress in sequencing technologies, whole genome sequencing (WGS) has become an effective tool in surveillance, tracking of pathogens and in outbreak investigation. WGS allows identification of the source of infection and to elucidation of transmission chains, which enables authorities to implement timely and appropriate interventions.

1. Introduction

Members of the genus *Brucella* are Gram-negative, aerobe, non-spore-forming spherical to rod-shaped bacteria. *Brucella* spp. cause brucellosis in a wide range of animals and humans. The clinical symptoms of brucellosis are infertility and abortion in wild and domestic animals [1], and flu-like illness including fever, sweats, weakness and pain in muscles, joint and back in humans [2]. Bacterial cells can colonise virtually all organs and tissues, can persist for years intracellularly and may cause recurrent infections, that – if untreated – can progress to a chronically incapacitating disease with severe focal complications such as spondylitis, neurobrucellosis or *Brucella* endocarditis [3]. Four out of 12 currently known *Brucella* sp. [4], namely *B. melitensis* (sheep and goat), *B. abortus* (cattle), *B. suis* (pig), and *B. canis* (dog), are pathogenic for humans [5]; *B. melitensis* and *B. abortus* are responsible for most of the reported clinical human cases [6].

With an estimated incidence of 500,000 cases per year, brucellosis is a frequent and widespread zoonotic disease [7]. Travelling, globalization, illegal food imports and the occurrence of new variants can be considered as driving factors for the re-emergence of brucellosis in so far brucellosis-free countries [7,8,9,10,11,12]. *Brucella* can be transmitted from infected animals and contaminated tissues to humans through inhalation or through skin contact, so is a high occupational risk hazard for hunters, farmers, veterinarians and abattoir workers. For these occupational groups, where close contact with

animal carcasses cannot be avoided, appropriate personal protective measures are required. However, the major public health risk is due to the consumption of contaminated raw milk and raw dairy products, and the consumption of raw or undercooked meat [2,6]. Pasteurization or sterilization of raw milk before marketing or further processing into dairy products and sufficient cooking of meat are the most effective measures to prevent *Brucella*. Most European Union (EU) member states are free of bovine and ovine/caprine brucellosis, and brucellosis has become a rare zoonotic disease due to a strict eradication program. However, more than 380 cases of human brucellosis, mainly caused by *B. melitensis*, were reported in the EU in 2017, and more than 60% of patients required hospitalization [6].

Brucella is able to survive frozen storage conditions and can grow between 6°C to 42°C, between pH 4.5 to pH 8.8 and in up to 4% NaCl. In addition, foods with a high fat content have a protective effect, allowing a longer survival of *Brucella* [13,14]. Heating at 72°C for 15 seconds (or equivalent) is sufficient to inactivate *Brucella* [13,14].

2. Whole genome sequencing (WGS) based surveillance

Austria is officially recognised as being free from bovine brucellosis (OBF) and of small ruminant brucellosis, but a few human cases are still reported every year [15]. As *B. melitensis* infections in Austria are rare and usually imported, collecting as many details as possible is essential to better understand patterns of transmission. This includes epidemiological information on patient's history but also microbiological data on bacterial strains.

Whole genome sequencing (WGS) has been frequently used to identify outbreaks for various diseases. For such investigations, it is worth implementing molecular techniques with the highest possible discriminatory power, because they provide a higher probability that two isolates with similar genetic profiles are indeed related. In the case of brucellosis, a retrospective study in Portugal was able to identified likely "missed outbreaks" using WGS, as isolates with no documented links clustered together [16]. In Austria, WGS was used to investigate a cluster of brucellosis cases detected in May 2018 [10]. Following an epidemic of miscarriages, *B. melitensis* was isolated from aborted bovine material and milk. Two veterinarians and the farmer's child were hospitalized and diagnosed with brucellosis. The genetic similarity between the cattle and human isolates (<2 allelic differences in core genome multi locus sequence type (MLST)) confirmed that they all belonged to the same outbreak. This outbreak was the first proved zoonotic transmission of *B. melitensis* in Austria in more than 15 years.

WGS can also support clinical management of brucellosis patients. An Austrian patient diagnosed with brucellosis in 2017 developed a second episode of brucellosis in 2019. To determine if the second episode was a relapse or a reinfection, the two *B. melitensis* isolates were sequenced. The 2019 isolate showed only one allelic difference in core genome MLST to the 2017 strain, supporting the hypothesis of a relapse.

Finally, WGS can also be used to support the investigation of isolated and imported brucellosis cases. Indeed, identifying importation routes is of crucial importance to take adapted control measures. When the epidemiological investigation does not succeed, genomic data can be used to identify the most likely geographical origin of the cases. This can be achieved using environmental or animal samples, as done in a study from Germany [9]. They collected *B. melitensis* strains from Turkish sheep and were able to link these animal isolates to clinical isolates from travellers returning from Turkey or Turkish immigrants, suggesting that these cases were indeed imported from Turkey. When neither food isolates nor animal isolates are available, *B. melitensis* strains can be compared with each other, analysing how strains of unknown origin cluster with strains of known origin. Previous studies from Germany and Italy used this method, and were successful thanks to a large number of isolates with known origin [17,18]. When lower numbers of isolates are available, published strains for which the country of infection is reported can be used, as was done in Malta, Belgium, Norway and Sweden [19,20,21,22]. In Austria, we were able to identify a cluster of 12 Austrian patients. *B. melitensis* strains isolated by Serbian partners in animals grouped within this cluster, supporting the hypothesis that all cases of this cluster were infected in Balkan countries.

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Domestic chicken omega 3 – a product for promoting human health

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Domestic chicken omega 3 – a product for promoting human health

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Abstract: Literature data show that the relationship between two groups of polyunsaturated fatty acids in diet, omega 3 acids, whose basic representative is α-linolenic acid (C18: 3 n-3), and omega 6 acids, whose basic representative is linoleic acid (C18: 2 n-6), has a significant role in development of cardiovascular diseases in humans. The optimal ratio of omega 6 to omega 3 fatty acids is around 4:1. In monogastric animals, the fatty acids in feed are absorbed in the gastrointestinal tract largely unchanged. This means the fatty acid profile of the animal's diet directly reflects the fatty acid profile of the tissue. The daily intake of unsaturated fatty acids can be increased by an adequate animal nutrition strategy. Flaxseed contains ten times more unsaturated (32.26%) than saturated (3.66%) fatty acids. The largest amount of unsaturated fatty acids (about 70%) is α-linolenic acid (ALA), which is a precursor of the entire omega 3 series of fatty acids, and which makes flaxseed an ideal raw material for the production of a wide range of omega 3 enriched products. In order to obtain chicken meat rich in omega 3, an experiment was organized with a specific diet for broilers at fattening. Thanks to the designed animal feed, it was possible to get products (meat, breast, drumstick, liver, subcutaneous fat) with significantly higher amounts of omega 3 fatty acids compared to the same products obtained from broilers fed with conventional mixtures, or with almost the ideal ratio between omega 6 and omega 3 fatty acids.

1. Introduction

Meat products are high quality food, and have pronounced nutritional and biological properties. In addition to the quantitative increase in meat production in the world, it is necessary that meat has impeccable quality and long-term sustainability. Numerous medical findings show that in the development of cardiovascular and other chronic diseases in humans, a significant role is due to the relationship between two groups of polyunsaturated fatty acids in the diet: omega 6 acids, whose basic representative is linoleic acid (C18:2 n-6) and omega 3 acids, whose basic representative is alpha linolenic acid (C18: 3 n-3). Due to the many potential benefits of the presence of omega 3 fatty acids in the diet, consumer demands for omega 3 enriched foods of animal origin are also growing [13]. The reaction of desaturation and elongation of the chain of alpha-linolenic and linoleic acids, in which their derivatives, polyunsaturated fatty acids, are formed, is catalyzed by the same enzyme - desaturase [5]. Since the reaction is catalyzed by the same enzyme, there is competition between these essential fatty acids for this enzyme, so that increasing the concentration of

linoleic acid can inhibit the conversion of alpha-linolenic acid to its derivatives [2]. On the other hand, a diet rich in alpha-linolenic acid can reduce the production of linoleic or arachidonic acid derivatives, which disrupts the ratio of omega 3 to omega 6 fatty acids in the body. Although the ratio of omega 6 to omega 3 fatty acids in humans who lived in the Paleolithic era was 1:1, modern ways of eating and living have disrupted this ratio, which is often higher than 25:1 today. It is believed that in a proper diet, humans should have approximately two to six times more omega 6 than omega 3 fatty acids, so that the optimal ratio of omega 6 and omega 3 fatty acids would be 4:1 [13]. The production of omega 3 fortified poultry meat is very appealing for many producers and consumers. In monogastric animals such as poultry and pigs, the fatty acids present in feed are absorbed in the gastrointestinal tract largely unchanged, meaning that the tissue fatty acid profile directly reflects the fatty acid profile present in the animal's diet [6]. There are many examples in the literature showing the introduction of specific practices in the diet and breeding of production animals can increase the content of omega 3 unsaturated and other desirable fatty acids in meat. If an appropriate animal nutrition strategy is adopted, the results can be visible in a short period of time [11].

The daily intake of unsaturated fatty acids can be increased directly, by enriching food of animal origin with omega 3 unsaturated fatty acids or indirectly with a suitable animal nutrition strategy, but the source of omega 3 unsaturated fatty acids in the broiler diet could be different [3]. If we use only supplements of plant origin (flaxseed or oil), the amount of omega 3 fatty acids in the intramuscular adipose tissue will increase, while the amount of omega 6 unsaturated fatty acids will decrease [10]. It is important to note that fat and fatty acids in muscle tissue are located within and between muscle fibers, with fat within muscle fibers being concentrated in fat cells that are isolated or located in clusters along muscle fibers and consisting predominantly of triacylglycerols, phospholipids and cholesterol [8] As the composition of fatty acids in triacylglycerols changes primarily under the influence of feed/food, it is clear that also changes the composition of fatty acids in intramuscular adipose tissue [11].

In broiler diets, sunflowers are used as fat sources, as are other oilseeds that are a source of fatty acids from the omega 6 and omega 3 series (Table 1).

Table 1. The most important plant sources of omega 3 and omega 6 fatty acids

Omega 6	Omega 3
Sunflower oil	Linseed oil
Corn oil	Rapeseed oil
Pumpkin seed oil	Walnuts
Nuts	

Flaxseed contains an optimal fatty acid composition and, comparing it with other oilseeds, in terms of energy content, it is between soybean and sunflower, and in terms of crude protein content, it is similar to oilseed rape and cotton seed [3].Due to all these characteristics, flax has become an important part of animal feed. What makes it a nutritionally valuable feed is the fact that in addition to a large percentage of dietary fiber, it also contains ten times more unsaturated (32.26%) fatty acids compared to the amount of saturated (3.66%) fatty acids present [9]. The largest amount of unsaturated fatty acids (about 70%) is alpha-linolenic acid (ALA), which is a precursor of the entire omega 3 series of fatty acids, which makes flaxseed an ideal raw material for the production of a wide range of omega 3 fortified food of animal origin [1]. This distinguishes flaxseed from other conventional energy sources that are routinely used in broiler diets (corn,

soy, sunflower), which contain significantly higher amounts of omega 6 fatty acids than of omega 3 fatty acids (Table 2).

Table 2. Chemical composition of extruded flaxseed

Dry matter, %	90.0
Crude proteins, %	26.0
Crude fiber, %	9.0
Crude lipid, %	25.0
Crude ash, %	5.0
NDF, %	22.38
ADF, %	12.36
ADL, %	5.65
Starch, %	4.0
Sugars, %	5.0
Calcium, g/kg	5.0
Phosphorus, g/kg	8.0
Magnesium, g/kg	4.0
Potassium, g/kg	11.0
Sodium, g/kg	0.58
Chlorine, g/kg	0.64
Sulfur, g/kg	3.86
Manganese, mg/kg	38.20
Zinc, mg/kg	53.0
Copper, mg/kg	10.0
Iron, mg/kg	157.60
Selenium, mg/kg	0.44
Cobalt, mg/kg	0.04
Molybdenum, mg/kg	0.76
Iodine, mg/kg	0.28
C14:0, g/kg	0.2
C16:0, g/kg	14.0
C16:1, g/kg	0.3
C18:0, g/kg	10.0
C18:1, g/kg	44.0
C18:2, g/kg	38.0
C18:3, g/kg	136.0

Energetic value		Pigs	Sows	Poultry
DE	kcal/kg	4015	4210	
ME	kcal/kg	3845	3966	3390
NE	kcal/kg	2861	2927	

Vitamin E	mg/kg	5.60
Vitamin B1	mg/kg	2.16
Vitamin B2	mg/kg	2.50
Vitamin B6	mg/kg	4.40

Vitamin B12	mg/kg	--
Niacin	mg/kg	74.2
Pantothenic acid	mg/kg	3.60
Folic acid	mg/kg	0.33
Biotin	mg/kg	0.36
Choline	mg/kg	2615

2. Materials and Methods

In order to obtain products under the commercial name Domestic Chicken Omega 3, an study was organized with a specific diet for broilers at fattening, where the classic raw materials used in the diet of broilers in our area, rich in omega 6 fatty acids (soybean meal, and corn grain) were replaced with exuded flaxseed (source of omega 3 fatty acids), so producing broiler feed with an ideal fatty acid profile. After slaughter, the meat, breast, drumstick, liver and subcutaneous adipose tissue of free-range chickens fed conventional feed and chickens fed with the feed with addition of extruded flaxseed were subjected to detailed analyses which determined: average nutritional value, energy value expressed in kJ and kcal, protein content, polyunsaturated and monounsaturated fatty acid content, carbohydrate content, salt content, fatty acid profile, cholesterol, water/dry matter ratio, instrumental skin color determination, comparative sensory analysis and saturated fatty acid content.

3. Results and Discussion

Based on the performed analyses, the following results were obtained in Tables 3 and 4:

Table 3. Composition of meat from chickens fed with conventional feed

CHEMICAL ANALYSES	Method code	Unit of measure	Prescribed value	Result
Energetic value	/	kJ/100g	/	664.25
Energetic value	/	kcal/100g	/	158.99
Salt content	/	g/100g	/	0.15
Protein content	02H.01.012	%	/	19.11
Sugar content	02H.01.016	%	/	0.14
Carbohydrate content	02H.01.016	%	/	0.14
C18:2 n-6	02H.01.028	g/100g	/	2.56
C18:3 n-3	02H.01.028	g/100g	/	0.174
C18:3 n-6	02H.01.028	g/100g	/	0.021
C20:3 n-3	02H.01.028	g/100g	/	0.000
C20:5 n-3	02H.01.028	g/100g	/	0.003
C22:6 n-3	02H.01.028	g/100g	/	0.006
Ratio n-6/n-3 fatty acids	02H.01.028	/	/	14.38
Eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid content	02H.01.028	g/100g	/	0.01
Total monounsaturated fatty acids	02H.01.028	g/100g	/	3.76
Total n-3 fatty acids	02H.01.028	g/100g	/	0.18
Total n-6 fatty acids	02H.01.028	g/100g	/	2.59

Total polyunsaturated fatty acids	02H.01.028	g/100g	/	2.78
Total saturated fatty acids (C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C24:0)	02H.01.028	g/100g	/	2.57
Cholesterol content	02H.01.029	mg/100g	/	38.04
Sodium	02R.01.214	g/100g	/	0.06
Dry matter content	SRPS ISO 1442:1998	%	/	28.72
Water content	SRPS ISO 1442:1998	%	/	71.28
Lipid content	SRPS ISO 1444:1998	%	/	9.11

Table 4. The composition of meat from chickens fed with designed feed with the addition of extruded flaxseed

CHEMICAL ANALYSES	Method code	Unit of measure	Prescribed value	Result
Energetic value	/	kJ/100g	/	675.25
Energetic value	/	kcal/100g	/	161.75
Salt content	/	g/100g	/	0.18
Protein content	02H.01.012	%	/	18.56
Sugar content	02H.01.016	%	/	0.12
Carbohydrate content	02H.01.016	%	/	0.12
C18:2 n-6	02H.01.028	g/100g	/	3.24
C18:3 n-3	02H.01.028	g/100g	/	0.69
C18:3 n-6	02H.01.028	g/100g	/	0.022
C20:3 n-3	02H.01.028	g/100g	/	0.004
C20:5 n-3	02H.01.028	g/100g	/	0.007
C22:6 n-3	02H.01.028	g/100g	/	0.000
Ratio n-6/n-3 fatty acids	02H.01.028	/	/	4.57
Eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid content	02H.01.028	g/100g	/	0.021
Total monounsaturated fatty acids	02H.01.028	g/100g	/	3.34
Total n-3 fatty acids	02H.01.028	g/100g	/	0.71
Total n-6 fatty acids	02H.01.028	g/100g	/	3.26
Total polyunsaturated fatty acids	02H.01.028	g/100g	/	4.00
Total saturated fatty acids (C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C24:0)	02H.01.028	g/100g	/	2.33
Cholesterol content	02H.01.029	mg/100g	/	25.41
Sodium	02R.01.214	g/100g	/	0.07
Dry matter content	SRPS ISO 1442:1998	%	/	28.70
Water content	SRPS ISO 1442:1998	%	/	71.30

Lipid content	SRPS ISO 1444:1998	%	/	9.67

Thanks to the designed feed for broilers at fattening, the products (meat, breast, drumstick, liver, subcutaneous fat) had a significantly higher amount of omega 3 fatty acids compared to the same products obtained from broilers fed in the usual way, or they had an almost ideal ratio between omega 6 and omega 3 fatty acids (5-6:1). Figure 1 shows the carcass of broilers fed with designed feed, with the addition of extruded flaxseed (left), and broilers fed with conventional feed (right). Figure 2 shows drumstick plus thighs of broilers fed with designed feed, with the addition of extruded flaxseed (above), and broilers fed with conventional feed (below).

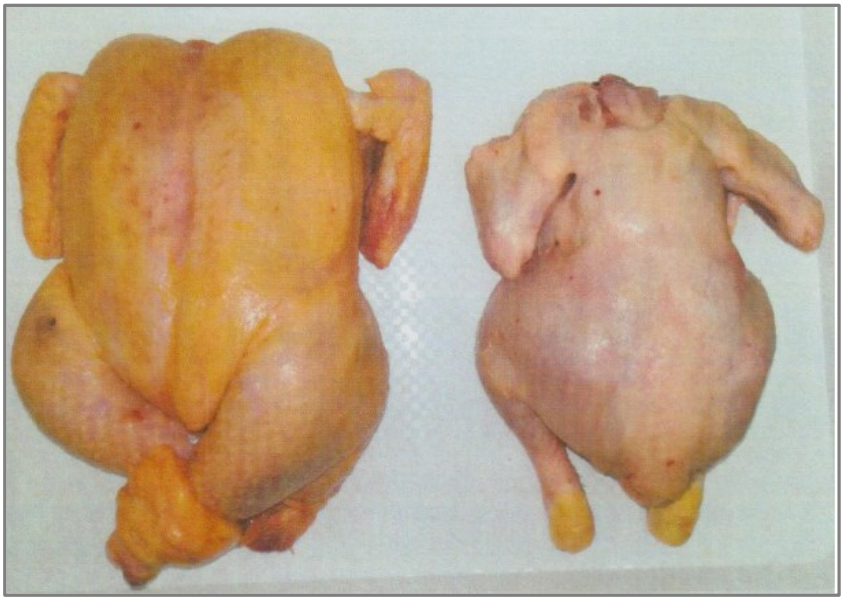

Figure 1. Carcass of broilers fed with designed feed, with the addition of extruded flaxseed, (left) and broilers fed with conventional feed (right)

Figure 2. Drumstick plus thighs of broilers fed with designed feed, with the addition of extruded flaxseed (above), and broilers fed with conventional feed (below)

The carcass fatty acid profile directly reflects the fatty acid profile in the animal's diet [4]. Since flaxseed has a desirable fatty acid composition, many producers are interested in using it in the final fattening of pigs and poultry, to improve the fatty acid composition of adipose tissue and pig meat. In the diet of pigs, soybean and sunflower are used, as well as other oilseeds that contain fatty acids from the n-3 series and fatty acids from the n-6 series [12].

Similar experiments on pigs were performed by [7], where the sources of fat in the diet of experimental groups were different in different fattening periods (from 30-60 kg and 60-115 kg), and accordingly after chemical analysis, the feeds were also of different fatty acid compositions. Thus, one experimental group received through feed saturated fatty acids (palmitic C16: 0 70-80%; stearic C18: 0 5-10% and oleic C18: 1 8-15%) in powder, and the other experimental group received through feed 10% flaxseed. Therefore, the content of total SFA, MUFA and PUFA differed between the experimental groups. Chemical analysis showed the experimental group that received flax had significantly less SFA (15.22%) and more PUFA (58.69%) than did the group that received SFA (75.02% and 13.13 %). In this experiment, the SFA/PUFA ratio was also influenced by feed components. Thus, the group receiving flaxseed had a significantly lower SFA/PUFA ratio (0.25) than the other group (5.71).

4. Conclusion

It is shown that using designed feed for broilers produces meat with significantly higher amounts of omega 3 fatty acids compared to the same products obtained from broilers fed standard diet. The enhanced products had an ideal ratio between omega 6 and omega 3 fatty acids (5-6:1). Based on the results, we can

conclude that the use of flaxseed in feed mixtures for broilers at fattening is medically, nutritionally and economically justified.

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Amino acids in animal feed: significance and determination techniques

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Amino acids in animal feed: significance and determination techniques

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Abstract. Amino acids are fundamental for animal nutrition. Their presence is necessary to maintain the normal structure and function of the intestine, and they are key in regulating metabolic pathways for improving health, survival, growth, development, lactation, and reproduction. The animal feed industry invests great resources and efforts to obtain optimal formulations in which the composition of amino acids plays a key role. In support of these aspirations in recent decades, much attention has been paid to the development and improvement of analytical techniques for the reliable, rapid and accurate determination of amino acid content in animal feed. This paper outlines different methodologies for the analysis of amino acid content in animal feed. Various methods, based on different analytical techniques, are presented for determination of amino acids in feed for nutritional and regulatory purposes.

1. Introduction

Amino acids (AAs) are building blocks for proteins and must be present in cells for synthesis of polypeptides [1]. AAs such as histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp) and valine (Val) are not synthesized by animals, so they are known as essential amino acids (EAAs) and must be included in diet. They have an important role in maintaining homeostasis of the whole body. The AAs cysteine (Cys) and tyrosine (Tyr) do not belong to the group of EAA, because they can be synthesized from Met and Phe in the liver. However, some animals cannot form the carbon skeletons needed for Met and Phe. This leads to the prevention of *de novo* synthesis of Cys and Tyr in these animals. The presence of these two AAs is necessary to maintain the normal structure and function of the intestine.

AA balance is crucial for animal growth. The fact is that the quality of feed, in addition to the animal hybrid and gender, largely affects the quality of meat [2]. Between the 1960s and 1990s, nutritionists developed the ideal protein concept (optimal proportions and amounts of EAA for chicken and pork diets based on the belief that all non-EAAs were sufficiently synthesized in animals [3-5]. This concept is currently being used by the National Research Council (NRC) [6,7]. AA-based diets are necessary for animals to maintain and increase protein content. Based on a large number of reports and papers published in scientific and professional literature over the past few decades, the concept of functional AAs has been developed. It defines AAs as key in regulating metabolic pathways for improving health, survival, growth, development, lactation, and reproduction [8]. The addition of AAs glutamine (Gln) and arginine (Arg) to a conventional diet thought to provide adequate AA intake can maximise growth

potential in young animals and prevent diseases (e.g. obesity, diabetes, necrotizing enterocolitis, and intrauterine growth retardation) in both animals and humans [9]. Declaring the AA composition and content in animal feeds is mandatory in many countries. AAs are also monitored by means of official analysis [10].

In this paper, we will analyse the significance of AAs in the diet from the aspect of animal growth, health and meat quality, especially pork and poultry. The methods for determination of AAs in animal feed will be discussed, as well as their advantages.

2. Effects of amino acids on meat quality

Different kinds of AAs play an important role in meat flavour. Adding AAs to animal feed can improve meat flavour [13]. The following AAs play a key role especially for the pork meat flavour: Trp, Thr, Arg, Lys and Leu. L-Trp is regarded as the third most important AA additive in animal feed after Lys and Met. Studies have shown that Trp improves the quality of pork meat by decreasing stress [14]. Stress before slaughter can affect meat quality and result in pale and soft meat. Trp reduces stress by stimulating the secretion of serotonin in the brain, which could be beneficial for the improvement of the pork quality. Thr, another EAA, is used for muscle protein synthesis [15]. Supplementation of Thr in the feed significantly increased the growth and daily gain. Arg is an important AA for protein synthesis. Supplementation of 1% Arg increased the body weight gain by 6.5% and the carcass skeletal-muscle content by 5.5% while decreasing the carcass fat content by 11% [13]. Lys increases appetite in animals, increases the resistance of livestock to different diseases and participates in fat metabolism. Leu is an EAA that must be supplied by the diet. Leu regulates the intracellular signal pathways of muscle cells, thus enhancing protein synthesis in mammalian skeletal muscle [16]. Based on this knowledge, it can be concluded that AAs play a significant role in improving the quality of meat.

2.1. Amino acids in poultry nutrition

Just like most animals, chickens need AAs as a source of protein for growth of muscles and development. The most important AAs for poultry are Arg, Lys, Met, Cys, and Trp. These are an absolute must to be included in poultry feed and play a critical role in the health of the birds. Non-EAAs such as Gly, His, Leu, Ile, Phe, Thr, and Val are often included in feed by several poultry feed brands and are believed to be important for chicken production. All deficiencies of EAAs result in retarded growth or reduced egg size or egg production [17].

A mixture of several AAs such as Cys, Gly, proline (Pro) and Glu, which are synthesized from pre-existing AAs (including EAA) by birds and had previously been thought to be non-EAA in chicken nutrition, was used in dietary formulations to yield better growth performance. There are many defined standards on the AA content of chicken diets during the first three weeks of their lives [11]. Reference values are given in the Dean and Scott Standard, the Huston and Scott Reference Standard, the modified Sasse and Baker Reference Standard, and Baker and Han's Ideal Chick Protein [12]. Common to all these standards that define the AAs in chicken diets are that their diet includes: (a) all EAA that are not synthesized by chickens; (b) several AAs (Cys, Glu, Gly, Pro, and Tyr) that are synthesized from either EAA or α-ketoglutarate plus ammonia by animals to various extents; and (c) no data on alanine (Ala), aspartate (Asp), asparagine (Asn), Gln, or serine (Ser) [1]. A few recent publications have challenged the NRC recommendations for AAs as being inadequate for current poultry strains. The Lys required each day by a white-egg-laying hen is 690 mg, or 0.69 g. Thus, the diet of a white-egg-laying layer eating 100 g of feed per day should have a Lys content of 0.69% [6].

Chickens have a high basal metabolism and require strictly balanced meals assembled from energy-rich foods [18]. Period of growth and gender can play an important role in defining the ratio of AAs in feed. Extensive studies with Lys, for example, have shown that males have higher requirements than females. Numerous studies have shown that only Lys is needed at a higher content in animal feed to achieve maximum weight gain in male poultry. That the Lys requirement is affected by gender but other AAs are not affected adds a complicating factor to use of ideal ratios for broiler chicks. Thus, for separate-sex feeding, female chicks having a 10% lower Lys requirement than male chicks means that

females would need to have ratios (to Lys) for all other indispensable AAs adjusted upwards by approximately 10%. The simplest solution to this gender difference in ratios is to use the male (gain:feed) requirement for Lys together with the male ideal ratios, i.e., for both sexes. The NRC model [6] shows these decreasing requirements in three growth periods: starter phase (0–3 weeks), grower phase (3–6 weeks) and finisher phase (6–8 weeks) [19]. Table 1 gives the AA requirements of poultry.

Table 1. Amino acids requirements of immature Leghorn-type chickens as percentages [6]

		White-Egg Laying Strains				Brown-Egg Laying Strains			
Amino acids	Unit	0 to 6 weeks	6 to 12 weeks	12 to 18 weeks	18 weeks to first egg	0 to 6 weeks	6 to 12 weeks	12 to 18 weeks	18 weeks to first egg
Lys	%	0.85	0.60	0.45	0.52	0.80	0.56	0.42	0.49
Met	%	0.30	0.25	0.20	0.22	0.28	0.23	0.19	0.21
Met +Cys	%	0.62	0.52	0.42	0.47	0.59	0.49	0.39	0.44
Thr	%	0.68	0.57	0.37	0.47	0.64	0.53	0.35	0.44
Val	%	0.62	0.52	0.41	0.46	0.59	0.49	0.38	0.43
Arg	%	1.00	0.83	0.67	0.75	0.94	0.78	0.62	0.72
Trp	%	0.17	0.14	0.11	0.12	0.16	0.13	0.10	0.11
Ile	%	0.60	0.50	0.40	0.45	0.57	0.47	0.37	0.42
Leu	%	1.10	0.85	0.70	0.80	1.00	0.80	0.65	0.75
His	%	0.26	0.22	0.17	0.20	0.25	0.21	0.16	0.18
Phe+Tyr	%	1.00	0.83	0.67	0.75	0.94	0.78	0.63	0.70
Gly+Ser	%	0.70	0.58	0.47	0.53	0.66	0.54	0.44	0.50
Phe	%	0.54	0.45	0.36	0.40	0.51	0.42	0.34	0.38

2.2. Amino acids in pig nutrition

Over the past two decades, there have been successful attempts to refine the patterns of some AAs in diets for lactating sows and pigs by addition of Arg, Gln, Glu, Pro, or Gly, or by determining mammary gland growth or changes of whole-body AA composition [1]. The AA composition in grower pig diet is listed in Table 2.

Table 2. Amino acid composition for grower pigs [6,7]

Amino acids	Content of AAs as a ratio of Lys (%)
Lys	100
Met	-
Cys	-
Thr	62
Val	68
Arg	42
Trp	19
Ile	54
Leu	102
His	32
Phe+Tyr	94
Met+Cys	57

AAs have been classified traditionally as nutritionally essential or nonessential based on growth or nitrogen (N) balance of animals. Nutritionally EAAs are those AAs with carbon skeletons that are not synthesized *de novo* and those AAs that usually are not synthesized in adequate amounts to meet the animal's needs and, therefore, must be provided in diets to sustain life [20].

Each body protein has its individual specific AA composition, turnover rate and maintenance needs, and the rate and priority of development of each proteinaceous tissue also changes, along with the other body tissues, as the animal grows. The AA composition of the whole body thus reflects the accumulated AA composition. The total quantity of AAs retained in all body components relates to a particular stage of development, but does or reflect the transfer between and within the various proteinaceous tissues or the pig's maintenance needs.

2.3. Amino acids in ruminant nutrition

Free AAs are not recommended as supplements in ruminant diets because they are degraded rapidly in the rumen. Balance must be achieved so that AAs protected from ruminal degradation are still available for intestinal absorption. These compounds should be stable, both when pelleted and when incorporated into silage-based total mixed rations in which the pH of corn silage can be as low as 3.6 [21].

3. Analytical techniques for the determination of amino acid composition

Due to their numerous implications in biological processes, AAs have been studied for decades. Before the appearance of mass spectrometry, numerous efforts were made to achieve the best possible resolution of AAs in complex matrixes using derivative reagents. Except for Tyr, Trp, and Phe, which are all AAs with an aromatic ring that allows them to be determined by UV spectroscopy in their native form, most of the AAs do not possess chromophore groups. Derivatization is, therefore, required for these compounds to convert them into a form suitable for UV or fluorescence detection. Since the discovery of the ninhydrin reaction for AA derivatization some decades ago, significant method developments have been achieved using a plethora of derivatization reagents. Briefly, the derivatization procedure can be performed using three main approaches: (i) the post-separation derivatization mode, commonly used in liquid chromatography (LC), (ii) the pre-separation mode used in (LC), gas chromatography (GC) and capillary electrophoresis (CE), and (iii) the in-capillary mode, restricted to CE [22].

3.1. Capillary electrophoresis

CE is a microanalytic technique for separating AAs. Its advantages over the high performance liquid chromatography method (HPLC) are its speed of application and the fact that gradient elution is unnecessary [23]. CE using indirect UV detection was applied for the analysis of nine AAs (Asp, Glu, Cys, Tyr, Asn, Pro, Gln, Leu and Try). AAs were prepared by dilution in distilled water from their powder forms, to a concentration of 150 μg/ml, for method development and reproducibility studies [24].

3.2. NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy uses the magnetic properties of the nucleus of the atoms for analysis and is one of the most powerful analytical methods for determining organic and inorganic compounds [25]. The complexity of the equipment and the price of the instrument mean this method is rarely applied for routine determination.

3.3. Gas chromatography

Different methods based on GC were employed for determination of AAs in heterogeneous samples, as well as in animal feed. GC-MS is one of the commonly used methods in AA analysis. The advantages of GC-MS over other chromatographic methods are high resolution and excellent productiveness. The main limitation of this method is that AAs are not volatile compounds, so they have to be transformed by chemical derivatives into vaporous compounds before analysis [26].

3.4. Liquid chromatography

High performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) with ultraviolet (UV) and fluorescence (FL) detection are commonly used for determination of

AAs [27-29]. UPLC is a suitable method for determining 17 AAs in low and high protein feed. UPLC is increasingly being utilized due to its rapid separation of AAs in approximately 35 minutes compared with 2 hours for a typical ion exchange chromatography (IEC) analysis. As mobile phases, they used mixtures of trifluoroacetic acid and water in different relationships, a mixture of methanol and water, acetic acid and acetonitrile. The derivative agents most commonly used are *ortho*-phthalaldehyde (OPA), (9H-fluoren-9-yl)methyl chloroformate (FMOC), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and phenyl isothiocyanate (PITC) [30]. Alternative methods have been developed to increase the selectivity. Within the last 15 years, LC-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) was developed for AA analysis. LC-ESI-MS/MS methods have been published for the analyses of underivatized or derivatized AAs. LC-ESI-MS/MS methods compare well with established AA analytical methods, although there are limitations with the measurement of some target analytes. Zhang et al [31] indicate that three methods, HPLC post column, UPLC and LC/MS/MS give similar performance (100% recovery) for most AAs.

3.5. Amino analyser

Amino analyser has become a widely accepted analytical technique for determining the AA composition in various research domains. The AAs are separated by IEC with a visible wavelength detector (IEC-VIS) and determined by reaction with ninhydrin using spectrophotometric detection at 570 nm (440 nm for proline). This method has been the most widely performed method for several decades and is still commonly used. This method is official by Commission Regulation (EU) No 152/2009. IEC requires derivatization of AAs, which can be performed by post-column derivatization using ninhydrin. Post-column ninhydrin derivatization has been the preferred method for many years by a majority of laboratories. Most systems are capable of resolving and quantifying roughly 40 AA peaks in a typical sample [32]. It is very important that the analysis of AA composition is simple and rapid. The quantitation of AA in matrices should be performed in relation to known reference or calibration standards. IEC-VIS can determine all AAs, with the exception of Trp, which is determined by HPLC with fluorometric detection.

4. Conclusion

The requirements for AAs in animal feeds are well defined in various sets of recommendations such as those of NRC. Requirements vary depending on the species and age of animals. AAs should be supplied either in the form of protein or crystalline AAs in feed to meet animals' requirements.

On the other hand, AAs affect not only the quality of meat and animal products as valuable foods, but they also contribute to food's colour, flavour and aroma. The content of AAs and quality of proteins in the meat of animals largely depends on AA content and ratios in feed. Also, the optimal content and ratio of AAs in animal feed depends on various factors, for example of the type, age and sex of the animals. Considering the significance of the animal feed industry, and the growing need for healthy and protein-rich food, it is necessary to focus all available resources in the development of reliable and accurate analytical methods for assessing the quality of food for nutritional and regulatory purposes.

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Potential utilization of emulsion gels and multiple emulsions as delivery systems to produce healthier meat products

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Potential utilization of emulsion gels and multiple emulsions as delivery systems to produce healthier meat products

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Abstract. In recent years the increase in consumers' demands for healthy food have accelerated the studies searching for innovative approaches in meat product formulations. Developing a healthier lipid profile and reducing fat are the most important goals in the meat industry. One of the main problems of animal fat replacement with plant oils is maintaining the technological and sensory properties of the products. Pre-emulsions provide a great opportunity to carry the healthier plant oils to meat systems for increasing mono and polyunsaturated fatty acid content, since adding liquid plant oils directly to product formulation can have technological and sensory problems. Using emulsion gels and multiple emulsions prepared with polyunsaturated oils could be a good option to achieve healthier meat products. This review addresses the emulsion gel and multiple emulsion properties and their use in meat products as fat replacers.

1. Introduction

Meat is a valuable food that should be included in the daily diet due to meat's biologically active proteins and essential minerals. Fresh meats are processed into meat products using various technologies and formulations to extend their shelf life and diversify consumption. Processed meat and poultry products like fermented sausages, emulsion-type sausages, and patties contain high amounts of saturated fat and cholesterol. However, it is very well known that excessive saturated fat intake has been associated with various chronic diseases, such as obesity, hypertension, and an increased risk of cardiovascular diseases [1,2] The increase in consumers' awareness of meat consumption and health has prompted the meat industry to develop healthier product formulations. Fat reduction and modification of fatty acids are the most important current approaches to the development of healthier meat product formulations [3].

For this purpose, scientists are reformulating meat products by reducing animal fat, improving the n-6/n-3 ratio, and increasing the amount of unsaturated fatty acids, to produce products with quality criteria are equivalent to products produced with standard methods [1]. It is possible to use those oils of plants and/or animal origin that contain high levels of unsaturated fatty acids, instead of saturated fat from animals. However, considering the product quality, reducing fat, and using plant oils instead of animal fat in the formulation is not a simple task. The fat used in meat products affects the technological quality and sensory properties such as flavor, juiciness, texture, and mouthfeel. Furthermore, liquid oils containing high levels of unsaturated fatty acids can cause undesirable flavor changes [4] and the formation of toxic compounds in the products due to their susceptibility to oxidation [5].

Thereby, to overcome the mentioned disadvantages, novel lipid modification strategies to stabilize and structure highly unsaturated liquid oils have been investigated. Structuring highly unsaturated oils exhibits a more feasible way of maintaining reformulated systems with properties equivalent to the meat products formulated with saturated fat [1,6,7]. Within this framework, the novel emulsion gels and multiple emulsion systems are emphasized to provide considerable advantages since they could provide structural reinforcement and thereby improve the stability, texture, and functional properties [7,8,9,10,11,12]. They could also improve oxidative quality [13]. The addition of structured oils to meat product formulations increased the textural, sensory, and technological properties and provided better

oxidative stability [14]. The main objective of this review is to demonstrate the properties of emulsion gels and multiple emulsions which can be used to reduce fat and modify the fatty acid composition of meat products, resulting in healthier formulations.

2. The use of gelled emulsions in meat products

Emulsion gels are emulsion systems with a gel network structure and solid mechanical properties as seen in Fig. 1 [8,15]. Production is carried out in two stages. In the first stage, the oil and water phases are emulsified with various proteins. Then, the liquid emulsion becomes viscous with the effect of clumping of emulsion droplets or gelling of the continuous phase as a result of heating, enzyme, or acidification processes [16,17]. An oil-in-water emulsion with a low viscosity continuous phase exhibits liquid-like properties unless it contains high amounts of oil. However, if the continuous phase is a viscous liquid or gel, the emulsion will also have viscoelastic behavior, indicating that the gel emulsions will also affect the texture of the product to which they are added [18]. Numerous proteins from milk, soy, and egg have been used in protein-stabilized emulsion gels, in which heat treatment, acidification, and enzyme treatment (trans-glutaminase) are the main protein gelation methods [16].

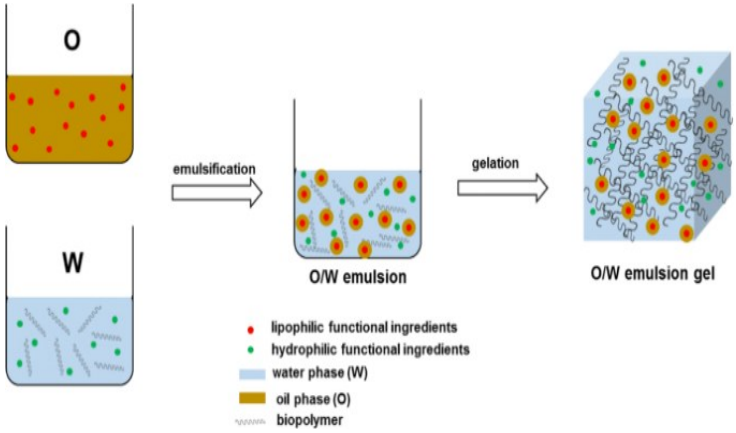

Figure 1. Preparation of emulsion gels (Lu et al., 2019)

In the production of emulsion gels, hydrocolloids and polysaccharides can be used as thickeners and stabilizing agents (apart from proteins) or because of their positive effects on health [19,20,21]. This feature of emulsion gels allows us to classify them as a method that can be used to solve the quality problems that arise when oil is used instead of animal fat in the formulation. With the addition of emulsion gel to the formulation, healthier product production is ensured, while the shelf life of the product is extended without changing the quality of the product.

The use of emulsion gels in various meat products has made it possible to obtain products with reduced fat content and improved fatty acid profile and n-6/n-3 ratio. Total fat reduction of up to 30% could be achieved in heat treated fermented sausages by replacing animal fat with hot-set emulsion gel formulated with linseed and peanut oil. Similar studies reported significant fat reductions in beef patties [22] and Bologna sausages [13] that were formulated with different emulsion gel systems as animal fat replacers. Table 1 shows the effects of using emulsion gels in meat product formulations. When the results of studies are examined, it is seen that using emulsion gels prepared with various plant oils in meatball and sausage formulations resulted in products with desired quality and technological properties [8, 20,23, 24]. Aleandre showed that when the pork fat of fermented sausage was replaced with an emulsion gel prepared with carrageenan and flaxseed oil, the energy value was decreased, the polyunsaturated fatty acid composition improved, and the n-6/n-3 ratio was obtained as 10.20 [7]. Better understanding of the behavior of emulsion gels in meat systems is important to guarantee the quality of the final product [20]. Incorporating emulsion gels into formulation can improve the technological

properties such as cooking yield, water, and fat binding capacity of meat products. Moreover, emulsion gels could be a more suitable alternative than simple oil-in-water emulsions to achieve better characteristics such as higher water holding capacity, better texture, and lower cooking loss in model system beef emulsions [20], in chicken patties [8], and fresh chicken sausages [11]. In chicken patties, replacement of beef fat with emulsion gel at a level of 50% resulted in similar cooking characteristics with control patties that contained 100% beef fat [8]. Meat products with incorporated mono or polyunsaturated oils are exposed to oxidation and quality deterioration. Therefore, these oils should be protected to make them more stable against oxidative changes during processing and storage [25] In this respect, emulsion gels create an opportunity to incorporate healthy oils into meat products to protect the oils against oxidation by gelled structure. Replacement of beef fat totally by a hot-set emulsion gel system retarded oxidation due to the capsulated oil droplets in the gel matrix acting as a protective barrier [26]. A similar pattern was shown in fermented sausages produced with emulsion gel prepared with fig seed oil, and 100% replacement of beef fat showed a more protective effect against oxidation compared with 50% replacement level [27].

Table 1. The use of emulsion gels in meat product formulation

Product	Emulsion gel	Results	Referer
Model system meat emulsion	Extra virgin olive oil, inulin, gelatin	Decreased pH Higher L* and b*, lower a*values Improved technological properties (water holding, emulsion stability) up to 50% replacement Lower hardness, gumminess and chewiness	[20]
Frankfurter	Olive oil, chia seed/flour, MTG, alginate or gelatin.	Improved oxidative stability Healthier and technologically higher quality product	[24]
Chicken patties	Extra virgin olive oil, inulin, gelatin	Detrimental effect on all investigated cooking characteristics when beef fat completely replaced with gelled emulsion Higher b* values observed with respect to GE concentration Lower hardness, gumminess and chewiness Similar scores awarded by the panelists in all the treatments except sample that is formulated only emulsion gel	[8]
Model system turkey breast emulsion	Olive oil, inulin and gelatin	Higher L* and b*, lower a*values Low fat content Equivalent emulsion characteristics in terms of emulsion stability, water-holding capacity and cook yield Increased expressible fluid when GE used as completely lipid source	[28]
Low fat beef patty	Microalgal oil and carregenan	Reduction of energy value Lower n-6 and increased n-3 Lower oxidation Acceptable sensory scores	[29]
Fresh sausage	Olive oil, chia flour and oat bran	Lower energy value due to reduced fat content Enhanced mono- and poly- unsaturated fatty acid content Low lysine/arginine ratio (benefits for heart and blood pressure) Reduced cooking losses Acceptable sensory properties Higher Kramer shear force	[21]
Harbin sausage	Camellia oil, sodium caseinate and carrageenan	No changes in protein content and textural properties Increased moisture content, L* and b* Modified fatty acid composition Reduced lipid oxidation	[30]
Reduced-fat beef patties	Microalgal oil, carregenan and blackthorn (*Prunus spinosa* L.) branch extract	significant fat reduction and improved lipid profile Reduced lipid oxidation and higher antioxidant activity with the addition of extract No significant changes in color parameters	[22]
Bologna sausage	Soybean oil, soy protein isolate, chia flour,sodium caseinate, inulin	Softer structure with the elaboration of gelled emulsion Reduced saturated fatty acid content Higher stability of the formulations with the addition of emulsion gel Less red and yellow sausages with the addition of gelled emulsion More organized (more continuous network, compact structure), with loss of its spongy structure, and more uniform distribution	[13]
Pork burger	Chia oil, chesnut flour and gellan gum	Improved cooking characteristics and sensory properties Decreased hardness Higher hypocholesterolemic / hypercholesterolemic and lower atherogenicity and thrombogenicity values Increased lipid oxidation	[31]
Fresh chicken sausages	Black cumin and flax seed oil, sodium caseinate, inulin and gelatin	Increment in moisture and protein contents, a decrement in fat content Reduced technological properties Darker sausages with the increasing amount of gelled emulsion Texture of samples was softened while total saturated fatty acid content reduced up to 52.61%	[11]
Sausage	Peanut and linseed oil, egg white powder, inulin and gelatin	Decreased total fat, saturated fatty acid and cholesterol contents Healthier lipid composition and improved nutritional ratios Better technological and acceptable sensory and oxidative features Increased hardness due to solid like properties of gelled emulsion	[12]
Heat-treated fermented sausages	Peanut and linseed oil, egg white powder, inulin and gelatin	Decreased saturated fats and improved nutritional ratios Protected polyunsaturated fatty acids Increased hardness with the use of cold set emulsion gels Advantages in sensory scores and storage stability	[26]
Bologna sausage	Soybean oil, inulin, and soy protein isolate,	Reduction of fat and sodium Increase in fiber content and polyunsaturated fatty acid Lighter and less red sausages Denser network Softer, more elastic, cohesive and resilient samples with a higher intensity of lipid oxidation	[32]

3. The use of double/multiple emulsions in meat products

Double or multiple emulsions are called “emulsion of an emulsion” [33]. Multiple emulsions with low thermodynamic stability are emulsions that combine both oil-in-water (W/O) and water-in-oil (W/O) systems. These emulsions have three phases, two water-oil interfaces and two separate emulsifiers [34]. There are two multi-layer emulsion systems (Fig. 2), W1/O/W2 (water-in-oil-water) or O1/W/O2 (oil-

in-water-oil). In W1/O/W2 emulsions, there is an oil-soluble emulsifier to stabilize the inner water droplets, and a water-soluble emulsifier to stabilize the oil droplets in the outer water phase [35].

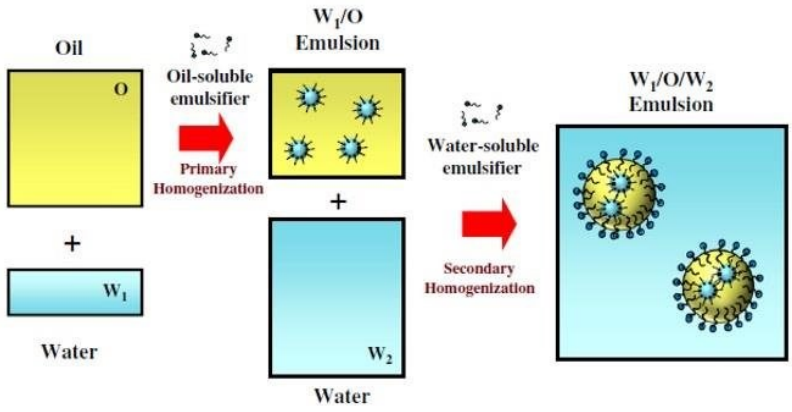

Figure 2. Preparation of W/O/W emulsions (McClements, 2012)

Replacing a significant portion of the oil in the droplets of an emulsion with small water droplets does not significantly alter the rheology unless the volume fraction of the outer droplets in a multiple emulsion is high. It is, therefore, a good option to use multiple emulsions for fat reduction applications in foods [36]. In meat products, W1/O/W2 emulsions are often used to reduce animal fat in the formulation, improve the fatty acid profile, reduce lipid oxidation, and encapsulate vitamins, minerals, bioactive and sensitive components [1, 37].

For this purpose, in various studies on meat products and model systems, sodium caseinate, whey protein, egg white powder, and beet juice were used as emulsifying agents, and olive oil, chia, perilla oil, and sunflower oil as oil sources. Thus, meat products with enhanced fatty acid profiles have been developed [34,37,38,39,40]. The effects of using double/multiple emulsions in meat products can be seen in Table 2. Model system beef emulsions produced with multiple emulsions prepared with olive oil and sodium caseinate had better stability and modified fatty acid composition compared with those produced with beef fat [34].

Table 2. The effects of using double/multiple emulsions in meat products

Product	W/O/W emulsions	Results	References
Sausage	Perilla oil or pork back fat, sodium caseinate	Modified fatty acid composition 60% reduction of fat content Similar thermal stability and higher processing loss Higher L* and lower a* value Equivalent hardness to reduced fat sausages Increased lipid oxidation	[39]
Model system meat emulsion	Olive oil and sodium caseinate	Decreased fat and increased protein content Enhanced fatty acid composition Reduced lipid oxidation	[34]
Model system meat emulsion	Olive oil and egg white powder	Increased mono- and poly- unsaturated fatty acid content Higher emulsion stability and water holding capacity Lower expressible fluid and fat	[40]
Pork patties	Perilla oil, sodium caseinate, gelatin, HXT	Less SFAs and higher proportions of LNA and ALA Increased Kramer shear force Greater oxidation values more than 1 after 3 days of Refrigeration More preferable by the panelists	[41]
Model system meat emulsion	Sunflower oil, whey protein isolate, beet juice	Elevated water and fat binding properties Reduction of caloric load and colour retention Softer structure however,more compact and better bound structure by the addition of emulsions with small droplets	[37]
Model system meat emulsion	Olive oil, flaxseed oil, fish oil, sodium caseinate and olive leaf extract	Improved oxidative stability by the addition of extract High oleuropein retention High contents of ω3 More balanced ω6/ω3 ratio Improved binding properties and harder texture	[42]
Reduced-fat meat batter	Soybean oil, whey protein concentrate and *Murraya koenigii* berries extract	Higher emulsion stability and cooking yield, hardness, G′, G″, and η* values Prolonged antioxidant activity No negative effect on microstructural properties Higher L* and b* while a* value decreased Excellent water and fat binding properties and emulsion stability Higher value for hardness and chewiness	[43]
Meat emulsion	Canola oil,whey protein and xoconostle extract	Lower fat and higher protein content Generally lower hardness values Higher Total phenols, ABTS, and DPPH. Higher malonaldehyde concentration when double emulsion was above 10% at the beginning, however, no significant changes was observed at the end of the storage.	[44]

4. Conclusion

Emulsion gels and multiple emulsions are the carriers of healthy oils in meat product formulations. These emulsion systems prepared with vegetable oil, proteins, and hydrocolloids can replace animal fat. Numerous plant oils or marine oils have been used to partially replace animal fat in meat products. They can be used successfully in meat product formulations to reduce fat and provide fatty acid modification. Their effects on the technological and sensory quality of meat products vary depending on the proteins, hydrocolloids in the formulation and the character of the oil carried into the system.

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Use of linseed and coconut flours in chicken patties as gluten free extenders

Use of linseed and coconut flours in chicken patties as gluten free extenders

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Abstract. Patties were extended with gluten free flours (linseed flour: LF, coconut flour: CF and their combination: LC) at a level of 5%. Control sample (B) was formulated with the same level of breadcrumbs. Using gluten free extenders did not change the water holding capacity (WHC); however, improved cooking yield resulted ($P<0.05$). Similarly, LF, CF and LC patties had lower diameter reduction and thickness change compared to the control sample ($P<0.05$). Due to color differences between breadcrumbs and gluten-free flours, color values of patties were affected significantly by the extender type ($P<0.05$). LF patties had the lowest L* and b*, the highest a* values within all patty groups. Different trends were observed in TBARS values of patties during storage, but in any case, all patties had TBARS values lower than 2.0 mg MA/kg throughout the storage. No significant differences were observed in patties' sensory properties.

1. Introduction

Celiac disease is a major immune-mediated public health problem which is characterized by intestinal inflammation from gluten ingestion [1]. The prevalence of celiac disease is estimated at 0.7-1.4% of the population [2]. When consumed by a person with celiac disease, gluten triggers an autoimmune response that results in damage to intestinal villi, nutritional deficiencies, cancer and other autoimmune diseases [3]. The main precaution for a person with celiac disease is adhering strictly to a gluten-free diet, which means not only scratching gluten-containing raw materials from the diet but also all products that contain them [4].

Nowadays, chicken patties show great economic and industrial importance and are widely consumed. This type of product contains different binder and extenders to improve stability, water holding capacity (WHC) and sensory properties, to entrap lipids and reduce the cost [5]. Moistened or dried breadcrumbs are usually used as binders and extenders in patties [6]. However, use of breadcrumbs obtained from gluten-containing products in patties is a limiting ingredient for people with celiac disease. For this reason, gluten-free alternatives could be better options for patty formulations.

Linseed (*Linum usitatissimum* L.) has become an emerging functional ingredient in recent years because of its fatty acid composition, phytochemicals, fiber content, and potential health benefits [7]. Previous studies have been carried out on the use of linseed oil and flour in different meat products [8]–[11]. Coconut (*Cocos nucifera*) is another emerging functional food due to its high fiber content, low

glycemic index, low amount of digestible carbohydrates, other nutrients, and it has no gluten [12]. Thus, coconut flour has become a good alternative for a healthy, gluten-free diet.

Even though different meat products containing coconut flour or linseed flour exist, no study was performed on these flours as breadcrumb replacers in chicken patties. Therefore, the main aim of this study was to investigate technological and oxidative effects of using coconut and/or linseed flours individually or their mixture as breadcrumb replacers in chicken patties.

2. Materials and Methods

Four different patty formulations were prepared as shown in Table 1. Chicken breast and skin were purchased from a local butcher, and breadcrumbs (Bağdat Baharat, Turkey), coconut flour (8.52 fat, 19% protein, 39% dietary fiber), linseed flour (4.13% moisture, 34,48% protein, 5.35% fat, 12.31% dietary fiber) were obtained from a local market. Chicken breast and chicken skin as a fat source were separately ground through a 3 mm plate with kitchen type grinder (Arnica, Turkey). Ground meat and skin, extenders (depends on formulation), salt and other ingredients were mixed for 1 min. Chicken patties were formed with stainless steel molds (d: 9 cm, h: 1.5 cm). After forming, the patties were cooked on an electric grill (Premier, Turkey) for 3.5 min each side at 180°C until the core temperature reached 72°C.

Moisture and ash contents of raw and cooked patties were determined according to AOAC procedures [13]. Protein content was determined using an automatic nitrogen analyzer (FP 528 LECO, USA) based on the Dumas method. Fat content was evaluated according to Flynn and Bramblet [14]. pH was measured in triplicate using a pH-meter (WTW pH 3110 set 2, Germany), equipped with a penetration probe. Cooking yield (CY) was determined by calculating weight differences for patties before and after cooking. To measure the diameter and thickness at the same locations before and after a cooking, three points on per patty were marked. The diameter and thickness of raw and cooked patties were recorded using Vernier calipers and calculated according to Serdaroglu & Demircioglu [15]. WHC was determined according to Hughes et al. [16]. Energy value (kcal/100 g) was calculated based on the European Parliament regulations [17]. Thiobarbituric Acid Reactive Substances (TBARS) value was measured according to Witte et al. [18]. A portable colorimeter (CR400, Konica-Minolta, Japan) was used to determine the surface color of the patties according to the Commission International d'Eclairage (CIE) standards. Patties were randomly assigned for sensory evaluation, were served warm to a ten-membered panel (graduate students of Ege University Food Engineering Department), and were subjected to sensory evaluation for appearance, color, texture, fattiness, juiciness, flavor and general acceptability. The experiment was performed twice, all analyses were carried out in triplicate, and the data was evaluated by one way and two-way analysis of variance (ANOVA) using SPSS software version 23.0. Differences among the means were compared using Duncan's Multiple Range Test.

Table 1. Formulation of chicken patty samples

Sample	Chicken meat (%)	Chicken skin (%)	Breadcrumbs (%)	Linseed flour (%)	Coconut flour (%)
B	81.5	10	5	-	-
LF	81.5	10	-	5	-
CF	81.5	10	-	-	5
LC	81.5	10	-	2.5	2.5

* Sample denomination: B (chicken patties formulated with breadcrumbs), LF (chicken patties formulated with linseed flour), CF (chicken patties formulated with coconut flour), LC (chicken patties formulated with coconut and linseed flour).
** 1.5% salt, 1% onion powder, 0.5% garlic powder, and 0.5% black pepper powder added to all of chicken patties

3. Results and Discussion

Chemical composition of raw and cooked patties in terms of moisture, protein, lipid and ash contents, total energy and pH values are shown in Table 2. Total moisture, protein, fat and ash in raw samples ranged between 67.15-67.89%, 5.49-6.43%, 16.90-17.40%, 2.41-2.86%, respectively. No significant differences were observed in moisture and protein contents of patties. The highest fat content was found in LC patties, which could be related to the oil content of coconut and linseed flours. The lowest ash content was found in B sample ($P<0.05$). The pH of the patties was between 6.16-6.26, and addition of linseed flour and coconut flour increased the pH of the patties.

Total moisture, protein, fat and ash in cooked patties were between 63.47-64.83%, 6.47-6.93%, 19.32-21.75%, 2.77-3.21%, respectively. Higher moisture content was recorded for CF samples compared to B after cooking, even though WHCs of these groups were similar ($P<0.05$). This behavior might be the result of better water absorption ability of coconut flour. Similar findings were recorded by Afoakwah et al. [12]. No differences were recorded among the fat contents ($P>0.05$). The lowest protein content was found in CF while LC had the highest protein content ($P<0.05$). Similar to raw patties, in cooked patties, the lowest ash content was found in B while higher ash contents were observed in LF, CF and LC samples ($P<0.05$). The energy contents of patties ranged between 161.26-167.87 kcal/100 g, and the lowest energy content was found in CF ($P<0.05$). In a previous study, blending coconut flour with wheat flour resulted in lower energy values compared to 100% wheat flour [19]. The pH of cooked patties ranged between 6.46-6.50, and the highest pH value was observed in CF treatment ($P<0.05$), while the rest of the patties had similar (lower) pHs ($P>0.05$).

Table 2. Chemical composition of raw and cooked chicken patties

Sample	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Energy (kcal/100 g)	pH
Raw						
B	67.80±0.47	5.49^{c}±0.01	17.25±0.52	2.41^{c}±0.02	146.22^{b}±1.86	6.16^{c}±0.01
LF	67.58±0.19	6.16^{b}±0.10	16.90±0.73	2.86^{a}±0.07	149.01^{ab}±1.41	6.25^{b}±0.01
CF	67.68±0.58	6.24^{b}±0.11	17.10±0.23	2.58^{b}±0.09	150.14^{a}±2.40	6.26^{a}±0.01
LC	67.15±0.24	6.43^{a}±0.07	17.40±0.61	2.83^{a}±0.04	152.21^{a}±0.74	6.18^{c}±0.01
Cooked						
B	64.07^{b}±0.19	6.93±0.55	20.12^{bc}±0.05	2.77^{d}±0.02	167.31^{a}±3.01	6.48^{b}±0.01
LF	64.48^{ab}±0.40	6.80±0.30	21.06^{ab}±0.87	2.85^{c}±0.03	164.70^{ab}±3.24	6.46^{b}±0.01
CF	64.83^{a}±0.04	6.47±0.51	19.32^{c}±0.32	2.95^{b}±0.07	161.26^{b}±2.11	6.50^{a}±0.01
LC	63.47^{c}±0.19	6.91±0.30	21.75^{a}±0.40	3.21^{a}±0.02	167.87^{a}±1.96	6.47^{b}±0.01

All values are means ± SD of three replicates.
Means within the same column with different superscripts (a-d) are different

Technological characteristics and color parameters of patties are summarized in Table 3. WHC of patties was between 93.81-94.90%, and no significant differences were found ($P>0.05$). This might be the result of similar moisture and protein contents in the patties, since moisture/protein ratio is an important parameter for WHC of meat products [20]. Use of gluten-free extenders in chicken patty formulations increased the cooking yield significantly even though the WHC of the patties was similar ($P<0.05$). Therefore, it could be said that linseed and coconut flours might help to retain fat within the matrix of during the cooking process [21, 22]. Significant differences in diameter and thickness changes among the extenders were identified ($P<0.05$), patties extended with breadcrumbs had highest values, and no difference was shown among the patties formulated with other extenders. Similar results were observed by Cocaro et al. [23] in chicken burger patties where linseed flour was used.

Color parameters (L*, a*, b*) were affected by the extender type ($P<0.05$), and this finding may be attributed to the different colors of the extenders. L*, a*, and b* values of patties ranged between 45.86-61.78, 0.68-4.55, and 16.40-22.83, respectively. Use of LF resulted in darker colored chicken patties while CF and B patties were similar ($P>0.05$). This observation is supported by the work of Hautrive et.al [24], who found that linseed flour in hamburgers resulted in darker and more reddish products.

Yellowness of LC patties was similar to that of B patties (P>0.05). CF patties had the lowest a* and the highest b* values within all treatments, due to the characteristic white color of coconut flour (P<0.05). Ayandipe et al. [20] reported that using coconut flour in chicken sausage as a filler resulted in higher L* and lower b* values.

Table 3. Technological characteristics and color parameters of patties

	Technological characteristics				Color parameters		
Sample	WHC (%)	CY (%)	DR (%)	LT (%)	L*	a*	b*
B	$93.81^{a}\pm0.67$	$86.25^{c}\pm1.41$	$13.75^{a}\pm1.41$	$4.18^{a}\pm0.46$	$60.59^{a}\pm0.82$	$1.54^{c}\pm0.21$	$19.04^{b}\pm0.61$
LF	$94.90^{a}\pm0.38$	$91.61^{ab}\pm0.41$	$8.38^{b}\pm0.41$	$1.81^{b}\pm0.50$	$45.86^{c}\pm0.44$	$4.55^{a}\pm0.16$	$16.40^{c}\pm1.01$
CF	$94.20^{a}\pm0.87$	$92.09^{a}\pm0.45$	$7.90^{b}\pm0.45$	$1.40^{b}\pm0.26$	$61.78^{a}\pm0.53$	$0.68^{d}\pm0.16$	$22.83^{a}\pm0.32$
LC	$94.13^{a}\pm0.42$	$90.08^{b}\pm1.08$	$9.38^{b}\pm0.55$	$1.34^{b}\pm0.03$	$52.66^{b}\pm0.79$	$3.40^{b}\pm0.07$	$18.15^{b}\pm0.30$

All values are means ± SD of three replicates.
Means within the same column with different superscripts (a-d) are different
WHC: water holding capacity, CY: cooking yield, DR: diameter reduction, TC : thickness change

Lipid oxidation is one of the main chemical changes that results in physical, sensory and nutritional quality deterioration in meat products. TBARS values of the chicken patties are shown in Table 4. Initial TBARS values ranged between 1.09-1.86 mg MA/kg. As presented in Table 4, extender type and storage time had significant effects on TBARS values. Initial TBARS values of B and CF treatments were similar and lower than in LF and LC treatments (P<0.05). Similar to our results, Hautrive et al. [24] stated that using linseed flour as a pork backfat replacer in hamburgers can increase TBARS values, probably due to the high polyunsaturated fatty acid profile of linseed flour. Samples incorporated with breadcrumbs had similar TBARS values until day 4, but however, significant increment was observed on day 6 (P<0.05). In contrast, TBARS values of LF decreased throughout the storage and were significantly lower compared to day 0 (P<0.05). The highest TBARS values of CF and LC samples were found on day 4. After that, significant decrement was observed in these samples, probably the result of decomposition of secondary lipid oxidation products (P<0.05). At the end of storage, CF patties had the lowest TBARS value (P<0.05), while other samples had similar (higher) values (P>0.05). In any case, all patties had TBARS values lower than 2.0 mg MA/kg throughout the storage. Any changes in product formulation can affect flavor, mouthfeel, and texture of the meat products, and in some cases, can greatly alter the sensory characteristics.

Sensory properties of chicken patties are shown in Figure 1. Appearance, color, texture, fattiness, juiciness, taste, and general acceptability of the samples ranged between 6.88-7.47, 6.76-7.35, 6.80-7.40, 6.72-6.78, 6.20-6.80, 6.42-7.3, and 6.76-7.34, respectively. The sensory properties of patties were found similar to each other (P>0.05), in contrast to Cócaro et al. [23]. All patties had acceptable scores, and this finding is an indicator of the promising potential of using as extenders linseed and coconut flours in gluten-free chicken patties.

Table 4. TBARS values of samples (mg MA/kg)

	Storage Time (Days)			
Sample	0	2	4	6
B	$1.08^{b,Y}\pm0.03$	$0.92^{d,Z}\pm0.01$	$1.03^{c,Y}\pm0.05$	$1.53^{a,X}\pm0.05$
LF	$1.86^{a,X}\pm0.03$	$1.79^{a,XY}\pm0.05$	$1.63^{b,Y}\pm0.22$	$1.48^{a,Z}\pm0.05$
CF	$1.12^{b,Y}\pm0.02$	$1.15^{c,Y}\pm0.01$	$1.91^{a,X}\pm0.09$	$0.97^{b,Z}\pm0.02$
LC	$1.85^{a,X}\pm0.01$	$1.56^{b,Y}\pm0.01$	$1.85^{ab,X}\pm0.05$	$1.58^{a,Y}\pm0.07$

All values are means ± SD of three replicates.
Means within the same column with different superscripts (a-d) are different
Means within the same row with different superscripts (X-Z) are different

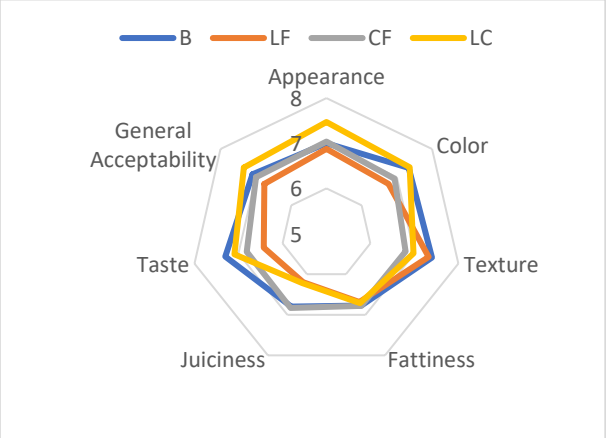

Figure 1. Sensory properties of chicken patties.

4. Conclusion

The present study indicates that use of linseed flour, coconut flour or their combination as extenders in chicken patties presented favorable impacts on technological and sensory characteristics. The physical properties suggested that cooking yield was lower and changes in diameter and thickness were higher in patties formulated with breadcrumbs. Sensory analysis showed that extender type did not negatively affect the evaluated parameters. As a result, using linseed and coconut flours as breadcrumbs replacer in chicken patty formulation could have a great potential in gluten-free diets for people with celiac disease without the patties undergoing technological and sensorial deterioration.

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Estimation of fat cover of bovine carcases by means of computer vision system (CVS)

Estimation of fat cover of bovine carcases by means of computer vision system (CVS)

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Abstract. The aims of this study were to obtain percentages of meat and fat cover for SEUROP classification system reference images using a computer vision system (CVS) and to calculate classification intervals which could be used in the future for construction of cheap and easy to use classification devices for small slaughterhouses. Lowest percentages of fat cover were found for the first class marked as "low" (the lowest fat content) and they gradually increased to the last class marked as "very high" (the highest fat content). Based on the obtained results, decision making intervals were proposed. In the present study, classification only refers to classification of adult bovine animals based on fat cover.

1. Introduction

In 2013, global beef consumption remained at the same level (around 9.5 kg/capita) as it was in 1961 [1]. During the same time, the world population increased from around 3.3 billion to 7.3 billion with annual population growth rate of 1.2 % (from 2003 to 2013) [2]. As a result, world production of beef increased during 1961-2019 from 28.76 million tons to 72.60 million tons [1]. However, average beef consumption per capita in Serbia in 2017 was 5.15 kg while the number of cattle reduced from 1949 to 2017 from 1,946,000 to 899,000, respectively [3, 4]. For improvement of cattle numbers in Serbia, it is necessary to implement agro-economic policy and strengthen farmers' associations [5, 6]. In addition, it is necessary for beef producers to follow new trends on the market and try to differentiate their products on the basis of geographical origin and specific nutritional characteristics [7]. According to Grunnert et al. [8], competitiveness on developed food markets is associated with the ability of companies to develop new differentiated products that reveal differences in consumer preferences and move away from price-based competition.

In 1981 in Europe, Regulation No 1208/81 was brought into force. The regulation refers to classification of carcases of adult bovine animals, widely known as the SEUROP system [9]. A few months later, in order to ensure uniform classification, it was necessary to define carcase conformation and fat classes more precisely [10]. Briefly, regulations refer to classification of both, carcase

conformation and fat cover. For the classification of fat cover, the five following classes were defined: 1 – low (L) ("no fat within the thoracic cavity"), 2 – slight (S) ("within the thoracic cavity the muscle is clearly visible between the ribs"), 3 – average (A) ("within the thoracic cavity the muscle is still visible between the ribs"), 4 – high (H) ("the seams of fat on the round are prominent, the muscle between the ribs may be infiltrated with fat") and 5 – - very high (VH) ("the round is almost completely covered with fat, so that the seams of fat are no longer clearly visible, within the thoracic cavity the muscle between the ribs is infiltrated with fat") [10].

In the beginning, classification of beef carcasses was optional, but in 1995 it became mandatory [5]. At first, the regulation was used for the purpose of monitoring market prices, while today, it is used to form the price of cattle sold to the slaughterhouse [5]. However, it was difficult to establish a unique means of classification between different slaughterhouses in Europe because the classification was done manually by the food operators. Although visual classification has long been used by the industry, it is a method of subjective nature depending on the experience and intuition of the operator. Furthermore, this means of classification required greater manual labor, and consequently, it increased the production cost. Many years later, in developed countries, automated classification systems have been developed and applied by the industry. On the other hand, in developing countries, the SEUROP classification system is not being used, mostly because it requires manual labor and trained operators. In addition, automated systems are, in most cases, too expensive for small slaughterhouses.

In recent years, there have been numerous studies on the application of computer vision systems (CVS) in the analysis of different food products [11-17]. Milovanovic et al. [18] measured the color of twenty-seven different milks and milk products by means of CVS and found that the method was more reliable and accurate compared to traditional devices for color measurement. Nyalala et al. [19] reported a number of studies which investigated the possibility of weight and volume estimation of poultry and eggs using CVS-based methods. In addition, Pietro Cavallo et al. [20] used a computer vision-based method for quality evaluation of table grapes, which showed accuracy of 92% and 100% in classifying the cultivars Victoria and Italia, respectively. Apart from being more accurate, computer vision-based methods are usually non-destructive, which is one of the reasons for their increasing popularity.

The aims of this study were to obtain percentages of meat and fat cover of SEUROP classification system reference images using CVS and to calculate classification intervals that could be used in the future for constructing cheap and easy to use classification devices for small slaughterhouses. In the present study, classification only refers to classification of adult bovine animals based on fat cover.

2. Materials and Methods

2.1. Carcase images

The study was carried out on reference images for classification of carcases of adult bovine animals (see https://op.europa.eu/en/publication-detail/-/publication/248996ec-f513-4852-a2a5-6fd307692b0a). The images were downloaded and imported into Adobe Photoshop 2020 (Adobe Inc., San Jose, CA, USA), where they were cropped and converted into PNG format. Reference images for each class are provided for both the inner carcase part (in the thoracic cavity) and the outer carcase part (outside of the carcase) (Figure 1).

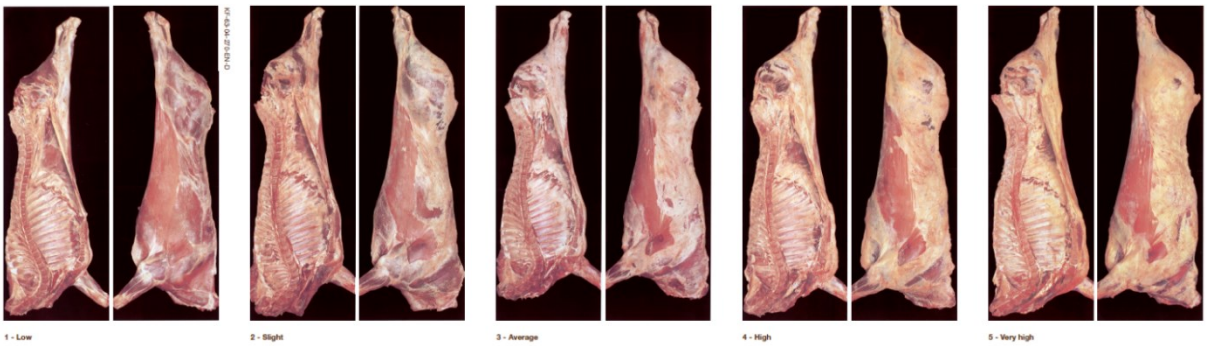

Figure 1. Reference images for classification of carcases of adult bovine animals

2.2. CVS analysis

As the background of the reference images is black and significantly differs from color of meat and fatty tissue, it was not necessary to change it. However, the images were imported into Adobe Photoshop 2020 (Adobe Inc., San Jose, CA, USA) software in which the color of the entire image was changed to black and white in the way that there was a clear color difference between meat and fat parts (Figure 2). This procedure was repeated for each of the reference images. The images were then imported into ImageJ (National Institutes of Health, Version 1.45 K) software which was used for the purpose of color segmentation, wherein three clusters were defined. The first cluster represented the color of meat, the second indicated the color of fatty tissue while the third referred to the background color.

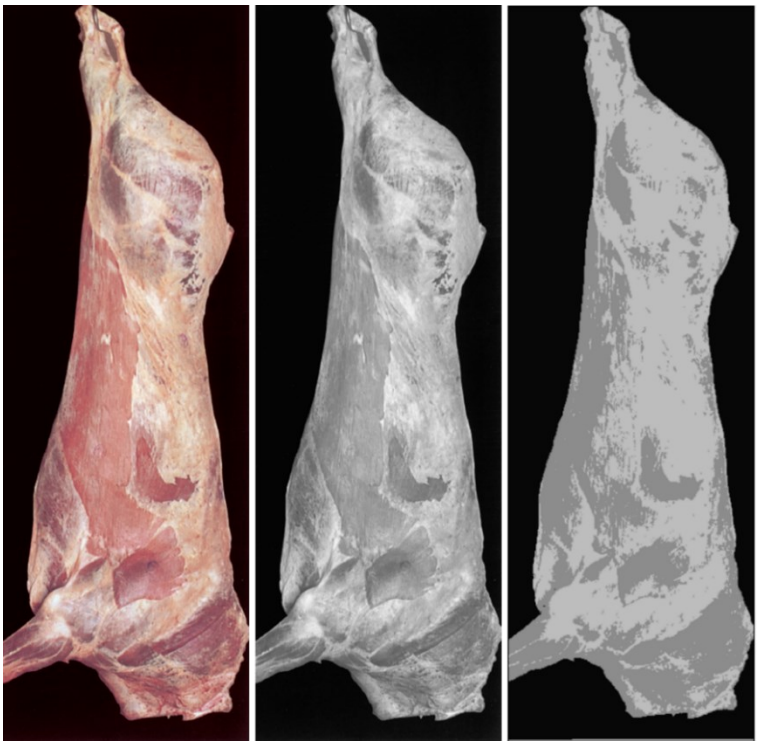

Figure 2. Computer vision analysis of bovine carcases: original image (left), black and white adjustments (middle) and color segmentation (right).

2.3. Statistical analysis

For the calculation of percentages of each cluster, percentages of fat cover of bovine carcases and proposed intervals for decision making, MS Excel (Microsoft, Redmond, WA, USA) was used.

3. Results and Discussion

Table 1 shows results of estimated fat cover of reference images for classification of adult bovine animals obtained by means of CVS. Lowest fat content was found for the first class i.e., carcase images with the lowest fat content in the thoracic cavity and on the outside of the carcase. Estimated fat content in both images, in the thoracic cavity and outside of the carcase, gradually increased from the first class marked as "low" to the last class marked as "very high". The highest difference in estimated fat cover between the following groups for images of the outside of carcase was observed between the first ("low") and the second class ("slight"), which showed fat content of 47.51% and 56.07%, respectively. On the other hand, the difference in fat cover of these two classes in images of the inner carcase part was 1.21%. Based on the percentages of fat cover obtained for each reference image, decision making intervals were proposed (Table 2). However, the main challenge in developing a mathematical model was classification of those carcases for which results of fat cover for inner and outer parts of carcase were not in accordance. It was observed that during CVS analysis of images representing inner carcase parts, the method has difficulties distinguishing between the color of bones and fatty tissue. This was especially obvious for the rib region, which was classified in the fat cluster in all of the analyzed images. For this reason, images of the outside of carcase should be of greater importance when it comes to classification. In the future, it is necessary to determine the importance of factors which would take into account the color similarity of bones and fatty tissue in order to facilitate decision making.

Table 1. Percentages (%) of meat and fat of bovine carcases in the thoracic cavity and outside of the carcass of reference images obtained by CVS

		Categories				
		L	S	A	H	VH
Outside of the carcase	Meat	52.49	43.93	40.23	38.97	36.42
	Fat	47.51	56.07	59.77	61.03	63.57
In the thoracic cavity	Meat	42.66	41.45	39.38	33.83	30.50
	Fat	57.36	58.54	60.62	66.17	69.50

As is the case with pork fatty tissue and pork, the tallow price is a few times lower than the beef price. Heifers naturally contain more tallow than bulls, which is the reason for their lower price. On the other hand, bulls are characterized by a high meat content, which is why they are more appreciated on the market. This indicates the economic importance of fat cover classification and why producers should improve meat yield. Implementation of fat cover classification would encourage producers to improve diets and breeding conditions in order to increase the meat content in their cattle, which would have positive economic effects for both producers and slaughterhouses. There are many studies focusing on development of different diets and breeding conditions for cattle in order to improve yield, growth performance and marbling [21, 22]. One of the important quality characteristics of beef that directly affects consumer purchase decisions is marbling [23]. Marbling is defined as "intramuscular fat and dispersion of fat within the lean meat" [24]. Liu et al. [24] investigated the correlation between European conformation and fat scores and marbling scores obtained by sensory panel. However, SEUROP fat scores only showed weak correlation with marbling ($P<0.001$; $r=0.45$), while conformation scores were not related with marbling.

Table 2. Proposed intervals in percentages (%) for different categories of carcase fat cover

		Categories				
		L	S	A	H	VH
Outside of the carcase	Meat	>48.21	48.21-42.07	42.08-39.59	39.60-37.66	<37.67
	Fat	<51.79	51.79-57.91	57.92-60.39	60.40-62.30	>62.30
In the thoracic cavity	Meat	>42.05	42.05-40.40	40.41-36.59	36.60-32.16	<32.16
	Fat	<57.95	57.95-59.57	59.58-63.38	63.39-67.83	>67.83

4. Conclusion

The study showed the possibility of CVS application in fat cover estimation of reference images for classification of bovine carcasses based on fat content. However, the limitation of this study was in the lack of a reference method for carcass classification based on which results could be compared. In addition, CVS showed difficulties in distinguishing between color of fatty tissue and color of bones and connective tissue. In the future, there is a need for development of a mathematical model that could be incorporated in classification software.

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Contribution of veterinary profession to the response to COVID-19 pandemic conveyed through experiences of the Veterinary Faculty Sarajevo

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Contribution of veterinary profession to the response to COVID-19 pandemic conveyed through experiences of the Veterinary Faculty Sarajevo

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Abstract. The International Organisation for Animal Health (OIE), from the onset of COVID-19 pandemic, promoted One Health in global and national responses. The OIE accentuated the role of the veterinary profession due to testing capacity of animal health laboratories and expertise. Veterinary Faculty Sarajevo through its Veterinary Institute participates in the national veterinary service with diagnostic and advisory roles. It has proactively enhanced the scope and quality of laboratories, including strengthening the interdisciplinarity and internationality. Development achieved through earlier pandemic threats resulted in having laboratory and technical facilities for molecular SARS-CoV-2 detection in the wake of the unveiling COVID-19 pandemic (early 2020). From confirmation of the first COVID-19 cases in Bosnia and Herzegovina (BiH), our staff participated in crisis response teams and, so far, held over sixty media addresses promoting public awareness and science based information. Our laboratories were included in the official detection system and were the first to sequence SARS-CoV-2, then to establish the Alpha COVID-19 variant in BiH human samples and to substantiate one-way virus transmission from humans to pets. The aim of this paper is to describe our activities as a participant in the response to the COVID-19 pandemic, alongside faced challenges and gained experiences.

1. Significance of veterinary profession in COVID-19 response

The dominant public image of a veterinarian is related to clinical practice dealing with small companion or farm animals, even though knowledge, skills and attitude gained through formal and continuous education form the basis for much broader contributions. Veterinary medicine education uniquely routes its graduates to comparative medicine, despite market forces driving it recently more towards companion veterinary medicine and thus sidelining the veterinary profession's foundational societal values [1]. Nevertheless, the veterinary profession has a longstanding and continuously expanding role in improvement of human and public health by improving animal production and welfare and health and food systems, participating in biomedical and comparative medical research, addressing zoonotic diseases, contributing to environmental and ecosystem health, and thus helping manage 21st century public health challenges [2, 3]. The veterinary profession is globally recognized as an invaluable asset due its public service in response to the current, and preventing future pandemics [4]. The current and potential contribution of public health veterinarians in combating COVID-19 includes expertize in epidemiological surveillance and disease management, laboratory capacities for diagnostics and

characterization of SARS-CoV-2 and other zoonotic pathogens, testing of human and animal samples for COVID-19, animal research and development of vaccines and transboundary disease risk assessment [3].

1.1. Experience and expertise with zoonotic emerging diseases

If we consider that most human infectious diseases are zoonotic in origin, out of the estimated 1.7 million unknown viruses of mammalian and avian hosts, 850,000 could have potential for animal to human spill-over [5]. The zoonotic provenance of SARS-CoV-2 is well documented, as is the potential role of animals in virus maintenance [3, 6]. Even before COVID-19, veterinary professionals gained vast experience in the emergence of viral diseases from wild animal populations and epidemics caused by emerging or exotic agents in naïve domestic animal populations. Global collaborative projects, such as PREEMPT, PREDICT and EPT, dealing with these emergencies thrived with the contribution of veterinarians. This led to veterinary epidemiologists being involved even as lead persons in COVID-19 task forces in many countries, while veterinary virologists experienced in virus evolution helped in identifying and predicting important SARS-CoV-2 mutations and their implications [3].

1.2. Animal disease surveillance toolkits

Policies and standards around "disease freedom status" in animal populations based on provisions in the Sanitary and Phytosanitary Agreement (SPS) of the World Trade Organisation (WTO), with the OIE as a globally recognised standards body, have been implemented for decades now. This led to development of a variety of tools and approaches in veterinary control strategies with proven success. Moreover, policy making regarding animal health is commonly required to be evidence based, leading to expansion of the contribution made by veterinary science and research. Hence, many of the COVID-19 measures, such as lockdowns and movement bans, differentiation of countries by disease status, health certification, risk-based sampling strategies, traceability, diagnostic protocol standardization and biosafety have long been globally harmonized and implemented by veterinarians in preventing and combating animal diseases and epidemics worldwide. Unfortunately, the aftermath of initially loosely harmonized and haphazardly introduced COVID-19 public health and containment measures between EU countries prompted approaches as seen earlier in the regulation of animal health. The EU had developed the Joint European Roadmap for coordinated phasing out of containment measures, as well as guidelines for standardized testing approaches [7].

1.3. Diagnosis and molecular characterisation of SARS-CoV-2

The lack of testing capacities and resources for COVID-19 diagnostics was evident worldwide, especially in undeveloped and developing countries with chronically neglected and under-capacitated health care systems. This, particularly in the early days of the pandemic, contributed to poor control of disease transmission. In line with guidelines and recommendations issued by the OIE, many veterinary laboratories were involved in testing human samples, but also supported development of common diagnostic and data exchange protocols and provided surplus personal protective equipment and other expendables [3, 8]. Information on human coronaviruses were lacking until the emergence of SARS-CoV and MERS-CoV, while veterinary medicine has extensive experience with animal coronaviruses, particularly regarding virus evolution and changes in tropism, virulence and inter-species transmission [9]. Veterinary medicine's contribution follows in our expanding knowledge on SARS-CoV-2 origin and spread, and in development of immunogenic and safe vaccines and effective antivirals [9].

2. Veterinary Faculty Sarajevo's role in veterinary public health

Veterinary Faculty Sarajevo is a recognized institution in the region, Europe and beyond, with tradition, expertise and capacities for veterinary education, support to the veterinary service, promotion of animal health and biomedical research. As the only higher education institution for veterinary medicine in the country, we provide education through degree programs or continuous education towards achieving high standard competences of our trainees in line with national and international demands and standards [10].

In addition, the Faculty has been continuously committed towards development of research, expertise and diagnostic capacities. Veterinary Faculty Sarajevo is involved in all regular and incident activities regarding endemic, exotic and emerging diseases in animal populations (domestic and wild), including animal disease transmitted by food/feed or vectors [11, 12, 13, 14, 15].

The Veterinary Institute of the Veterinary Faculty is a subsidiary unit that merges all our laboratory services within five Centres and with a separate unit for Inter- and Intra-laboratory quality control and standardization. The Institute's laboratories are accredited according to the BAS EN ISO/EC 17025:2018 [16] and are appointed as national reference laboratories for several important OIE listed diseases. The buildings of the Institute were purposefully designed, constructed and equipped during the last decade following the highest standards of biomedical laboratory safety (Figure 1 and 2). All laboratories within the Institute can operate under BSL2+ requirements, while infrastructure is in place for a BSL3 subunit. Our laboratories are integrated in the official system of animal disease and food safety control, supporting domestic trade and export certification, veterinary inspection official controls, and as subcontractor, for internal control for food/feed producers. Our "tailor made" laboratory information management system follows up all laboratory protocols and provides the basis for operational management, audits and reporting.

Picture 1: Processing samples at the Laboratory for Molecular-Genetic and Forensic Research within the Institute

Picture 2: Analysing viral genome sequences in the same Laboratory within the Institute

From the early 2000s, our Faculty recognized the emergence of needs for enhancing expertize in veterinary epidemiology, disease surveillance, disease diagnostics and risk analysis, towards strengthening the capacity to implement OIE standards by provisions of the SPS of the WTO. Our role in veterinary public health was particularly emphasized during the COVID-19 pandemic, since experiences and capacities developed through earlier pandemic threats resulted in ready available laboratory and technical facilities for molecular SARS-CoV-2 detection in the wake of the unveiling COVID-19 pandemic (early 2020).

3. Achievements and experiences of the Veterinary Faculty Sarajevo during the COVID-19 pandemic

From laboratory confirmation of the first COVID-19 cases in Bosnia and Herzegovina (5 March 2020), our staff participated in crisis response teams and, so far, held over sixty national and regional media addresses promoting public awareness and dissemination of evidence/science based information. Our laboratories were included in the official laboratory detection system, first to sequence SARS-CoV-2 [17], then to establish the Alpha SARS-CoV-2 variant of concern (B.1.1.7) in BiH human samples [18] and to confirm and substantiate one-way transmission of the virus from infected humans to pets [19]. Since international quality accreditation (ISO/EC 17025) is not required for human diagnostic

laboratories in BiH, at times, only our laboratory results for molecular COVID-19 diagnostics were acceptable as a requirement for travelling to Austria, Germany and China. Similarly, many national sport bodies and diplomatic offices in BiH choose our laboratory services in providing testing and test results for their international travel needs. However, the recognition received and achievements accomplished did not go hand in hand with necessary and expected support from the relevant government health authorities. Despite proven capabilities, capacities and expertize supporting the official response to the COVID-19 pandemic and even ongoing contributions to other important public health issues, veterinarians in BiH are faced with suspicion and prejudice, shown to various extents by policy makers, the medical community and the public. This discouraging fact tends to be a common experience of veterinary professionals elsewhere as well [3, 4]. Most of our investment in laboratory equipment, facilities, expendables and hiring and training of additional staff came from internal resources, either by self-financing or from self-negotiated donations and partnerships. As a public institution, we faced additional challenges due to public expenditure cuts, slow and administratively demanding public procurement procedures, an employment ban and lag of the public administration, even more accentuated during the pandemic and consequent economic crisis. The Veterinary Faculty managed to triple SARS-CoV-2 molecular testing capacities from March 2020. Currently we are capacitated to run 320 COVID-19 samples daily using PCR-based methods. In regards to whole genome sequencing, we are able to sequence up to one hundred samples per week.

Out of the annual laboratory workload, 80% was accomplished in the second half of 2020, which consequently put a strain on the laboratories but also on other departments. The Faculty simultaneously and promptly needed to adapt to new educational processes (online), and we managed to complete curriculum plans in full. Hence, the most valuable asset in facing and solving the above challenges were our human resources, academic, technical and administrative, people who managed through joint efforts to meet and surpass their tasks, despite facing personal and professional risks and uncertainties along with direct pandemic consequences.

4. Lessons learned

Even though the COVID-19 pandemic provided an opportunity for better general perspectives of the veterinary profession in BiH and strengthened the significance and positive image of the Veterinary Faculty Sarajevo, such perception could be lost without strategic follow-up. This crisis brought to light the preparedness, self-reliance and adaptability of many other veterinary institutions worldwide, but also underlined a chronic lack of recognition and underinvestment for effective integration of our capacity, knowledge and possibilities in public health services. The most profound pandemic effects lie in endangerment of global food security, and disruption of food chains and markets, whereas veterinarians are uniquely placed, not just for assisting in the first response, but for continuous roles in combating lasting and broad consequences of existing and new emergencies [20]. Food security is an essential cornerstone of health and economic growth and, thus, is undeniably linked to global security [21]. Even more important than recognizing external factors impeding the veterinary profession's public image, appreciation and significance, we are called to better exploit our internal strengths and capacities as well as to oversee the building of our future professional perspectives. The COVID-19 pandemic proved that interdisciplinary actions are an imperative, since only joining experiences, expertise and resources will prevent future pandemics and build capacities for socio-economic resilience and public health infrastructure in facing the challenges before us.

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Essential oils as natural additives in dry-fermented sausages

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Essential oils as natural additives in dry-fermented sausages

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Abstract. The usage of food additives is one of the key methods for inhibiting microorganisms' growth and delaying oxidative changes in dry-fermented sausages (DFS). However, they have numerous negative health effects, so the novel meat industry is oriented towards different natural alternatives while focusing on plant extracts, including essential oils (EOs). EOs are interesting since they are generally recognized as safe (marked as GRAS) and have a broad acceptance from the consumers. Their activity depends on numerous parameters including the method of extraction, concentrations and possible synergistic effects of their bioactive compounds. Various conventional (hydrodistillation and organic solvent extraction) and novel (microwave-assisted, ultrasound-assisted and supercritical fluid extraction) extraction techniques are being applied for EO recovery, and optimization of these process is an essential step towards cost-effective production of high-quality extracts. Generally, it can be concluded that EOs are added into DFS to delay or prevent lipid oxidation, retard microorganisms' growth, improve colour stability and extend the shelf-life.

1. Introduction

Dry-fermented sausages (DFS) have been produced in countless countries all around the world. Nowadays, consumers are becoming increasingly aware of these meat products for their significant health benefits and unique odour and flavour [1]. DFS are manufactured using meat (e.g. pork, beef) and fat, salts, spices, starter cultures and food additives [2,3]. Due to the relatively high fat content (20-30%), various raw resources and absence of high temperature during processing, DFS are very disposed to quality deterioration [2,3].

Lipid oxidation is one of the main non-microbial factors of quality deterioration in meat processing [4]. It should be noticed that lipid oxidation leads to loss of colour, rancidity and accumulation of volatile compounds (e.g. aldehydes, ketones) with negative implication for human health [4,5].

Spoilage and pathogenic microorganisms induce numerous problems in food [6]. The spoilage microorganisms could produce slime and gas, affects to discoloration, off-odours and off-flavours. Moreover, pathogenic bacteria are mainly responsible for foodborne diseases and food poisoning via meat and meat products. Hence, lipid oxidation and microbiological deterioration of meat and meat products can be marked as major limitations in the modern meat industry [7].

The usage of food additives is one the best solution in order to slow down the quality deterioration of and prolong their shelf lives of DFS. Sodium and potassium nitrite are the most common food additives used in DFS processing, mainly as an inhibitor of pathogenic bacteria (e.g. *Clostridium botulinum* and *Listeria monocytogenes*). Additionally, these additives possess a strong antioxidative potential and contribute to development of typical reddish-pink colour and flavour in DFS [8]. However, these additives are deemed as unhealthy due to potential formation of nitrosamines, compounds with strong

toxic and carcinogenic potential. It is well known that nitrosamines could be shaped as a consequence of interaction among nitrites and secondary amines [8-10].

Subsequently, the interest of many scientists is motivated on discovery substitutes for these additives from natural resources [11-20]. It is noteworthy that several studies suggested different plant extracts, including essential oils (EOs), which could contribute to customers' health status and might even have stronger antioxidant and antimicrobial capacity than synthetic antioxidants [21]. EOs are defined as volatile oils with high percentage of terpenoids with strong antioxidative and antimicrobial potential [21].

In addition, several studies showed that EOs isolated from diverse plants and vegetables could be used as novel antioxidants and antimicrobials in meat processing [2,4,7, 9-11].

2. Essential oils (EOs)

EOs contain complex mixtures of terpenoid compounds, which can be present in different parts of herbs, (e.g. waxy channels, glands and trichomes). These compounds present very complex mixtures of organic compounds such as terpenoids, aldehydes, esters, ketones, acids and alcohols, where the major constituents commonly compose up to 85% of the EO, while the minor compounds and trace elements constitute up to 15%. The chemical status of EOs highly depends on several factors: climate, geographical origin, plant cultivar, development stage, cultivation conditions, post-harvest processing of plant material and applied technique for their isolation [22].

Terpenoids are the most important class of organic compounds present in the EOs. They are recognized as isoprene (5 carbon atoms) oligomers and depending on the number of carbon atoms they can be classified as monoterpenes (10 carbon atoms), sesquiterpenes (15 carbon atoms) and diterpenes (20 carbon atoms). Terpenoids with more than 25 carbon atoms (sesterpenes, triterpenes and tetraterpenes) are mainly present in fatty oils rather than EOs. Terpenoids from EOs could also have different degrees of oxidation and they can be divided in hydrocarbon and oxygenated compounds, while nitrogen and sulphur are rarely present in EO constituents [22].

Importantly, terpenoids possess specific physico-chemical properties in terms of odour, polarity, volatility and bioactivity which directly reflect the approach for their isolation, purification and further application mainly in food, cosmetics and pharmaceutical sectors. EOs are commonly screened for their *in vitro* antibacterial and antifungal activity [21]. This suggests their potential application as direct natural antimicrobials for human and animal pathogens. This effect also ensures that EOs might be used as additives in foods and cosmetics and could be used as alternative for synthetic preservatives. The specific odour of EOs is responsible for their wide use in the perfume and fragrance industry, and for their ability to improve aromas in food, beverages or drugs [6,9,10]. EOs are often recognized as potent antioxidants, which additionally enables their utilization as natural additives together with their antimicrobial effects. Large variations in their chemical profiles further influences their bioactivity, and it has been reported that certain EOs might exhibit antiparasitic, allelopathic, anticarcinogenic, anti-obesity, immunomodulatory, anti-inflammatory, and antispasmodic effects along with some other biological activities [21,22]

Since various factors can strongly affect the chemical profile of EOs, which further alters their bioactivity, standardization is one of the main issues with these natural products, and their legislation is of great importance. EOs are permitted for use in food packaging in the EU, while EOs are also listed as flavouring ingredients by the Food and Drug Administration and have generally marked as safe in the United States [21,22].

2.1. Essential oil extraction technique

EOs and plant extracts could be isolated using diverse extraction techniques. Generally, there are two types of extraction techniques: conventional (e.g. Soxhlet - SX, hydrodistillation - HD) and emerging (e.g. supercritical fluid extraction - SFE, microwave-assisted - MA, ultrasound-assisted – UA, subcritical

water - SW extraction and high-pressure assisted extractions - HPAE). HD and SFE have been commonly used for lipophilic fractions, while diverse emerging techniques such as MA, UA and SW extraction have been used for polyphenolic fractions recovery [20-22].
It is well known that SX with organic solvents and HD extraction techniques have some disadvantages. High temperatures during process of HD affect to degradation of thermosensitive compounds, which outcomes in difference of the chemical shape. Moreover, HD demands huge energy consumption. The high toxicity of solvent residue and its poor selectivity are the main drawbacks of SX technique. Consequently, new trends of "green" chemistry demand application of "green" extraction techniques. Moreover, it has been highlighted that green extraction techniques, such as SFE provided some advantages in relation to chemical shape and selectivity over conventional extraction [5,20-22].

2.1.1. Supercritical fluid extraction (SFE)
SFE is a progressive and environmentally friendly alternative to conventional solid-liquid or liquid-liquid solvent extraction both for analytical sample preparation and for production scale applications. In this technique a clean solvent such as carbon dioxide is used instead of toxic organic solvents. The advantages of SFE are the opportunity of tuning the solvent power of the fluid by changes in pressure and temperature, while modifying, at the same time, other physico-chemical properties such as diffusivity, viscosity and density. In general, transport properties are favoured under supercritical conditions, and therefore, extraction processes are faster and provide higher extraction yields [20-22].
Yousefi et al. [25] suggested the resulting SFEs are clean and pure, of high quality, and have a great similarity to the aroma of the original plant before the extraction process. The temperatures used in SFE (around 35 °C) allow it to be used for thermally sensitive compounds, maintaining the quality of the final product.

3. Application of EOs in DFS
Recently, many scientific researchers observed the usage of EOs as emerging antioxidants and antimicrobials in DFS [7, 9, 26-33]. EOs were isolated from different pharmaceutical plants and spices (e.g. basil, caraway, juniper, oregano, sage, thyme) and applied as emerging additives in order to extend the shelf-life and improve quality of DFS [7-9].

Basil (*Ocimum basilicum* L.) is a plant belonging to the *Lamiaceae* family [26]. This plant contains a relative high content of terpenoid and phenolic compounds (linalool, methylchavicol, eugenol and/or methyl cinnamate) with strong antibacterial potential [26,27]. Hence, the usage of basil and its EO has a significant potential in meat industry [26,27]. Rattanachaikunsopon and Phumkhachorn [26] added basil essential oil (BEO) in order to decline the growth of *Salmonella* Enteritidis strain in fermented sausages. The results of this study suggested that the usage of BEO at 50 ppm efficiently decreased the population of a clinical *Salmonella* Enteritidis strain in fermented sausages after two days of refrigerated storage. Moreover, Gaio et al. [27] reported that usage of BEO in similar levels leads to decreased the population of *Staphylococcus aureus* in DFS.

Caraway (*Carum carvi* L.) of the Apiaceae family is one of the oldest aromatic and medicinal plants all over the world [28]. The seeds of caraway contain a more than 40% of EOs, with significant nutraceutical, antioxidant and antimicrobial potential [28-29]. Additionally, numerous researches evaluated the effect of caraway EO (CEO) on the shelf-life and quality of DFS [28-30]. Krkić et al. [29] suggested that CEO in mixture with chitosan biopolymer powerfully enhanced the oxidative stability of DFS during five months of storage. In other studies, strong antibacterial [28] and antifungal [30] activity of CEO in preservation of DFS were established.

Juniper (*Juniperus communis* L.) and its EO (JEO) are broadly applied in different industries: food, cosmetic and pharmaceutical. Regarding high content terpenoids (e.g. α-pinene, limonene and myrcene), JEO shows a strong antioxidant, antibacterial, antifungal, and anti-inflammatory properties [7]. In our earlier study [7], we showed that JEO (0.01 μL/g) enhanced the colour and retarded lipid oxidation in DFS. Also, the results revealed the important antioxidative activity of JEO, and subsequently, its high potential as a partial substitute for nitrites in DFS [7].

Oregano (*Origanum vulgare*) EO (OEO), comprising two significant active terpenoid compounds (thymol and carvacrol), had antioxidative effects in different meat products [31].

Sage (*Salvia officinalis* L.) and its EO (SEO) are broadly applied in different food processing, including novel meat industry [10, 32], regarding relatively high content of terpenoid compounds (e.g. eucalyptol, α-thujone and camphor) with significant antibacterial and antioxidant activity. Šojić et al. [10] detected that SEO powerfully decreased the level of lipid oxidation and the inhibited mesophilic bacteria growth in DFS stored at 15 °C for 225 days. Also, the results of aforementioned study showed that SEO (0.05 μL/g) could be applied as effective replacement for sodium nitrite in DFS.

Wild thyme (*Thymus serpyllum* L.) EO (TEO) established the presence of various bioactive compounds (e.g. flavonoids, tannins, terpenoids and other compounds) with strong antimicrobial, antioxidant, antitumor, hypoglycaemic and haematological effects [11, 33]. Huang et al. [33] investigated the application of TEO in DFS produced using horse meat. The results suggested that TEO can be considered as a natural additive in DFS production since it inhibits growth of pathogenic bacteria and accumulation of biogenic amines, and this improves the quality of DFS [33].

4. Conclusions

The results present in this reviews show that the use of EOs obtained from different plants (basil, caraway, juniper, oregano, sage, thyme) can extend the shelf life of DFS, delaying the deterioration (lipid oxidation and microbiological growth) and enhanced the quality of final products. Also, EOs contain different bioactive compounds with strong health benefit for human. Conventional extraction techniques affect to degradation of thermosensitive compounds and have negative ecological impact. Hence, the need to find more sustainable, ecological and viable extraction processes has led researchers to develop alternative techniques permitting to the “green” extraction concept, included SFE, UA, MW and HPAE. Additionally, these techniques allow the valorization of the by-products obtained from agri-food industry, underwriting to the reducing the environmental impact of food production.

Along with green” extraction concept, the recommendations of health international organizations to decrease the usage of synthetic additives (e.g. nitrites) are becoming gradually important, forcing meat industry to reformulate meat products to obtain healthier products. Therefore, the combination of these strategies is important to align the production of healthy meat products with sustainable actions. Finally, prior to addition in meat products, it is also essential to determine the optimal level and evaluate the toxicity of EOs, as well as their bioavailability in the human body.

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