Methods and Protocols in Food Science

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Functional Meat Products

Methods and Protocols in Food Science

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Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science.

Volumes and chapters will be organized by field and presented in such way that the readers will be able to reproduce the experiments in a step-by-step style. Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol will be clarified.

Functional Meat Products

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Preface to the Series

Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science. The series is unique as it includes protocols developed, validated, and used by food and related scientists as well as theoretical basis are provided for each protocol. Aspects related to improvements in the protocols, adaptations, and further developments in the protocols may also be approached.

Methods and Protocols in Food Science series aims to bring the most recent developments in research protocols in the field as well as very well-established methods. As such the series targets undergraduate, graduate, and researchers in the field of food science and correlated areas. The protocols documented in the series will be highly useful for scientific inquiries in the field of food sciences, presented in such a way that the readers will be able to reproduce the experiments in a step-by-step style.

Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol is clarified. Then, an in-depth list of materials and reagents required for employing the protocol is presented, followed by a comprehensive and step-by-step procedures on how to perform that experiment. The next section brings the do's and don'ts when carrying out the protocol, followed by the main pitfalls faced and how to troubleshoot them. Finally, template results will be presented and their meaning/conclusions addressed.

The Methods and Protocols in Food Science series will fill an important gap, addressing a common complain of food scientists, regarding the difficulties in repeating experiments detailed in scientific papers. With this, the series has a potential to become a reference material in food science laboratories of research centers and universities throughout the world.

Campinas, Brazil ***Anderson S. Sant'Ana***

Preface

Meat production has tripled over the last four decades and increased 10% in the last 10 years. In 2020, meat production was around 328 million tons (Mt). Over the next decade, the worldwide consumption of meat proteins is projected to increase by 14, primarily driven by income and population growth. Thus, the global meat supply will expand over the projection period, reaching 374 Mt by 2030. Protein availability from beef, pork, poultry, and sheep meat is projected to grow by 5.9%, 13.1%, 17.8%, and 15.7%, respectively, by 2030. However, meat and by-product consumption are often related to non-transmissible chronic diseases, such as cardiovascular issues, diabetes, and intestinal and colorectal cancer. This relation occurs mainly because some kinds of meat and processed meat present a large amount of saturated fatty acids, cholesterol, sodium chloride, and other additives, such as nitrite and nitrates, that could be responsible for nitrosamine formation. Thus, the meat industry has been reviewing animal genetics and diets and also reformulating meat products in order to develop healthier formulations. The processed meat reformulation moves toward the decrease of fat, sodium, or cholesterol content. In addition, a better composition of unsaturated fatty acids, natural additives utilization, and even the incorporation of functional ingredients has been tested and stimulated.

Some fibers or prebiotics have been used to develop meat products with reduced saturated fat content, as they contribute to the stabilization of meat emulsions and improve the product's yield and texture. Prebiotics could also be selectively used as substrate in fermented meat products and, thus, could be considered an attractive strategy to increase healthiness by stimulations of beneficial bacteria, such as probiotics. Probiotics are able to produce health-improving compounds, usually via the hydrolysis of polysaccharides, proteins, and fats, creating biologically active compounds such as bioactive peptides, organic acids, vitamins, and conjugated linoleic acid. Additionally, enrichment of meat products with vitamins, unsaturated fatty acids, natural additives, and minerals are important approaches within the context of the development of functional meat products. These functional ingredients could have beneficial effects on human health while meeting consumer expectations for nutritionally improved meat products. On the other hand, there are several details to be observed in the reformulation of meat products with functional ingredients, including chemical, physical, microbiological, and sensory analyses stability.

The purpose of this book is to give a comprehensive introduction to methods and procedures related to the manufacture of functional meat products. To reach this goal, scientists from different disciplines like Food Engineering, Food Technology, Food Microbiology, Chemistry, Sensory Analyses, Pharmaceutics, and Nutrition will work in chapters to provide comprehensive protocols in this field. This book follows the highly successful Methods and Protocols in Food Science (MeFS) series format. All chapters include introductions to the respective topic, lists of all necessary materials and reagents, step-by-step, readily reproducible protocols, and notes giving tips on troubleshooting and avoiding pitfalls in the methodologies.

Chapter 1—Probiotic Fermented Meat Products—explores the world of probiotics in meat production. Readers will learn about the incorporation of beneficial bacteria into fermented meat products, promoting not only enhanced flavor but also potential health

benefits. Chapter 2—Probiotic Emulsified Meat Products—delves into the development of emulsified meat products that incorporate these beneficial microorganisms. Readers will discover how to develop products that marry the qualities of emulsified meats with the health advantages of probiotics. Chapter 3—Prebiotic Meat Products—explores how prebiotic ingredients can be used in meat products to stimulate the growth of beneficial gut bacteria, resulting in improved health outcomes. Chapter 4—Symbiotic Fermented Meat Products—focuses on the synergy of probiotics and prebiotics. Readers will learn how to create fermented meat products that harness the power of both probiotics and prebiotics to enhance flavor and health benefits.

Chapter 5—Fermented and Structured Meat Products with Fibers for Reducing Fat Content—delves into the fascinating world of using fibers in meat products. Readers will discover how to create structured meat products that incorporate fibers, reducing fat content while maintaining texture and taste. Chapter 6—Emulsified Meat Product with Fibers for Reducing Fat Content—narrows the focus to emulsified meat products. Readers will explore how fibers can be used in emulsified meats to reduce fat content while retaining desirable qualities. In Chap. 7—Emulsified Meat Product with Oleogels for Reducing Fat Content—readers will learn how to incorporate oleogels into emulsified meat products to reduce fat content without compromising texture or taste.

Chapter 8—Analysis of Thiamine, Riboflavin, and Nicotinic Acid in Meat—describes insights into techniques for accurately measuring essential B-vitamins in meat products. In Chap. 9—Natural Additives in Meat Products as Antioxidants and Antimicrobials—readers will explore the use of natural additives in meat products, particularly for their roles as antioxidants and antimicrobials, helping to extend shelf life and ensure safety. Chapter 10—In Vitro and In-Model Evaluation of the Antimicrobial Activity of Lactic Acid Bacteria Protective Cultures to Replace Nitrite in Dry Fermented Sausages—discusses the intriguing possibility of replacing nitrite with protective cultures. Readers will learn about in vitro and in-model evaluation techniques for assessing the antimicrobial effects of these cultures in dry fermented sausages.

Chapter 11—Sodium Reduced Meat Products—is considered a critical health concern. Readers will discover methods and strategies for developing meat products with reduced sodium while preserving taste and safety. Chapter 12—Direct Method for Simultaneous Analysis of Cholesterol and Cholesterol Oxides by HPLC in Meat and Meat Products—provides a precise analytical approach to simultaneously measure cholesterol and cholesterol oxides in meat, aiding in nutritional assessment and quality control. Chapter 13—The Long-lasting Potential of the DNPH Spectrophotometric Method for Protein-derived Carbonyl Analysis in Meat and Meat Products—delves into a long-lasting method for analyzing protein-derived carbonyls in meat products, a valuable tool for quality control and research.

Finally, Chap. 14—Functional Molecules Obtained by Membrane Technology—explores membrane technology to obtain functional molecules from meat. Readers will discover how this innovative approach can yield valuable compounds for various applications. And Chap. 15—Bioactive Peptides Obtained from Meat Products—explores the world of bioactive peptides derived from meat products. Readers will learn about methods to isolate and utilize these peptides, potentially unlocking their health benefits.

The focus of this special volume is to address the latest relevant state-of-the-art protocols to manufacture functional meat products. In addition, this book combines as comprehensibly as possible well-established protocols and procedures being used by several laboratories in academia and industry. It will introduce the broad field of protocols that

can be used for functional meat products production to Graduate Students, Postdoctoral Associates, and all researchers who are either still at the beginning of their academic careers or scientists who are in search of new challenges in a new field hitherto unfamiliar to them. In summary, this book covers a wide spectrum of topics within the realm of functional meat products, ranging from the incorporation of probiotics and prebiotics to analytical methods, innovative fat reduction techniques, and the utilization of natural additives and bioactive compounds. It provides a comprehensive guide for researchers and professionals in the food industry looking to explore and contribute to the development of healthier and more innovative meat products.

Florianópolis, Santa Catarina, Brazil *Silvani Verruck*
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Contents

Preface to the Series v
Preface vii
Contributors xiii

1 Probiotic Fermented Meat Products 1
Ruben Agregán, Marcelo Rosmini, José Ángel Pérez-Álvarez, Paulo E. S. Munekata, Laureano Frizzo, Paulo C. B. Campagnol, and José M. Lorenzo

2 Probiotic Emulsified Meat Products 13
Denis Baranenko, Irina Shestopalova, Yulia Broyko, Malikakhon Kurbonova, Nikita Tyutkov, Elena Lemeshonok, and Bazhena Artemova

3 Prebiotic Meat Products 25
Paulo Eduardo Sichetti Munekata, Cynthia Farias Manassi, Rubén Domínguez, Mirian Pateiro, Silvani Verruck, and José Manuel Lorenzo

4 Synbiotic Fermented Meat Products 39
Lujuan Xing, Jiaming Cai, Ming Ju, Jian Zhang, and Wangang Zhang

5 Fermented and Structured Meat Products with Dietary Fibers for Reducing Fat Content 57
Paulo Eduardo Sichetti Munekata, Rubén Domínguez, Mirian Pateiro, Eva Maria Santos, Alfredo Teixeira, Marcelo Rosmini, and José Manuel Lorenzo

6 Emulsified Meat Product with Oleogels for Reducing Saturated Fat Content 69
Camila de Souza Paglarini, Ana Karoline Ferreira Ignácio Câmara, Thais Lomonaco Teodoro da Silva, and Marise Aparecida Rodrigues Pollonio

7 Emulsified Meat Product with Fibers for Reducing Fat Content 81
Vivian Feddern, Eduardo Huber, Vanessa Biasi, and Vicky Lilge Kawski

8 Analysis of Thiamine, Riboflavin and Nicotinic Acid in Meat 95
Ceres Mattos Della Lucia and Lívya Alves Oliveira

9 Natural Additives in Meat Products as Antioxidants and Antimicrobials 111
Noemí Echegaray, Marcelo Rosmini, Juana Fernandez, Paulo E. S. Munekata, Laureano Frizzo, Paulo C. B. Campagnol, and José M. Lorenzo

10 In Vitro and In-Model Evaluation of the Antimicrobial Activity of Lactic Acid Bacteria Protective Cultures to Replace Nitrite in Dry Fermented Sausages 125
Eva Hierro, Xavier F. Hospital, Sergio B. Mano, and Manuela Fernández

11 Reduced-Sodium Meat Products 137
Maria Lúcia Guerra Monteiro, Eliane Teixeira Mársico, and Carlos Adam Conte-Junior

12 Direct Method for Simultaneous Analysis of Cholesterol and Cholesterol Oxides by HPLC in Meat and Meat Products 151
Vanessa Sales de Oliveira, Geni Rodrigues Sampaio, Elizabeth Aparecida Ferraz da Silva Torres, and Tatiana Saldanha

13 The Long-Lasting Potential of the DNPH Spectrophotometric Method for Protein-Derived Carbonyl Analysis in Meat and Meat Products 169
Rebeca Cruz, M. Madalena C. Sobral, and Susana Casal

14 Functional Molecules Obtained by Membrane Technology 181
Giordana Demaman Arend, Claudio Malaghini, Maicon S. N. dos Santos, Carolina E. Demaman Oro, Marcus V. Tres, and Katia Rezzadori

15 Bioactive Peptides Obtained from Meat Products 193
Mirella Romanelli Vicente Bertolo, Josemar Gonçalves de Oliveira Filho, Sinara Teixeira do Brasil Morais, Keila Nascimento Cavalcante, Daniel Rodrigues Cardoso, and Stanislau Bogusz Junior

Index *209*

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Chapter 1

Probiotic Fermented Meat Products

Ruben Agregán, Marcelo Rosmini, José Ángel Pérez-Álvarez, Paulo E. S. Munekata, Laureano Frizzo, Paulo C. B. Campagnol, and José M. Lorenzo

Abstract

The fermentation of meat is an ancient culinary tradition worldwide used mainly with the intention of extending meat shelf life and diversifying. Plenty of products with their respective recipes have been developed throughout the history of civilization. Spain is a country with historical tradition in the production of fermented meat products, highlighting chorizo and salchichón. Specifically, the latter can be divided into different varieties according to aspects, such as size. Thus, products, such as longaniza, fuet, secallona, or didalets, can be classified and named according to the length and width of the piece. The ingredients used for elaboration are practically the same between these salchichónes. In the present chapter, the production of fuet is described in depth since it represents one of the most traditional and consumed fermented meat products in Spain and it is also being internationalized to other countries. On the other hand, the addition of probiotic cultures to meat dough is increasingly practiced, which has potential health benefits. Therefore, the production of fuet with probiotic microorganisms might help to develop novel and healthy alternatives to the traditional recipe. Ingredients including pork lean and belly, spices and other additives incorporated in the form of commercial mixes, and starter and probiotic cultures are used in the elaboration of the fuet proposed in this chapter, throughout different steps, which can be classified as mincing, mixing, stuffing, fermentation, curing, and conservation.

Key words Fermented meat product, Fermented sausage, Probiotic, Fuet, Elaboration process

1 Introduction

The elaboration of fermented meat products is a culinary tradition perpetuated over time by generations in different parts of the world, such as Europe, where a wide and varied offer of these products can be found [1]. Although the technology of these products has undergone significant modifications throughout the history of humankind [2], the purpose of fermenting meat has always been the same, extending shelf life and diversifying [3]. The development of fermented meat products involves dynamic and complex chemical processes, in which lactic acid

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bacteria (LAB) stand as the main muscle-transforming microorganisms, causing the acidification of the medium. This drop in pH helps to stabilize the product, delaying deterioration processes and preventing the development of pathogenic bacteria. In addition, acidification positively impacts on sensory attributes, increasing the final product acceptance [1].

The fermentation of meat can be done in two ways, allowing the indigenous muscle microflora to act alone or using predefined microorganisms (known as starter cultures) to initiate and carry out the transformation processes. These starter cultures mainly consist of one or several LAB species, micrococci, and staphylococci [4], but also yeast and molds can be used [5]. They are specifically designed to meet the food safety criteria specified by the regulatory entity and the technological and organoleptic specifications of the company. In this way, the fermentation can be controlled, and the process standardized, yielding safe and high-quality meat products [6]. The current market trend towards healthier products has led to the research of other microbial cultures capable of exerting health benefits. In this context, special attention has been paid to probiotics, living organisms capable of modifying the gut microbiome and improving health when consumed in adequate amounts [7]. Immunomodulatory effects and anticancer, antimicrobial, antidiabetic, and anti-inflammatory properties have been associated with the consumption of these microorganisms [8–12].

Different studies have been searching for good probiotic candidates to be used in the preparation of fermented meat products (Fig. 1). Strains such as *Bifidobacterium longum* KACC 91563 [13], *Enterococcus faecium* CECT 410 [14], *Lacticaseibacillus casei* ATCC 393 [15], *Lactobacillus paracasei* DTA83 [16], *Lactobacillus rhamnosus* LOCK900 [17], *Lactobacillus acidophilus* CRL1014 [18], and *Lactobacillus sakei* 23 K [19] have been recently assessed in this regard, showing a good ability to produce quality fermented meat products since they are able to satisfactorily colonize the meat dough, reach a reasonably high number of counts, and barely affect sensory attributes, pH, and oxidative status [7]. As can be seen, there are many potential probiotic cultures to be used in the development of fermented meat products, which opens the door to multiple industrial and commercial possibilities [20].

For the elaboration of a fermented meat product, different and varied recipes can be followed since there are innumerable products of this type with very different characteristics, linked to geographical areas of the world [21, 22]. In Spain, there is a long tradition of making these food products and varieties, such as chorizo and salchichón, which can be tasted throughout the different territories of this country. Several classes of salchichón can be found according to parameters, such as size. Thus, products, such as longaniza, fuet, secallona, or didalets, with different lengths and widths are

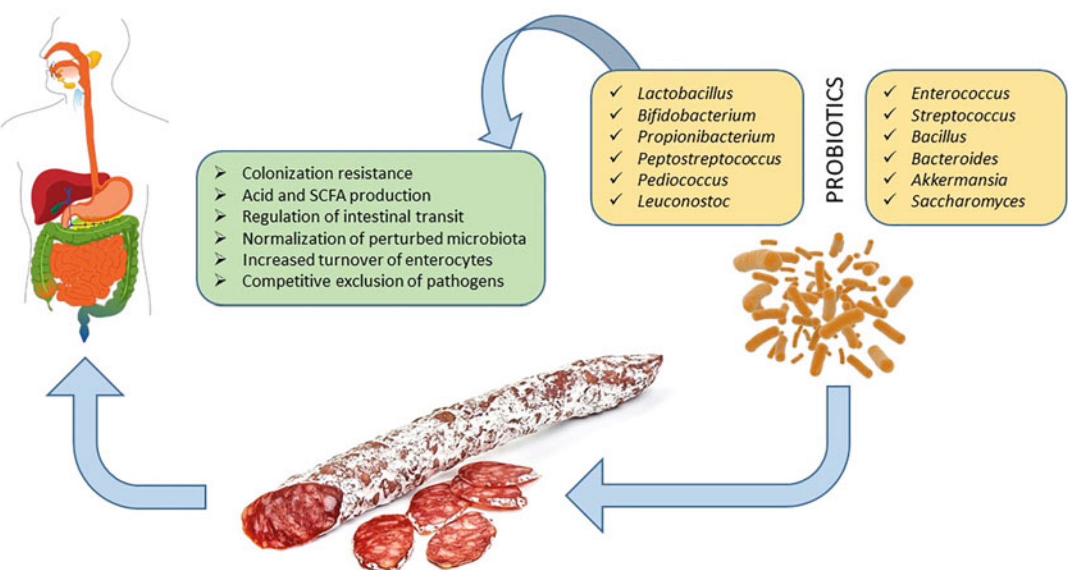

Fig. 1 Potential health benefits of probiotic fuet consumption and possible bacteria involved [24, 25]. *SCFA* short-chain fatty acid

commercialized. In this chapter, we selected the "fuet" as the base product to show its production process in depth. Fuet is a traditional product from the region of Catalonia widely consumed in Spain. Only in 2019 that consumers of this country spent more than 250 million dollars on this meat product and longaniza. In addition, fuet product is becoming international, and its consumption has spread to other neighboring countries such as France and also to the entire European continent and the United States, where it is prized for its presumed high-quality ingredients, exceptional flavor, and superior wholesomeness compared to similar Mediterranean-type sausages [23].

2 Materials

2.1 Ingredients

Fuet is made mainly with pork and fat. In addition, salt, spices, and sugar are also used. Different preparations of this fermented sausage are possible, so in order to avoid conflicts between the multiple existing formulations, we have decided to compile some of the most relevant recipes recently published in the scientific literature to develop our own concept of fuet. Pork lean is a fundamental part of the traditional recipe, but other meats are currently replacing pork in some novel manufacturing protocols. After chopping and blending the lean and fat, salt and ground pepper are added. Other species such as garlic can also be incorporated. These seasonings work as flavor enhancers and can help in the stabilization process by

Table 1
Ingredients and proportions in the elaboration of the probiotic fuet

Ingredients	Proportion (%)
Pork lean	60
Pork fat	30
Water	4
Commercial mix	4
Commercial starter culture	1.98
Commercial probiotic strain	0.02

Commercial mix: salt, dextrin, dextrose, stabilizer (sodium phosphate (E-451)), spices and spice extract, flavor, antioxidants (sodium ascorbate (E-301) and sodium citrate (E-331)), and preservatives (potassium nitrate (E-252) and sodium nitrite (E-250)). Starter culture: *Pediococcus* (50%), *Staphylococcus xylosus* (25%), and *Staphylococcus carnosus* (25%). Probiotic strain: LGG® (*Lacticaseibacillus rhamnosus* GG). Data are based on the studies carried out by Bis-Souza et al. [29], Zamora et al. [30], and Peñaranda et al. [31]

exhibiting antioxidant and antimicrobial properties [26–28]. Differences between fuets, both commercial and homemade, can be found at this point of preparation. The type of meat and the fermentation process also have a significant influence on the final product.

Other ingredients, including stabilizer (e.g., phosphate), antioxidants (e.g., ascorbate and citrate), preservatives (e.g., nitrate and nitrite), dextrin, dextrose, and flavorings, are also added to the meat matrix. These compounds, together with the spices, are usually incorporated in the form of commercial mixes. A starter culture consisting different type of species, including LAB, and a probiotic culture are then inoculated. Finally, the formed dough needs to be completed with water up to a certain percentage of humidity. Artificial pig casings are used to stuff the dough obtained, but natural pork casings are also commonly used, and a food-grade *Penicillium candidum* mold is applied to the surface of sausage to protect it from the invasion of spoilage molds during storage. Moreover, it adds a touch of flavor and extra aroma to the final product. Finally, fermentation and drying processes complete the production protocol. Ingredients and proportions in the preparation of the probiotic fuet are detailed in Table 1.

There are many commercial mixes available on the market, but we suggest using the one sold by Catalina Food Solutions S.L. (El Palmar, Murcia, Spain) [30]. This mix is made up of salt, dextrin, dextrose, stabilizer (sodium phosphate (E-451)), spices and spice extract, flavor, antioxidants (sodium ascorbate (E-301) and sodium citrate (E-331)), and preservatives (potassium nitrate (E-252) and sodium nitrite (E-250)).

Regarding the starter culture, the commercial mix used by Zamora et al. [30] (Microsan-R), also from Catalina Food Solutions S.L. (El Palmar, Murcia, Spain), was chosen for the elaboration of the probiotic fuet. The genus *Pediococcus* at a concentration of 50% and the species *Staphylococcus xylosus* and *Staphylococcus carnosus* at concentrations of 25% each compose the starter culture. On the other hand, the probiotic strain LGG® (*Lacticaseibacillus rhamnosus* GG), marketed by the company Chr. Hansen (Hørsholm, Denmark) (*see* **Note 1**) and successfully tested in the preparation of a salchichón [29], was the one chosen to colonize the fuet. Finally, as previously mentioned, a layer of *Penicillium candidum* spores is applied to the product after stuffing. There are different commercial brands on the market that provide this mold. In this case, we propose the one marketed by the company Danisco S.A. (Barcelona, Spain), according to the elaboration carried out by Marcos et al. [32].

2.2 Equipment

The equipment for making fuet is an essential part of the production protocol since inadequate material can lead to undesirable results. Thus, we have suggested a series of elements and brands that can adequately satisfy the needs of manufacturers during the different stages of production (Table 2). Photos of this machinery are shown in Fig. 2.

A mincing machine is the first piece of equipment necessary for the fuet production process as it allows the chopping of meat and fat, which will form the base of the sausage. For this, a refrigerated mincer from La Minerva di Chiodini Mario (Bologna, Italy) with a 6 mm mincing plate can be used. For fine grinding and mixing of both raw materials, along with the commercial mixes of additives and microorganisms (starter culture and probiotic strain), an Industrial Fuerpla (Benetusser, Valencia, Spain) vacuum grinder is

Table 2
List of suitable industrial equipment for the elaboration of the probiotic fuet and the corresponding brands (*see* Notes 2 and 3)

Equipment	Brand
Mincing machine	La Minerva di Chiodini Mario (Bologna, Italy)
Vacuum grinder	Industrial Fuerpla (Benetusser, Valencia, Spain)
Semiautomatic stuffer	Sia Suministros Industriales (las Torres de Cotillas, Murcia, Spain)
Sausage tying machine	Andher-*Comercial Eliseo Andújar S.L.* (Alcázar de San Juan, Ciudad Real, Spain)
Air-drying chamber	–
Conservation chamber	–

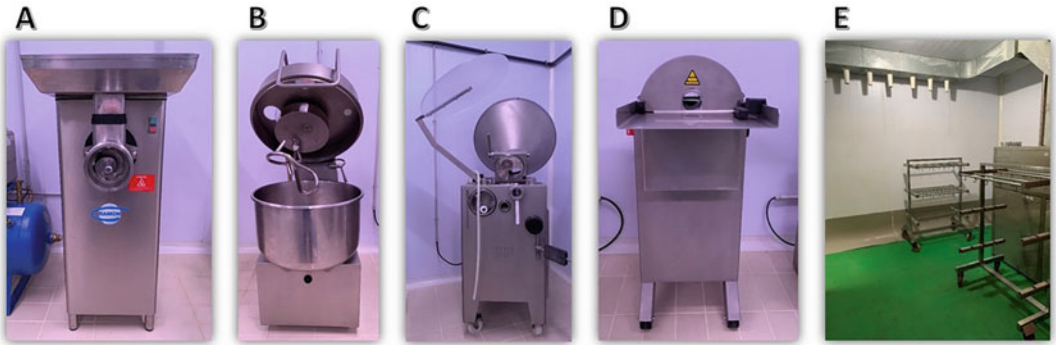

Fig. 2 Suggested equipment for the elaboration of a probiotic fuet. (**a**) mincing machine; (**b**) vacuum grinder; (**c**) semiautomatic stuffer; (**d**) sausage tying machine; (**e**) air-drying chamber

recommended. A conventional or industrial refrigerator will be necessary, depending on the amount of dough produced, to store it and allow the compaction to occur. Stuffing can be performed using a Sia Suministros Industriales (Las Torres de Cotillas, Murcia, Spain) semiautomatic stuffer and a 45 mm diameter artificial casings (Edicas, Salamanca, Spain). The sausages formed can be divided and tied using an Andher sausage tying machine (Alcázar de San Juan, Ciudad Real, Spain). Finally, the fermentation and drying processes can be carried out in an air-drying chamber. After elaboration, the product can be moved to another automated chamber to preserve it until consumption.

3 Methods (*See* Note 4)

Fuet requires a strict production protocol and good raw materials (lean meat and belly) to obtain a quality product. Once these elements are minced and mixed with the commercial mix and both the starter and probiotic cultures, the dough formed is stuffed, fermented and dried, and conserved (Fig. 3). This highly summarized production procedure consists of a series of detailed stages that will be described in depth in the following paragraphs.

3.1 Chopping of Raw Materials

The first step when making fuet is to obtain good-quality raw materials. A product made with meat and fat of little aptitude for the preparation of sausages will affect the quality of the product. The classical recipe for fuet uses pork lean and pork belly. However, recently modifications of this model recipe have been appearing, which attempt to diversify this product by incorporating proteins from different animals. Meat from animals, such as goat, sheep, beef, and other less common, such as duck, foal, or game, can be used in the production of salchichón [33–35]. In our case, both the meat and fat for making fuet will be from pork.

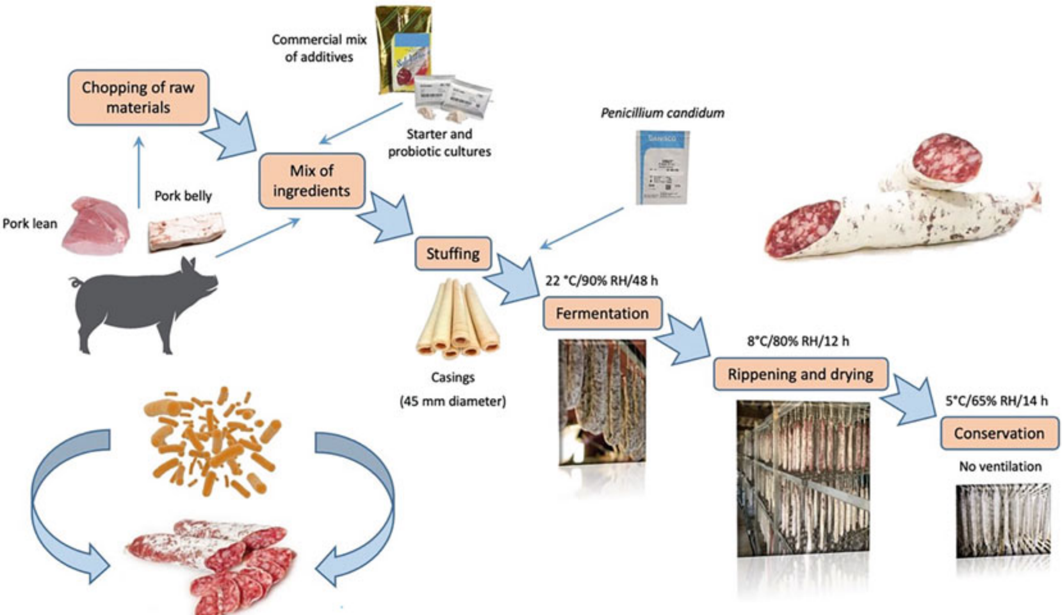

Fig. 3 Production scheme of probiotic fuet

The previously refrigerated pork lean and pork belly are cut into chunks of between approximately 10 × 10 and 20 × 20 cm to allow their easy passage through the mincer tube. The meat and fat are placed separately in the mincer's loading hopper and are pushed by an auger towards the hole that ends in a perforated disc that grinds the raw material. The chopped lean and fat are then collected in a clean stainless steel container. In this operation, the sharpness of the blades must be adequate; otherwise, the mincing may be poor, causing tears in the meat and overheating. This leads to problems in the ripening and drying stage, giving rise to fuets with poorly defined short surfaces.

3.2 Mix of Raw Materials with Additives and Starter and Probiotic Cultures

After the mincing process, the lean and fat are properly mixed under refrigerated (< 4 °C) vacuum. The absence of air is essential to prevent subsequent problems, such as discoloration and a higher development of microorganisms. At this point in processing, the commercial mix of additives in powdered form, incorporating salt, spices and spice extract, dextrin, dextrose, and flavoring, along with a stabilizer, antioxidants, and preservatives, is poured over the meat mixture and fat. This operation should last the time enough to allow the formation of the most uniform paste as possible. Around 5 min would be needed to process 20 kg of dough. During the mixing process, both the starter and probiotic cultures are added. The moment and the order of addition are indifferent. Specifically, the commercial starter culture will be incorporated in the form of lyophilized powder (commercial presentation), being previously

rehydrated for 8 h (approximately 7 g per 100 mL of water) (adaptation of the preparation carried out by Zamora et al. [30]). On the other hand, the probiotic culture will be prepared and added according to the instructions for use provided by the trader. Along with all the mentioned ingredients, water will be added according to the proportion indicated in Table 1.

Once the different ingredients are mixed, the dough formed is stored under refrigeration for 24–48 h. This storage favors the obtaining of a higher-quality dough by allowing better integration of the aromas in the meat and fat. Similarly, this period helps salt penetration. In this way, protein coagulation can be increased, and dough with superior rheological characteristics can be formed.

3.3 Stuffing of Meat Dough

The meat dough adequately prepared is introduced into artificial casings by means of a stuffer, trying to avoid the presence of air as much as possible. The mixing of ingredients under vacuum conditions makes it possible to considerably reduce the formation of air cavities in the dough, but this condition could be reversed if the stuffing process is not carried out properly. Insufficient fill pressure could add air into the casing and form small air-filled spaces that may cause discoloration, abnormal coloring, moldiness, and other abnormalities. In addition, the dough must be kept away from any source of moisture as it could also cause abnormal coloring. At the end of the stuffing process, casings are divided into portions about 30–35 cm long that are tied with a string specially indicated for this use. Finally, all the formed pieces must be washed with clean water to remove any kind of leftover material from the surface.

The artificial casings used in this protocol should be soaked for about 2 h before stuffing to avoid breakage during the filling process. A small handful of salt can be added to provide an extra touch of saltiness to the sausage.

3.4 Addition of Protective Mold on the Surface of Sausage

Just after stuffing and before fermentation, a layer of specific mold is added to the surface of sausages to prevent possible contamination with spoilage molds. A spore solution of the mold *Penicillium candidum* is prepared according to the manufacturer's instructions, and the sausages are immersed in the liquid [32].

During the drying stage, the internal water of the sausage slowly escapes to the outside, leaving the surface of the product wet. Under these conditions, molds can proliferate and spoil the fuet, causing possible health problems. For this reason, spores of certain types of mold should be added in order to protect the sausage during storage. Furthermore, the white layer formed by these microorganisms helps control water loss, promoting more uniform dehydration, and adds aroma and taste to the final product, being actually considered an important quality attribute.

3.5 Fermentation Process

The stage after stuffing and adding mold is fermentation. During this brief period of time, fundamental biochemical processes for the proper sensory development of fuet will take place. The sausages are transferred in a drying chamber under special humidity and temperature conditions. According to the parameters used by Zamora et al. [30], temperature and relative humidity (RH) will be set at 22 °C and 90%, respectively, for 48 h. In this stage, the starter culture will colonize the meat substrate and metabolize the sugar, transforming it into lactic acid. This will cause a drop in pH, which will lead to a series of phenomena, such as protein and fat hydrolysis, color changes, and texture modification, among others.

3.6 Ripening and Drying Period

The post-fermentation period, commonly known as the curing stage, encompasses the most prominent changes (chemical, physical, physicochemical, microbiological, and sensory) in the sausage matrix. In fact, one of the most important events that occur in this stage is the redness of sausages. This phenomenon is produced by the formation of nitropigments, favored by the conversion of nitrates into nitrites due to the action of reducing microorganisms. After 48 hours of fermentation, the RH and temperature values will be reduced to 80% and 8 °C, respectively, and the sausages will be maintained for the next 12 days in these new environmental conditions. At the end of this time, adequate microbiological stability and unique organoleptic properties will be achieved.

On the other hand, the ripening and drying period is characterized by the loss of moisture in the dough, known as "merma." Thus, a salchichón can suffer a reduction of at least 30% of the initial content [35]. During post-fermentation, the sausages must remain hung in the darkness to avoid the appearance of rancidity in the crust due to the action of light. However, they should not be hung too close together because proper ventilation is prevented. This promotes the accumulation of humidity between pieces, which may lead to mold on the product.

3.7 Conservation

At the end of the production protocol, the freshly finished fuets must be kept under specific storage conditions that maintain their unique sensory quality and also prevent microbiological spoilage. Therefore, they are transferred from the curing chamber to the conservation chamber, using a lower refrigeration temperature and RH than those used during the previous stage. Specifically, fuets should be stored at 5 °C and 65% HR in the absence of ventilation for 14 days.

4 Notes

1. The probiotic culture proposed in the fuet elaboration was highly researched, and the most notable results discovered can be consulted on the website of the trading company.
2. The models of the machines suggested for the elaboration of the probiotic fuet were not provided on purpose because the model may vary depending on the amount of product. Only the proportions of the ingredients were given, leaving the decision about the production size in the hands of the reader.
3. The trademarks of the ingredients and machines provided in this chapter are only indicative. They were suggested due to previous satisfactory experience by the authors.
4. All the processes before fermentation have to be done under refrigeration temperature. This implies that the work rooms employed need to be prepared to produce cold. Otherwise, microorganisms can grow over the recommendable limits, spoiling the fuet prematurely.

Acknowledgments

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Chapter 2

Probiotic Emulsified Meat Products

Denis Baranenko, Irina Shestopalova, Yulia Broyko, Malikakhon Kurbonova, Nikita Tyutkov, Elena Lemeshonok, and Bazhena Artemova

Abstract

Probiotics are live microorganisms that provide health benefits to the host, including improving gut health, boosting the immune system, and reducing inflammation. However, incorporating probiotics into emulsified meat products can be challenging due to factors affecting their viability, such as heat, pH, and bile salts. To improve probiotic viability, promising methods include selecting resistant strains, using protective coatings, and adding prebiotics. Incorporating probiotics into emulsified meat products can enhance their safety, shelf life, nutritional value, and sensory characteristics. However, it is essential to select the right probiotic strains and employ techniques to improve their viability and functionality in the final product. Thus, this chapter presents a protocol to add viable probiotics to emulsified meat products and also discusses the main difficulties of the process.

Key words Probiotic pate, Meat emulsification, Encapsulation

1 Introduction

Probiotics aim to restore the gut microbe ecosystem, promoting host health [1–7]. To ensure probiotic health benefits, bacteria must be stable in the gut and adequately present in food, at a recommended level of 10^6–10^7 CFU/mL [8, 9].

Meat products offer a probiotic carrier for lactose-intolerant individuals [10–13], with the addition of probiotics providing health benefits and improving storage stability by suppressing harmful microorganisms. The appropriate strain selection for each probiotic product is crucial, considering factors such as resistance to low pH, bile acids, adhesiveness, colonization ability, safety, and storage stability [1, 14–16]. Representatives of lactic acid bacteria (such genera as *Lactobacillus*, *Bifidobacterium*, *Streptococcus*) and *Propionibacterium* are commonly used in meat production [17–21].

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When selecting (LAB) strains for meat products, lactic acid production, temperature, and water activity should be considered [20, 22]. LAB strains prevent oxidative processes, reducing glucose and total cholesterol and normalizing metabolism [17–19, 23].

Bifidobacteria and propionic acid bacteria offer benefits in sausage production, including inhibiting harmful microorganisms, enhancing intestinal microbiota, and producing fatty acids, antimutagenic substances, and vitamin B12 [3, 18, 19, 24, 25]. Not only bacteria can be used as probiotic cultures in the production of meat products but also yeast, such as *Saccharomyces cerevisiae* strains [26].

Adding probiotics to cooked and emulsified meat products poses challenges due to high temperature, salt, and acid levels that can affect strain viability and product characteristics [27]. Various methods can be employed to enhance the viability of probiotics in emulsified meat products, such as careful strain selection, implementation of protective coatings, utilization of prebiotics, and optimization of processing conditions. Encapsulating probiotics protects them during food processing and storage, maintaining viability and preventing premature release or loss in emulsified meat products (Table 1).

The main techniques used to encapsulate probiotic cells are extrusion [33–38], emulsion [39–42], spray drying [30, 43–46], spray chilling [47–50], and fluidized bed [36, 51]. Encapsulation coats probiotics in a protective matrix, using materials like chitosan, alginate, and xanthan, which are vital for stability and safeguard *Bifidobacterium bifidum* and *L. acidophilus* in both probiotic carrier foods and the GI system. This technique preserves probiotics in fermented meat and improves quality and health benefits of emulsified meat [52–55].

Probiotics require protection during GI tract passage, and immobilization technology offers this by enclosing them in a matrix [56]. Natural water-soluble polysaccharides are favored for immobilizing probiotics due to their safety and biodegradability [43, 57]. Edible films (e.g., Na-alginate edible films) successfully delivered probiotics to sliced ham, maintaining stability during storage, with or without high-pressure processing [58–60]. Thermostable probiotics, with strains isolated from heat-treated meat, are also ideal for high-temperature processed meat products [12, 27, 30–32, 61–63]. Therefore, inoculated thermotolerant LAB strains enrich emulsions and ensure dominant probiotic microbiota during shelf life [64]. It can also be promising to use meta-, post-, and paraprobiotics.

Metabiotics, which include paraprobiotics and postbiotics, is a newer concept in microbiology referring to nonviable microbial products that can confer positive health effects [36, 65]. Paraprobiotics and postbiotics offer advantages over probiotics in food, as

Table 1
Encapsulated probiotics in meat products

No.	Meat product	Probiotic strains	Description	References
1	Cutlets	*Lactiplantibacillus plantarum* Lp-115, *Bifidobacterium animalis* spp. *lactis* Bb-12, *Lactobacillus acidophilus* La-5	The microparticles (alginate, β-cyclodextrin, and xanthan gum) demonstrated good stability during cutlet thermal processing. They played an important role in protecting probiotic bacteria, which led to their survival during consumption and stimulated GI (gastrointestinal) digestion	[27]
2	Cutlets	*Lactobacillus plantarum* SP-A3	No significant survival rate difference was found between capsule sizes of 200–300 μm and 400–600 μm (74.7 ± 0.7% and 68.8 ± 1.2%). Encapsulated cells had higher survival rates than unencapsulated cells (0% in three experiments) with up to 75.4% survival. Encapsulation is a potential method for adding probiotics to heat-treated meat products	[28]
3	Semi-finished meat	*Lactobacillus rhamnosus* GG	Capsules frozen with the semi-finished meat product had a value of 9.28 ± 0.15 lg (CFU/ml). The encapsulated microorganisms had a 59 ± 2% viability after heat treatment, allowing such semi-finished meat products to be classified as probiotic products	[29]
4	Meat batters	*Aerococcus viridans* UAM21, *Enterococcus faecium* UAM10a, *Lactobacillus plantarum* UAM17, *Pediococcus pentosaceus* UAM11	Spray-dried LAB strains increased its count and reduced the number of *Enterobacteria* in samples, indicating effective protection by the encapsulation. The resulting particles can be used in other emulsified heat-treated meat products and as bioprotective cultures to enhance microbial quality	[30]
5	Sausages	*E. faecium* UAM1, *P. pentosaceus* UAM2	Cactus pear peel flour was found to have prebiotic potential and reduces oxidative rancidity in cooked sausages when used as a functional ingredient with inoculated thermotolerant LAB strains. Adding cactus fruit peel flour to cooked meat products with LAB strains can be a viable option for symbiotic meat products	[31, 32]

they are more stable and demonstrate technological benefits [36]. They mainly include short-chain fatty acids, enzymes, peptides, teichoic acids, and organic acids [65–67]. Postbiotics have direct immunomodulatory and clinically relevant effects and can improve overall health and alleviate symptoms in a number of diseases. Additionally, they can help to maintain high concentrations of viable probiotic cells in products and improve their effectiveness in colonizing the colon mucosa [26, 66, 67]. They could potentially improve the safety and shelf life of meat by inhibiting the growth of harmful bacteria and reducing spoilage [68]. Studies have shown that metabiotics can help prevent the growth of pathogenic bacteria, such as *E. coli* [69] and *Salmonella* spp. [10, 70, 71], which are common in meat products. Paraprobiotics also have shown promise in improving the microbiome composition and function, modulating the immune system, and reducing inflammation.

Metabiotics derived from probiotic microorganisms could potentially improve the safety and shelf life of meat by inhibiting the growth of harmful bacteria and reducing spoilage [68, 69]. However, further research is necessary to understand the effects of metabiotics on meat products and to ensure their safety and efficacy.

The addition of probiotics to meat products enhances gut health and improves storage stability of products. Choosing the right probiotic strain is crucial, and techniques such as encapsulation and the use of edible films can protect probiotics during processing and storage. In cooked meat products, thermostable strains can maintain their prevalence, while thermotolerant strains, isolated from cooked sausages, and *Bacillus* spores have demonstrated their ability to sustain through cooking and storage in sausage production. It should be noted that probiotics can be added aseptically to emulsified products with a spreadable consistency after heat treatment of the main raw material. For emulsified products, it is possible to apply probiotics as part of edible coatings or use thermostable forms of microorganisms that tolerate heat treatment and subsequent storage. This chapter provides a protocol for obtaining a liver pate spread. It should be noted that the nuances of the product formulation, as well as its type, may differ. The main step in creating a probiotic-emulsified product is the introduction of microorganisms at the appropriate stage and in the appropriate form. This chapter presents a technique to add viable probiotics to emulsified meat products (on the example of pate), which is less explored compared to fermented meat products.

2 Materials

- Liver 55% (beef, pork, or lamb).
- Brain 10% (beef or pork).
- Back fat (lard) 30%.
- Onions sauteed with butter 3.1%.
- Sodium chloride 1.3%.
- Sugar 0.4%.
- Ground black pepper 0.04%.
- Ground allspice 0.04%.
- Ground nutmeg 0.04%.
- Ground cinnamon 0.04%.
- Ground carnation 0.04%.
- Probiotic culture (Table 1) (*see* **Note 1**).

2.1 Equipment

- Scales (accuracy and limit of measurement depend on the scale of production).
- Grinder with a grid hole diameter of 2–3 mM.
- Cutter-blender.
- Cooking boilers (*with aseptic filling unit for 3.2.4* or 3.2.6**) or other blanching and heat treatment equipment.
- Encapsulator (laboratory type, B-390, BUCHI, Switzerland) or another co-extruding industrial encapsulator.

3 Methods

The protocol for making a probiotic liver pate is illustrated in Fig. 1.

3.1 Preparation of Encapsulated Microorganisms

Encapsulation may be performed on a co-extruding industrial encapsulator same to laboratory B-390 apparatus (BUCHI, Switzerland):

1. Prepare a 3% sodium alginate solution and a microbial suspension.
2. Mix the sodium alginate solution and the microbial suspension in a 9:1 ratio.
3. Gently stir the mixture for 30 min.
4. Feed the suspension dropwise through a nozzle into the quenching solution that contains 0.1 M calcium lactate.

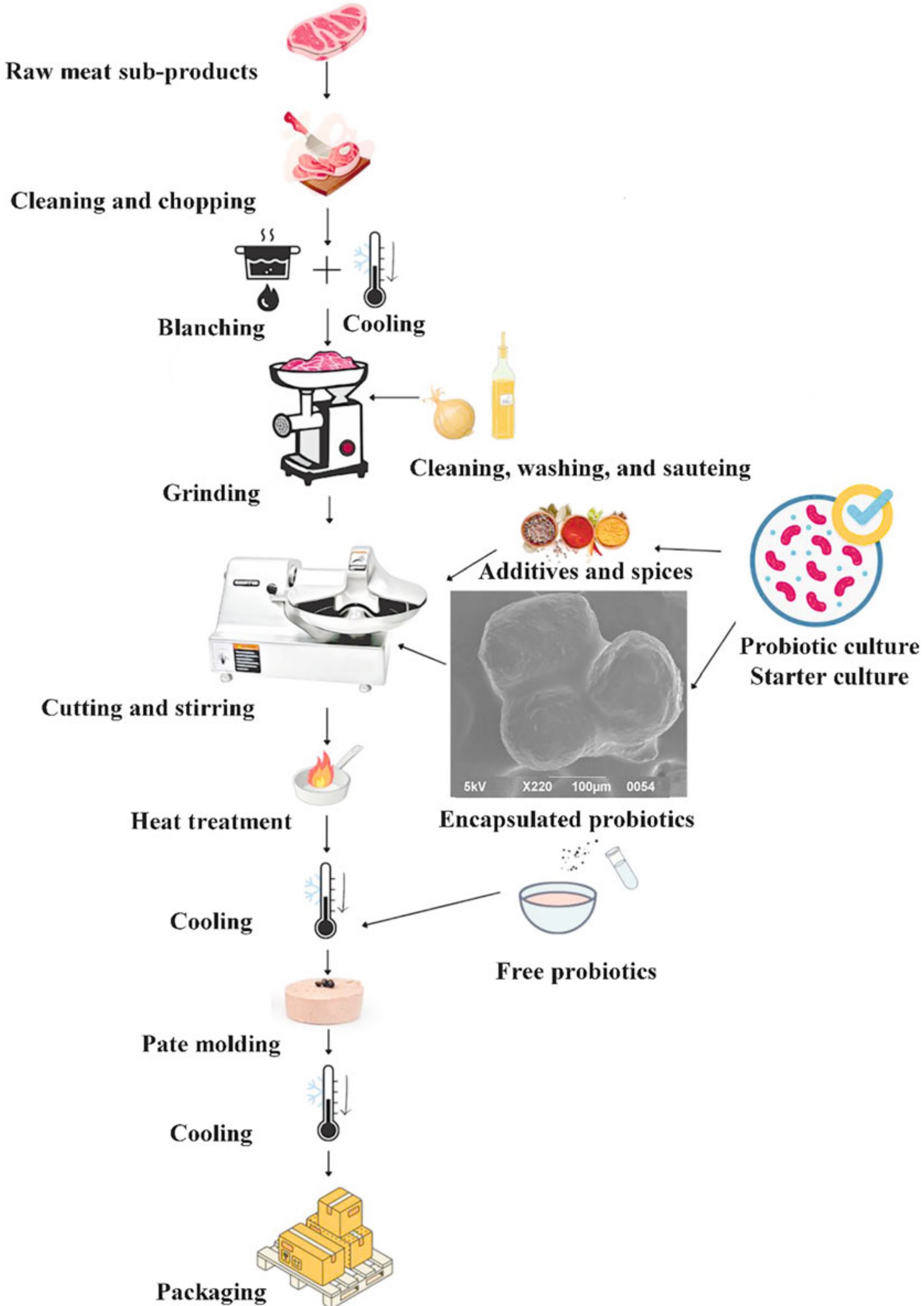

Fig. 1 Process flow diagram of liver pate production

5. Allow capsule formation to occur due to the formation of ionic cross-links.
6. Stir the resulting capsules in a calcium lactate solution for 40 min to solidify the polymer structure (*see* **Note 2**).
7. Filter the capsules through a 0.18 mM mesh size filter.
8. Wash the capsules by centrifugation twice with sterile distilled water.

3.2 Liver Pate Production

1. *Raw Material Preparation*. The liver is prepared by removing large blood vessels, residual fatty tissue, lymph nodes, and bile ducts. It is then washed in cold running water and cut into pieces weighing 300–500 g. The liver is blanched by boiling it in open two-wall cauldrons with a liver-to-water ratio of 1:3 for 15–20 min or until it is decolorized. After blanching, the liver is cooled in cold running water or on racks until its temperature reaches no higher than 12 °C.

   The brains are washed and blanched in boiling water for 10–15 min and then cooled in thin layers in bowls or on racks until they reach a temperature of no higher than 12 °C.

   To replace part of the liver and fat with meat sub-products, follow these steps:

   (a) Remove large glands, lymph nodes, bruises, impurities, and bristle residues from the pork cheeks.

   (b) Blanch the pork cheeks and fatty pork in boiling water for 15–20 min, stirring periodically.

   (c) Cut the heart in half, and remove any blood clots. Wash the heart pieces in cold water, and boil them at 95 °C for 3–4 h until softened. Then, cool them to a temperature no higher than 12 °C.

   (d) Halve the pork heads, wash them with cold water, and boil them for 3–4 h until softened. Cool the boiled heads to a temperature not lower than 50 °C, separate the fleshy part from the bone, and cool it to a temperature no higher than 12 °C.

   (e) Prepare sub-products such as pork skins by boiling and disassembling them.

   (f) Soak the lungs for 2 h, wash them, peel, and boil them for 2–4 h until softened.

2. *Onion preparation*. Onions are prepared in a separate room. Onions are peeled, all the defective parts are removed, and the remaining is washed in cold water. Onions are sauteed with butter until golden brown. After that, the onions are mixed with the boiled meat to be chopped in a grinder. It is possible to use dried onions.

3. *Preparation of minced meat*. Fat, onions, cooled liver, and brain (and other sub-products) are ground on a grinder with a grid hole diameter of 2–3 mM. The spices, sugar, and salt are added to the mixture before everything is being treated with a cutter-blender for 5–8 min to obtain a homogeneous ointment-like mass.
4. * *Introduction of encapsulated probiotics*. Encapsulated probiotics are added to the meat mixture at the end of the stirring process. The cutter should be put into stirring mode to minimize damage to the capsules. Stirring is performed for 3–5 min (*see* **Notes 3–6**).
5. *Heat treatment*. Prepared pate mass is sent for heat treatment in a stirrer–heater. Thermal processing of pate mass is carried out at 80–85 °C at continuous agitation for 60 min until reaching a temperature of 72 °C in the center of the product.
6. * *Introduction of free probiotics*. After cooling the mixture inside the stirrer, free probiotic cells are added aseptically within a water suspension, followed by stirring for 5 min (*see* **Notes** 7 and **8**).
7. *Filling*. Pate mass after heat treatment is packed in portions of 100 and 200 g in aluminum foil and other packaging materials approved by health authorities. Packed and wrapped in foil, pate is sent to the cooling chamber.
8. *Cooling*. Cooling is carried out at a temperature of 0–4 °C for no more than 10 h until the temperature in the center of the product decreases to 0–8 °C.

4 Notes

1. The successful incorporation of probiotics into emulsified meat products depends on various factors, including the selection of suitable probiotic strains, optimization of the emulsification process, and evaluation of the product's sensory and microbiological quality. There are several approaches to make a successful functional meat product, including encapsulation of probiotics, selection of heat-resistant probiotic strains, and usage of metabiotics. Despite the promising results obtained so far, further research is required to explore the potential of probiotic emulsified meat products and address the challenges associated with their production and commercialization.
2. Additionally, it is possible to use a solution of chitosan for additional fixation of the capsules.
3. *Lactobacillus acidophilus* encapsulated with calcium alginate microbeads in beef meat pates can withstand cooking at least at 120 °C (72 °C in the pate's center) [33].

4. To improve the stability of capsules, multiple coating technology can be used. Each layer of such capsule will provide additional protective feature [28, 29, 72].
5. Coating materials should be nontoxic and protect the encapsulated microbial cells in an environment that promotes cell damage. Among the available encapsulation materials, sodium alginate and chitosan are widely used because these materials are nontoxic, economical, and easy to handle [73].
6. Encapsulation of bacteria leads to their dilution by a factor of ten, since they are mixed with sodium alginate solution at a ratio of 1:9. To achieve the required concentration of bacteria in the product, the number of capsules should be selected based on the initial concentration of bacteria in the suspension and product organoleptic characteristics. If the capsule size does not exceed 300 microns, it is acceptable to add them to the product (up to 5% of the total product mass). For example, if the initial concentration of bacteria is 10^{11} CFU/g in the suspension, their encapsulation and addition to the product capsules at a concentration of 5% give the number of microorganisms in the product within 10^{8} CFU/g.
7. Free bacterial cells can be added to the mixture aseptically after the heating process. This will exclude the necessity to shield them from heat damage.
8. There are several things to consider when bacteria are added in free form. Their activity during the storage may lead to accumulation of metabolites (e.g., lactic acid), which may change the sensory characteristics of the final product. On the other hand, free probiotic cells can improve product shelf life and safety by suppressing the development of pathogenic microflora through bacteriocin production [20, 74–76].

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Chapter 3

Prebiotic Meat Products

Paulo Eduardo Sichetti Munekata, Cynthia Farias Manassi, Rubén Domínguez, Mirian Pateiro, Silvani Verruck, and José Manuel Lorenzo

Abstract

The consumption of meat products has been done since ancient times due to its high nutritional value, but recent studies have been pointing out the importance of rethinking their formulation and processing to obtain healthier and functional meat products, especially with the addition of prebiotics. Prebiotics are food components that are fermented by select gut microbiota and generate health-related physiological responses in host.

Inulin is a natural polymer that fits this concept and has a main backbone and branched chains composed of fructofuranosyl sub-units. In addition to the numerous studies supporting its health benefits, the use of inulin has been tested in many meat products with relevant results regarding the level of incorporation and impact in product quality. This chapter describes the preparation and incorporation of inulin in a traditional fermented sausage and mortadella.

Key words Inulin, Functional meat products, Fermented sausage, Mortadella

1 Introduction

The nutritional importance of meat products is supported by a list of essential nutrients found in these foods, which includes a high protein content and an elevated amount of essential amino acids as key aspects, coining meat and meat products as the main elements of modern diet [1]. Moreover, the presence of vitamins (especially B complex vitamins) and minerals (such as iron, zinc, selenium, and phosphorus) reinforces the recommendation to consume meat and meat products [1, 2]. Although these characteristics are essential for the global population, recent scientific evidence have been strengthening the correlation between the consumption of meat products and the risk of developing diseases such as cardiovascular diseases and cancer [3].

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The increasing concern and willingness to improve the quality of widely consumed meat products have been pushing researchers and professionals of the meat sector to rethink the formulation and processing of meat products. From this perspective, important terms have gained the spotlight in the discussion and developments of meat products: "functional," "healthier," and "reformulated" [4]. Moreover, the knowledge about the incorporation of health-promoting ingredients in meat products has markedly increased and lead to important advances to meet the demand for functional and healthier meat products [4, 5].

The inclusion of prebiotics has a great importance due to the low content of components capable of inducing prebiotic effects in current commercial meat products. Although the term is still under discussion for a comprehensive concept and definition, prebiotics are considered as food components that are selectively used to generate health benefits to the host microorganism, which are not limited to the intestinal area and are not necessarily viable nutrients for the host [6]. It is relevant to comment that the term "prebiotics" has been traditionally linked with fibers (e.g., β-glucans, pectins, dextrins, and inulin), but recent discussion have been generated to expand the concept in order to include other compounds such as phenolic compounds and unsaturated fatty acids [7, 8]. In this sense, the concept of "prebiotic" is expected to be a major topic of discussion among the scientific community and the society in the following years.

One key prebiotic is inulin that can be found in foods consumed around the world (such as leek, onion, and garlic) and is currently commercially explored from rich natural sources (e.g., chicory and Jerusalem artichoke) to produce concentrates with designed properties [9]. Inulin is composed of fructofuranosyl sub-units connected by β-(2,1) linkages and branched chains originating from β-(2,6) position. The characteristic degree of polymerization observed in inulin varies between 2 and 60, which differentiates from other fructooligosaccharides (degree of polymerization in the range of 3–10) [9, 10].

The non-digestibility of inulin is a key aspect related to the health benefits of this natural polymer [10, 11]. Many studies support the consumption of inulin to induce the immune system and the proliferation of beneficial gut microbiota [12] and reduce post-prandial glycemic response [13], for instance. Due to the relevant aspects supporting the development of functional meat products and the relevance of inulin as prebiotic, this chapter aims to describe, step by step, the incorporation of prebiotic (particularly inulin) into widely consumed meat products (fermented sausages and mortadella).

2 Materials

A prebiotic meat product is produced by incorporating a prebiotic ingredient into a conventional formulation of meat product. Many studies indicate that several sources of probiotics can be explored as well as that many types of meat products can be reformulated with these probiotics (Table Error! Reference source not found.). The incorporation of prebiotics (especially inulin) is generally limited to up to around 10% of formulation in most meat products (*see* **Note 1**).

In the present chapter, the selected prebiotic is inulin, and the meat products are a fermented sausage and mortadella. Inulin has been tested up to 7% (w/w) for fermented sausage formulation and around 6% (w/w) for mortadella formulation (Table 1). In order to improve standardization, the use of commercial concentrates of inulin can be of great value. In this sense, some products have been tested in scientific studies in the meat products area: Frutafit® Inulin TEX, Raftiline® ST Inulin, and Orafti®.

The major ingredient of meat products added with inulin remains lean meat and fat (fat can be replaced; please check the studies in Table 1), followed by other ingredients that characterize the sausage and/or with technological importance [14–17]. Specifically, the lean pork meat is used and accounts for more than 50% (w/w), and fat may be used at up to 20% (w/w) of formulation. Other important ingredients to obtain the expected characteristics of meat products are as follows:

- *Sodium chloride* that is involved for the solubilization of myofibrillar proteins, salty taste, and improved microbial stability.
- *Sodium nitrite* that is responsible for the formation of characteristic pink pigment along with myoglobin, inhibition of spoilage microorganism, slow lipid oxidation, and conferring cured flavor.
- *Sodium tripolyphosphate* that improves the retention of moisture and glossy visual (important quality attribute for mortadella).
- *Ice (water)* that facilitates the dispersion of ingredients in the dough and, when in solid state, assists in the preservation of the low temperature during processing.
- *Seasoning* that characterizes the sausage and may include pepper, garlic, onion, nutmeg, herbs, and others.

2.1 Equipment

The modern production of fermented sausages and mortadella relies in the use of process-specific equipment. The list below indicates the main equipment used and their role for these meat products.

Table 1
Prebiotics and potential prebiotics in meat products

Product	Prebiotic or potential prebiotic	Formulation (g/kg)				References
		Lean meat	Fat[a]	Prebiotic	Other ingredients[b]	
Low-fat mortadella	Wheat fiber (Vitacel WF200), oat fiber (Vitacel HF600), and inulin (Raftiline HPX)	600.0	50.0–85.5	50.0	270.0–300.0	[14]
Low-fat, low-salt mortadella	Inulin (Orafti GR), fructooligosaccharides (FOS) (NutraFlora P95), polydextrose (Sta-Lite® III), and resistant starch (Hi-Maize 260)	600.0	200.0	15.0–60.0	140.0–185.0	[15]
Low-fat mortadella	Inulin (Orafti GR or Orafti® HP)	443.0	187.0	33.0–67.0	183.0–339.0	[16]
Mortadella sausage with fat replacement	Inulin (Fibruline XL, Cosucra)	600.0	180.0	30.0	190.0	[17]
Fat substitute in salt-reduced Bologna sausage elaborated with chicken meat	Inulin-based emulsion gels [EG was prepared with 4% soy protein (DuPont®, Paulínia, SP, Brazil), 50% soybean oil (Liza®, Primavera do Leste, MT, Brazil), 16.5% inulin (ORAFTI GR, Clariant, Pemuco, Diguillín Province, Chile), and 29.5% water]	700.0	0–100.0	100.0–200.0	92.0–192.0	[34]
Low-fat chicken bologna sausages	Inulin powder (MyProtein) or oat fiber (Vitacel HF 200)	540	50–54	34–37	370	[37]
Low-fat dry fermented chicken sausage	Inulin (Raftiline HP-Gel)	768.0	87.0	66.0	80.0	[18]
Dry fermented sausage with fat replacement	Inulin (chicory product, Cosucra)	750.0	90.0	40.0	119.75	[19]
Low-fat fermented sausage	Fructooligosaccharides (NutraFlora® P95)	800.0	150.0	20.0	30.0	[20]
Low-fat fermented sausage	Inulin (natural powder from Jerusalem artichoke)	720.0	150.0	50.0	80.0	[21]

Low-fat Thai pork sausage	Inulin powder (Jebsen & Jessen Technology Ltd., Thailand)	611	86	36	267	[36]
Low-fat pork sausage	Hybrid hydrogel of inulin and microcrystalline cellulose	900	50	50	29	[38]
Low-fat Brazilian cooked sausage (Paio)	Orafti® inulin from Clariant and oat fiber from JRS Rettenmaier®	700.0	0.0	200.0	207.6–310.0	[33]
Restructured sausages	Dried chicory fiber powder (Lyntz Inc., Korea)	800.0	100.0	100.0	158.0	[32]
Cooked-emulsified sausages with total fat replacement	Inulin-collagen suspension [inulin 4% (Fibruline instant, Cosucra, Warcoing, Belgium) and collagen 1.65% (bovine collagen powder BPS 90; Protein Slovakia, Liptovský Mikuláš, Slovakia)]	500	0	56.5	451	[35]
Salami	Citrus fiber (Citri-Fi), inulin (Orafti HPX), and arabinogalactan (acacia fiber, Nexira)	n.i.	n.i.	20.0	n.i.	[22]
Fat replacer on beef burger	Chicory roots powder	800	50–150	50–150	400	[39]
Low-fat beef burgers	Fructooligosaccharide (Ingredion), inulin (Orafti), oat fiber (JRS Rettenmaier), and wheat fiber (Nutrassim)	700.0	100.0–200.0	30.0–60.0	190.0	[23]
Low-sodium burgers	Inulin (Orafti HP)	896.9–947.9	0.0	28.4–80.7	22.4–23.7	[24]
Meat pâté	Inulin (Orafti HPX)	300.0	112.5	125.0	462.5	[25]
Guinea fowl pâté	Inulin (Orafti HPX)	411.0	149.5	74.5	365.0	[26]

[a]Animal fat or vegetable oil

[b]Other ingredients depend on meat product formulation and may include poultry liver, ice, commercial seasoning, sodium chloride, sodium nitrite, sodium, tripolyphosphate, sucrose, and sodium erythorbate

- *Meat and fat grinder*: grind meat and fat; the size of plate can be chosen based on the expected characteristics of meat product.
- *Cutter*: consist in a rotating bowl with perpendicular blades that continuously comminute the meat, fat, and other ingredients into a homogeneous dough.
- *Stuffer*: mechanical or manual system that assists in the stuffing of dough into casings by pushing the dough through a small-diameter tube.
- *Carts*: sausages can be hanged in carts to facilitate transport from the processing area to the thermal treatment chamber.
- *Thermal treatment chamber*: a chamber with controlled temperature where sausages are heated following specific conditions to achieve pasteurization of sterilization.
- *Refrigerated chamber*: a chamber with controlled temperature where thermally treated sausages can be stored at a low temperature (preservation and shelf-life evaluation).

3 Methods

Production of Fermented Sausage

The production of fermented sausage with inulin can be performed following the method reported by Glisic et al. [19]. The formulation proposed for the prebiotic fermented sausage is indicated in Table 2. The production process (Fig. 1) is repeated in three different days.

Table 2
Formulation of fermented sausage with inulin as prebiotic

Ingredient	Proportion (g/kg)
Lean meat	750.0
Pork back fat	90.0
Inulin	160.0
Sodium chloride	23.0
Curing salt[1]	0.32
Spice mixture	4.0
Starter culture	*Lacticaseibacillus casei* and *Staphylococcus carnosus*

Adapted from Ref. [19]

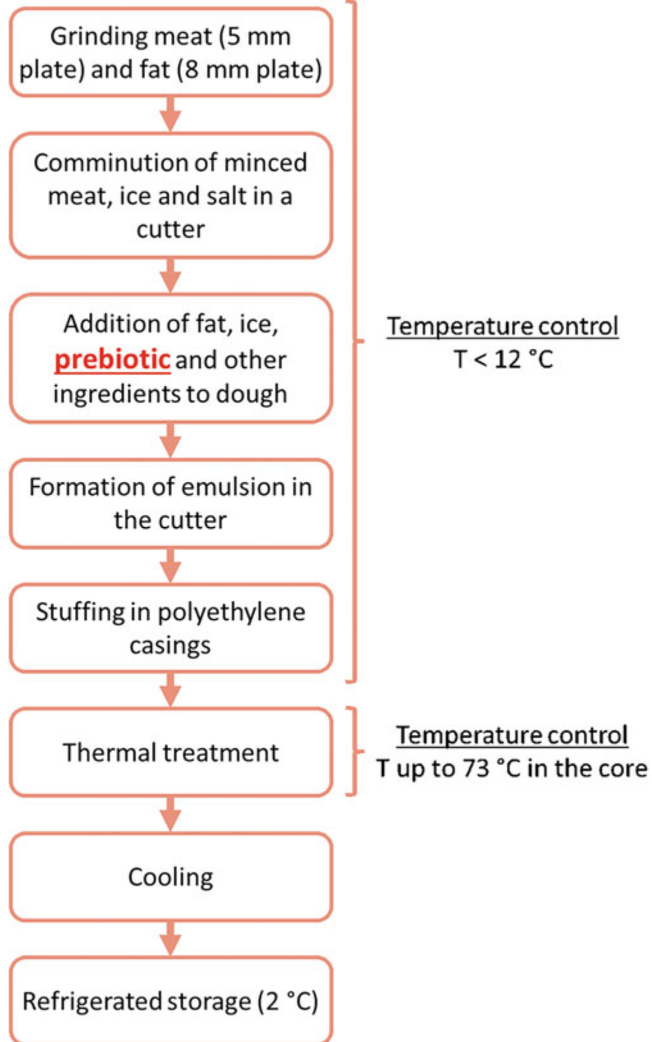

Fig. 1 Fermented sausage production flowchart

The processing initiate by chopping and grinding the meat and fat and mixing them. The following step involves the mixing of ingredients (lean meat, fat, prebiotic, and other ingredients). At this stage, inulin can be added in the form of powder or gel (*see* **Note 2**). Once the prebiotic is added, the remaining ingredients can be mixed for a couple of minutes until complete homogenization. The following step consists in stuffing of meat dough into casings. Natural casings have been traditionally used and provide the adequate conditions for the next stages of processing: fermentation, smoking, and ripening.

Fermentation is a crucial stage to ensure the growth of key microorganisms in the meat dough that improves standardization of quality among batches. This stage is characterized by a short

period, specific temperature (> 20 °C), and elevated relative moisture (>80%), which eventually favor the growth of microorganisms and modification of meat dough characteristics. The dough can be fermented at 24 °C and 91% relative humidity for 48 h. A pH drop is expected (down to values in the range of 5.0–4.5) during this period due to microbial activity [19]. Once the fermentation is complete, the sausages are smoked and ripened to specific conditions to promote important physicochemical changes and biochemical reactions.

Smoking is an important process that can modify the characteristics of sausages. It is important to remember that smoking is one of oldest methods to preserve meat and consists in the exposure of meat or meat products to the smoke of wood (condensation of vapor phase that deposits smoke-generated compounds on the surface of meat or meat products) to improve their preservation [27]. In the procedure indicated by Glisic et al. [19], this stage can be carried out at 21–23 °C with relative humidity of 85% for 8 h for 3 days.

The ripening stage is traditionally characterized by a long period where sausages are gradually and continuously dehydrated to reduce moisture and water activity. Physicochemical and biochemical reactions associated with the development of sensory properties (especially flavor and taste) are also promoted [27]. Sausages can be ripened for 28 days at 15 °C and relative humidity of 85% [19]. The final product (*see* **Note 3**) is characterized by a firm texture, dark red color, dried external surface, characteristic aroma and flavor of fermented and ripened sausage, reduced moisture (around 30%), pH below 5.4, reduced water activity (<0.85), and high counts of lactic acid bacteria and *Micrococcaceae* (> 6 log CFU/g) [19].

Production of Mortadella

The production of mortadella can be carried out using the methodology proposed by Biasi et al. [14]. The entire processing is performed thrice on different days. Table 3 indicates the proportion of ingredients.

The flowchart presented in Fig. 2 indicates step-by-step instructions to produce mortadella. The first step is to chop and grind the meat and fat, which can be carried out using a meat grinder with plates of 5 and 8 mM, respectively. After that, the ground meat and other key ingredients (salt and half of the total amount of ice) are mixed in a cutter until a homogeneous dough is obtained. This homogenization stage in the cutter takes few minutes to be accomplished.

Table 3
Formulation of mortadella with inulin as prebiotic

Ingredient	Proportion (g/kg)
Lean meat	600.0
Animal fat	14.5
Ice	237.0
Mortadella seasoning	4.3
Sodium nitrite	0.2
Sodium tripolyphosphate	3.0
Salt (NaCl)	25.0
Sodium erythorbate	0.5
Inulin	***50.0***

Adapted from Ref. [14]

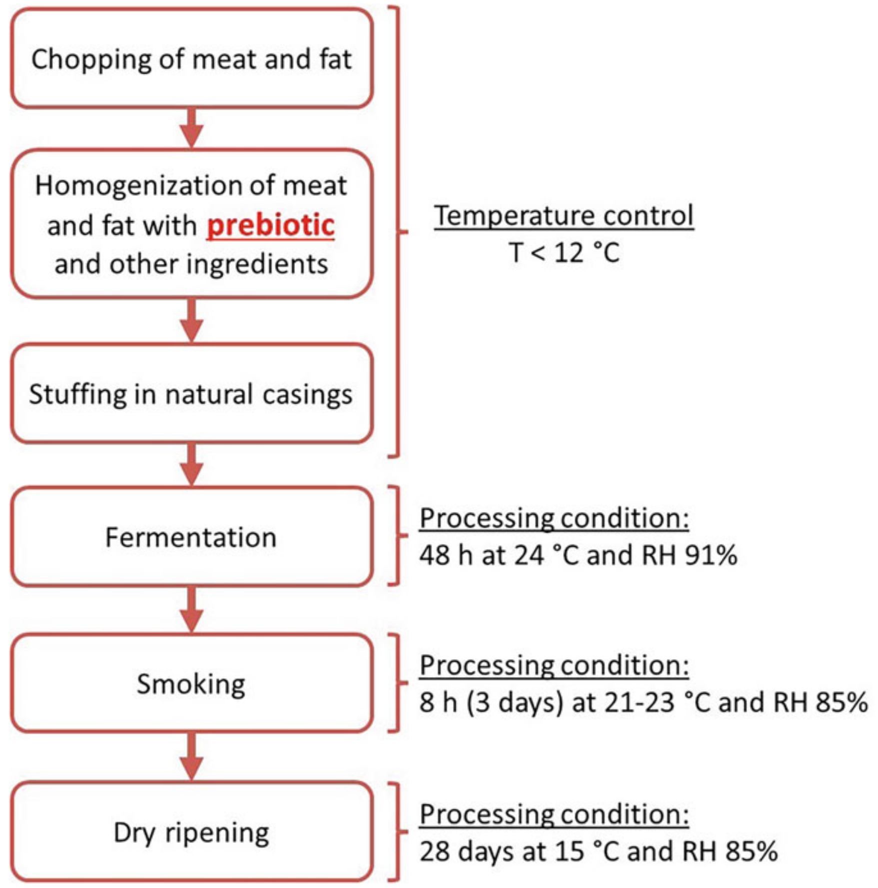

Fig. 2 Mortadella production flowchart

Then, the fat, remaining ice, prebiotic, and other ingredients (e.g., seasoning and additives) are added to the dough (*see* **Note 2**). Again, the dough is mixed in the cutter for few minutes to obtain a homogeneous aspect. After that, the mortadella dough is stuffed in artificial casings and hanged in cart for thermal treatment.

The thermal treatment is carried out in sequential stages where temperature is gradually raised: heat until 60 °C, hold for 30 min, and then heat at 80 °C until the core reaches 73 °C. As soon as the thermal treatment is finished, the sausages are cooled (cold water until reaching 15 °C) and stored at refrigeration temperature (2 °C).

The final product presents the characteristic pink color of mortadella, smooth internal surface when sliced, homogeneous continuous phase with glossy aspect, and (when added) visible ingredients such as cubes of fat. Additionally, it is relevant to comment that the form of inulin incorporation (powder vs. gel) can affect the properties of the final product (*see* **Note 4**).

4 Notes

1. Although a definitive concentration of inulin has not been defined, the recommend daily intake to obtain health benefits (especially in patients carrying chronic diseases) varies between *3.5* and *10 g/day* (up to 25 g/day in some cases). In the case of infants, lower daily consumption doses (around 0.8 g/day) seem to provide important health benefits (against immune diseases and atopic dermatitis and recovery and well-being during diarrhea episodes). Therefore, the concentration for the inclusion of inulin into meat products should consider the indicated range of concentration *(3.5–10 g/day*). Additionally, a high consumption of inulin (40–50 g/day) can generate side effects such as osmotic diarrhea [28].
2. The incorporation of inulin can be done in either powder or gel forms. In the case of powder form, the previous dilution into sterile water is recommended to facilitate the dispersion into the meat dough [18, 29]. When the incorporation of inulin is preceded by a gelification process (particularly relevant for incorporation of healthier oils such as linseed oil, rich in linolenic acid content), the gel containing inulin can be formed with a gelifying agent (such as animal gelatin) and then frozen [19, 29]. In this sense, the gelified inulin can be chopped and incorporated into the meat dough for sausage preparation. However, it is important to remember that gelification of inulin is dependent on polymer characteristics, especially the size of the main backbone chain and the highly branched structure [30, 31].

3. Scientific evidence suggests that the scores for sensory attributes of fermented sausages are also improved by the incorporation of gelified inulin, but the effect of inulin gel as emulsifier with a healthier oil in the sensory properties remains a challenge [19].
4. The inulin state should be considered when incorporating inulin into the meat dough. Adding inulin in powder form was found to improve the texture properties, while its incorporation in gel form did not affect the texture of cooked meat batters [29].

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Chapter 4

Synbiotic Fermented Meat Products

Lujuan Xing, Jiaming Cai, Ming Ju, Jian Zhang, and Wangang Zhang

Abstract

With the increase of health awareness, consumers are focusing on strategies to maximize health-promoting compounds in meat products. Therefore, the use of prebiotic and probiotic in meat products has grown considerably. Additionally, the application of novel functional compositions (probiotic microorganisms, fibers, polysaccharides) in meat products is a novel strategy that improves the fermenting and functional properties of meat products. In order to clarify the use of functional components in the processing of synbiotic fermented meat products, this chapter focuses on the characteristics of synbiotic components as well as the production methods of fermented meat products. The objective of the content is to provide references for the production of synbiotic fermented meat products.

Key words Meat product, Fermentation, Prebiotic, Probiotic, Function, Flavor

1 Introduction

Meat and meat products are essential nutritional resources in the human diet as they can supply high-quality proteins and vitamins as well as minerals of zinc and iron. Fermented meat, such as sausage, salami, and dry-cured hams, is an important part of meat products and has a special flavor because of the long or short terms of fermentation. During fermentation, the protein and fat in meat would be hydrolyzed by microorganisms or endogenous enzymes along with the generation of free amino acids, fatty acids, peptides, and other flavor substances. All over the world, the consumption of fermented meat has become extremely common for people [1]. During processing, the nitrate salts are usually supplemented with fermented meat as they can improve the sensory attributes and oxidative stability, as well as suppress microbial growth to extend the shelf life of meat product [2]. However, according to epidemiological investigation, the consumption of processed or fermented meat products will potentially increase the health risks as the saturated fatty acids, cholesterol, salt, and other additives inside are associated with the generation of chronic diseases, including

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diabetes, cardiovascular issues, and intestinal cancer [3]. Thus, consumers are also trying to seek safe and high-quality fermented meat products with less chemical compounds inside.

Generally, the fermentation process would rely on the starter cultures, such as lactic acid bacteria, staphylococcus, micrococcus, and yeast. To improve the nutritional and functional properties of fermented meat, synbiotic fermentation has attracted more attention in recent years. During processing, the probiotics and prebiotics would be supplemented together in the synbiotic fermented meat products, which are posed to be an excellent matrix to deliver living bacteria into the body. Without heat treatment, the consumption of fermented meat products would increase the survival rate of probiotics and then exhibit their health-regulating effects for consumers. In addition, prebiotics could also be added into meat products, such as inulin, κ-carrageenan, chia flour, and others, which are commonly used as replacements of pig fat and pose improvements to the texture properties of fermented products. Normally, the prebiotic has the capacity of resisting the human digestive process as it could not be digested by the human body, and thus the prebiotic can arrive at the colon and finally be utilized selectively by the intestinal microorganisms.

According to recent studies, the application of probiotics in fermented meat products, such as fermented sausages, dry-cured ham, fuet, and salami, has already been tested to be successful. On the other hand, the supplement of synbiotic strains will improve the flavor of meat and can be used as a strategy to produce healthier and safer products. In an in vivo study, the synbiotic swine sausages with prebiotics of inulin were shown to regulate the intestinal microbiota of healthy rats, which also changed the metabolome in fecal and plasma samples. Compared with the control, the abundance of *Bifidobacterium* was increased along with the improvement in the fecal concentration of AGCCs [4]. In another study, the addition of citrus fibers in salami showed to have an anti-inflammatory effect in mice as biomarker, c-reactive protein, and tumor necrosis factor-α (TNF-α) were suppressed than in controls [5].

With the development of microbiome science, the application of probiotics and prebiotics is also extending, which forms new formulas in the production of meat products. Based on this situation, the utilization of probiotics and prebiotics may also endow functional properties to fermented meat products. In this chapter, the types of probiotics and prebiotics and the function and flavor characteristics of fermented meat products are summarized, which is hoped to provide references for the production of synbiotic fermented meat products.

2 The Probiotics in Fermented Meat Products

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" FAO/WHO [6]. The majority of probiotics includes *Lactobacillus*, *Bifidobacterium*, *Escherichia*, *Saccharomyces*, and *Enterococcus* [7]. Probiotics have a wide range of health-improving properties, which include maintaining a healthy intestinal microflora, synthesizing vitamins, boosting the immune system, lowering cholesterol, and reducing lactose intolerance [8–10]. Here, *Lactobacillus* is the most widely used probiotic in processing fermented foods and also a crucial dominant flora in traditional fermented meat products, which can directly impact the overall qualities of meat products [11, 12]. During fermentation, lactobacilli can produce amounts of metabolites, including hydrogen peroxide, rhodopsin, bacteriocins, ethanol, acetaldehyde, lactic acid, acetic acid, and butanedione [13]. As a result, the application of lactobacilli showed to enhance the flavor components, lower biogenic amine content, and improve the antibacterial and antioxidant properties of fermented meat products [14–17]. In addition, the coccus bacteria isolated from fermented meat products are also considered to be potential candidates for probiotics. Identified from fermented meat products, *Staphylococcus* sp. *DBOCP0* was demonstrated to inhibit the growth of *E. coli MTCC 40* and remain active in the gastrointestinal environment [18]. Additionally, as conducted by Yuksekdag and Aslim [19], *Pediococcus pentosaceus* Z12P and Z13P were isolated from the traditional Turkish sausage Sucuk, which also showed to be potential probiotics [19]. In beef, the inoculation of the probiotic *Lactiplantibacillus plantarum* TN8 was shown to improve the color parameters of fermented beef, whereas the lipid and protein oxidation level was checked to be suppressed. Simultaneously, the variations in meat quality had a dosed manner with the concentration of inoculation of strains [20]. In Tunisian dry fermented sausage, the addition of potential probiotics, *L. plantarum* and *Staphylococcus xylosus*, inhibited the growth of Gram-negative bacteria and also increased the sensory properties of sausage, which may be related to the nitrate reductase, protease, and acidifying activity of these strains [21]. In Iberian dry fermented sausages, the inoculation with *Limosilactobacillus fermentum* HL57 increased the content of acetic acid and lipid degradation products, which promoted the sensory parameters of sausage [22].

Over the centuries of development, the fermentation of foods has evolved gradually from a process driven by unknown microorganisms naturally to the subjective selection of starter cultures with special functions [23]. Different types of starter cultures endow different functions to fermented foods. The primary performance of starter cultures relies on their ability to produce

bacteriocins to reduce the growth of spoilage and pathogenic bacteria [24, 25]. Some starter cultures can reduce the content of biogenic amines [26, 27]. In addition, the starter cultures can also endow unique flavor to fermented meat products by promoting the hydrolysis of fats as well as the formation of aromatic substances [28, 29]. Among different starter cultures, probiotics are receiving increasing attention because of their potential health benefits for humans [30, 31]. Some probiotics could also be used as the primary starter cultures to produce the fermented meats. For example, *Bifidobacterium longum* KACC 91563 was able to reduce lipid oxidation levels and increase the total unsaturated fatty acid content in fermented sausages [32]. *Lacticaseibacillus rhamnosus* CTC1679 reduced the number of *Listeria monocytogenes* and *Salmonella enterica* in fermented sausages [33]. In the dry fermented sausages, the *Bifidobacterium animalis* subsp. *lactis* BB-12, *L. rhamnosus* LOCK900, and *Lactobacillus acidophilus* Bauer were added separately to compare their effects on the meat quality, where the group with *Bifidobacterium* was checked to have a higher pH than other formulations [34]. In contrast, the group of *L. rhamnosus* and *L. acidophilus* exhibited a higher acidification rate, respectively, demonstrating the *Lactobacillus* was suitable to grow in a higher acidic condition. Similarly, the strain of *L. rhamnosus* LOCK900 was tested to be starter culture in fermented sausages and loin, where these two strains could compete with other microflora in meat products and maintain a stable content during the storage of 6 weeks [35]. In special, the content of lactic acid was elevated significantly, which was effective to suppress the growth of harmful microorganisms. Currently, some research is also focusing on the regulatory effects of probiotic fermented meat products on human health. Jahreis et al. [36] studied the effect of probiotic (*Lacticaseibacillus paracasei* LTH 2579) sausages on blood lipids and immunological parameters in healthy volunteers [36]. In fecal samples, a significantly increasing trend in *L. paracasei* LTH 2579 counts was observed in volunteers with a supplement of probiotic sausages. In addition, the expression of CD54 (ICAM-1) on lymphocytes decreased significantly after consuming probiotic sausage, but there was no significant influence on the cholesterol and triacylglyceride content in serum. Generally, the probiotics used in meat products appear to be suitable carriers to improve the quality as well as the function of fermented meat products, especially for non-heat treatment meat products.

3 Application of Prebiotics in Fermented Meat Products

As defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP), prebiotics is the substrate selectively utilized by host microorganisms conferring a health benefit [37]. By

definition, the prebiotics includes fibers, polyphenols, and polyunsaturated fatty acids (PUFA), as well as conjugated linoleic acid (CLA). The health-improving effects of prebiotics have all been reported, such as regulating the gastrointestinal tract to inspire the immune responses, helping the cardiac metabolism to control the blood lipid levels, and promoting the mental health by regulating the brain function, as well as increasing the bone fortification by lifting mineral bioavailability [38]. Dietary fibers, such as inulin and dextran, have been widely used as fat replacements in the production of new low-fat fermented meat products [29, 39]. From a health perspective, the combination of probiotics and dietary fibers (prebiotics) in producing fermented meat products can bring multiple benefits to consumers [40]. Sirini et al. [41] supplemented chestnut flour and *L. plantarum* to Spanish dry-cured sausage and found that the lactic acid bacteria content was improved without modifying the product flavor [41]. Coelho et al. [42] added *L. paracasei* LPC02 and lactulose to the fermented sausages and reported that the addition of probiotics and prebiotics significantly reduced the nitrite content without affecting the sensory attributes [42]. Defined by the ISAPP, synbiotics are a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confer a health benefit on the host [43, 44]. Yang et al. [45] reported that synbiotic formula (containing *Bifidobacterium*, inulin, etc.) significantly improved the intestinal ecosystem of rats; meanwhile, the digestive enzyme activity and the number of probiotic bacteria in the feces were all upgraded [45]. Inulin had the effect of promoting the growth of *Bifidobacterium* in the colon, which in turn improves the lactose intolerance in humans by secreting lactase into the intestine [46, 47]. Pérez-Burillo et al. [5] investigated the health benefits of salami fermented with *L. rhamnosus* HN001 and citrus fiber [5]. The 24 healthy volunteers were supplemented with a daily intake of 30 g of salami for 4 weeks. Compared with the control group, the salami showed an anti-inflammatory effect by suppressing the secretion of CRP and TNF-α in the serum of volunteers. At the same, the antioxidant biomarkers, as well as butyrate production, also increased with the supplement of salami. Given the positive impact of prebiotics or probiotics on organism health, the combination of synbiotics also offers a new direction for the development of fermented meat products. Thus, the extraction and selection of dietary fiber from plant by-products (fruit peels, leaves, etc.) in combination with prebiotics to form synbiotics is a sustainable solution to improve the health parameter of meat.

4 Bioactive Compounds in Synbiotic Fermented Meat Products

4.1 Bioactive Peptides

Bioactive peptides are generally consisted of 2–20 amino acids and possess diverse biological functions for human nutrition and health [48], such as antioxidant, antihypertensive, antidiabetic, and anti-fatigue [49]. During the fermentation, curing, ripening, and storage stages of meat products, the bioactive peptides may also be produced through the hydrolysis by microbial proteases and endogenous enzymes Keska et al. [50]. Probiotic starter cultures, such as *L. acidophilus* and *Limosilactobacillus reuteri*, can enhance the ACE inhibitory capacity of bioactive peptide extracts in fermented meat products [51, 52]. The mixture of *L. acidophilus* Bauer and *Bifidobacterium animalis* ssp. *lactis* BB-12 can increase the free radical scavenging abilities of the peptides (MW $<$ 3 kDa) isolated from dry-cured loins [53]. In addition, the lactic acid bacteria can not only contribute to the probiotic properties of fermented meat products but also improve the products' safety by producing antimicrobial peptides [54]. Bacteriocins is a classical antimicrobial peptide, of which nisin [55], curvacin A [56], and sakacin P [57] are widely used in the meat industry. Moreover, Pinto et al. [58] reported that the addition of 3% inulin and probiotics modified the proteolytic pattern of synbiotic fermented products and promoted the formation of diverse peptides by increasing enzymatic or chemical modifications [58]. These studies indicate that the addition of synbiotics can increase the generation of novel peptides derived from meat products and thus enhance the health benefits of fermented meat products.

4.2 Amino Acids

Nutritionally, meat product is abundant in essential amino acids, including lysine (Lys), tryptophan (Trp), phenylalanine (Phe), methionine (Met), threonine (Thr), isoleucine (lle), leucine (Leu), and valine (Val). The content of amino acids in meat products is influenced by the animal species, the processing conditions, and the type of starter cultures. Free amino acids, such as Trp, Tyr, and Met, have been reported to have an antioxidant capacity, of which Trp could also promote the intestinal barrier and immunomodulatory activity [59, 60]. Meanwhile, the composition of amino acids affects the biological function of peptides. Generally, the antioxidant peptides are abundant in hydrophobic amino acids [61]. The peptides contain Ala, Val, arginase (Arg), Pro, tyrosine (Tyr), and Trp at the C-terminal, which contribute to a high ACE inhibitory activity [62]. In the fermented meat products, the application of *Lactobacillus* and *Pediococcus* was shown to improve the protein hydrolysis along with the generation of small peptides and amino acids, which exhibited anti-inflammatory and antioxidant activities, and maintained energy balance [63]. As such, *Lactobacillus casei* LOCK 0900 could increase free amino acid and peptide

content of dry-cured pork loins, probably through the hydrolysis of intracellular peptidase and protease [64]. Meanwhile, the combination of *L. rhamnosus* LOCK900 and *Bifidobacterium animalis* subsp. *lactis* BB-12 changed the distribution of free amino acids in dry-aged pork loins by increasing the content of Ser, Asp, and His [65]. In addition, the essential amino acids, such as Met, Lys, Leu, and Phe, were increased by the addition of fructooligosaccharides (FOS) and *L. rhamnosus* GG in synbiotic low-fat Spanish *Salchichón* [66]. Therefore, the supplement of synbiotics changes the distribution of amino acids in food matrix proteins, which provides a new research idea for the improvement of the nutritional properties of fermented meat products.

4.3 Fatty Acid

The content of fat is an important factor affecting the sensory and nutritional properties of fermented meat products [67]. During the fermentation of meat products, the fat is gradually hydrolyzed into fatty acids by lipase. In addition, the fatty acid composition is also related to the health and nutritional characteristics of fermented meat products [68]. Based on the structure, the fatty acids are divided into saturated fatty acid, monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA). From a health point of view, the excessive consumption of saturated fatty acids can increase the content of low-density lipoprotein (LDL) in the body and raise the risk of cardiovascular diseases [69], while unsaturated fatty acids have been reported to decrease serum cholesterol and LDL cholesterol [70] and are considered health-promoting nutrients. Therefore, changing the fatty acid composition of fermented meat by supplementing symbiotic components is another strategy to improve the health attribute of meat products. Ozer and Kilic [71] reported that *L. plantarum* AB20-961 and *L. plantarum* DSM2601 were applied to increase PUFA content and produce a high yield of conjugated linoleic acid (CLA) in semidry fermented sausage [71]. The CLA and conjugated linolenic acid (CLNA) can contribute to human health, which have anti-obesity, antidiabetic, and anti-atherogenic functions and boost immunity [72]. Widely existed in fermented food, bifidobacteria, LAB, and propionibacteria all have been reported to produce CLA and CLNA [73], which also acted as the probiotic starter cultures to improve the nutritional value of fermented meat products. Inulin is a popular prebiotic that promotes the growth of probiotics and has special gelling characteristics. It can be applied in low-fat meat products (e.g., Bologna sausages) for reducing the harmful risks of a high-fat diet and also increasing the nutritional properties of meat products [74, 75]. In a randomized, double-blind, and parallel-design study, the synbiotic (*L. acidophilus* and inulin) could decrease saturated fatty acids and increase unsaturated fatty acids in the red blood cells of subjects [76]. The synbiotic containing FOS, inulin, and *Bifidobacterium lactis* LAFTI B94 has been reported to increase CLA

content in cheese [75], while a free fatty acid profile in synbiotic fermented meat products is still a new research area and needs further development for in vivo testing.

5 The Flavor of Synbiotic Fermented Meat Products

Flavor is a crucial quality index of meat products, and it largely decides the acceptance of consumers. The common volatile flavor compounds include aldehydes, ketones, alcohols, esters, and nitrogen-containing and sulfur-containing compounds, and the combination of these compounds together forms the unique flavor profile of meat products [77]. During processing, there are many factors influencing flavor generation such as temperature, humidity, culture of spices, and also production formula. Among those, the application of starter cultures is especially important since it can greatly contribute to the special flavor and aroma of the products by following four possible pathways. Firstly, the lipid β-oxidation reaction can be accelerated with the action of lipid enzymes secreted by bacteria, and the products of the reaction, like linear aldehydes, ketones, and alcohols, are crucial aroma contributors to fermented meat products [78]. Next, the esterase activities of bacteria are beneficial for the formation of ester compounds with fruity and sweet aroma [79]. In addition, amino acid catabolism caused by bacteria is also involved in the flavor development of fermented meat products. For example, sulfur-containing amino acids like methionine and cysteine can be degraded under the catalysis of bacteria into sulfur-containing flavor compounds which are considered as crucial aroma substances due to their relative low thresholds [80]. Lastly, carbohydrate fermentation is also an important origin for the generation of flavor, where the sugar can be hydrolyzed by bacteria to generate the flavor compounds like 2-butanone with a fruity aroma and acetic acid [81]. Till now, the effects of probiotics on the flavor profile of fermented meat products have been reported in traditional meat products [2]. Klingberg et al. [82] produced the Scandinavian-type fermented sausages with the addition of *L. plantarum* MF1291 and MF1298 and *Lactiplantibacillus pentosus* MF1300, respectively [82]. Finally, there were no significant differences among the probiotic and commercial starter culture groups (*Latilactobacillus curvatus* HJ5 as control), which implied that the addition of probiotics did not bring negative effects on the overall flavor of fermented sausage, and these three stains could be considered as ideal candidates of probiotic starter cultures for the manufacture of fermented sausages. A similar finding was also reported by Muthukumarasamy and Holley [83], where the flavor profile was not changed in fermented sausage produced by *L. reuteri* as a probiotic [83]. However, when the three strains of probiotic starter cultures (*L. rhamnosus* LC-705,

E-97800, or GG) were used to process fermented sausage, the sensory showed that *L. rhamnosus* E-97800 or GG group had a similar flavor profile as the control, while *L. rhamnosus* LC-705 had an unsatisfactory flavor when compared with the commercial starter culture, namely, *Pediococcus pentosaceus* [84]. The above reports all demonstrate that the addition of probiotic starter culture singly could not contribute to the flavor improvement of fermented meat products.

Recently, the combination of prebiotic saccharides and probiotic starter cultures was also applied in the production of fermented meat products since prebiotics can exert a synergistic effect to guarantee the viability of beneficial microorganisms in the human intestines [85]. The flavor of dry coppa processed with the addition of probiotics (*Bifidobacterium animalis* subsp. *lactis* BB-12) and synbiotics (BB-12 and inulin) was improved compared with the control group [86]. In another study, the influence of adding probiotics and/or prebiotics on dry fermented sausage was investigated during manufacture [42]. Four batches of sausages were processed: one was set as control, and the other three were treated with *L. paracasei*, lactulose, and the combination of lactulose and *L. paracasei*, respectively. The results showed that the supplement of probiotics and/or prebiotics did not impair the purchase intention and the sensory acceptance of final products. In the PCA model, the group of synbiotics was separated from the other groups, which was described as having characteristic aroma and acid and salty taste. Furthermore, the changes of specific flavor volatile compounds were evaluated in fermented sausages by supplying the synbiotic composed with fructooligosaccharides and two strains of probiotics (*L. rhamnosus* and *L. paracasei*) as a partial substitution of fat [30]. The study pointed out that the synbiotic supplement distinctively increased the ester compounds (especially butanoic and hexanoic acid ethyl esters) with pleasant aromas, which could be attributed to the esterase activity of lactic acid bacteria by accelerating the esterification reaction of alcohols and fatty acids. Meanwhile, the synbiotic supplement significantly reduced the content of hexanal with an unpleasant odor. To conclude, the synbiotic addition was potential for the flavor improvement of fermented meat products. However, reports about the effects of adding synbiotics or single probiotics on the flavor of fermented meat products are quite limited. Most of those published reports performed an overall flavor analysis by sensory evaluation, and only a few focused on the specific flavor compounds. Thus, more work needs to be done about the specific flavor compound changes induced by the addition of synbiotics or single probiotics in fermented meat products, and the involved influential mechanism should also be investigated and elucidated in the future.

6 The Production Process of Fermented Meat Products

6.1 Materials

6.1.1 Raw Meat

Generally, the material for the production of fermented meat is consisted of lean meat, fat, salt, additives, and spices as well as the casings. Meat and fat are the two major ingredients in most fermented meat products. Consequently, their characteristics strongly affect the sensory, nutritional, safety, and health aspects of the products. In special, pork is the main material for fermented meat products; the species such as beef, lamb, chicken, goat, and turkey and different types of game are also used (*see* **Note 1**).

6.1.2 Starter Cultures

Traditionally, fermented products depend on wild microorganisms, which usually do not conform to any specific species but are typically related to *L. plantarum*. In the United States, *L. plantarum*, *Pediococcus pentosaceus*, and *Pediococcus acidilactici* are the most commonly used starter cultures. In Europe, the most common include *L. plantarum*, *Pediococcus pentosaceus*, and *Staphylococcus xylosus*. Up to now, the combined starter cultures are available in which one organism produces lactic acid and another improves desirable flavors (*Micrococcaceae*, *Latilactobacillus brevis*, *Latilactobacillus buchneri*). Most of the starter cultures used in production are freeze-dried bacteria. In advance of the inoculation, the starter cultures are usually revived at room temperature for 18 ~ 24 h, and the inoculation concentration is generally 10^6 ~ 10^7 cfu/g.

6.1.3 Other Ingredients

Salt is the major additive in fermented meat products, and the supplement content is 2–4%, which allows LAB to grow and inhibit several unwanted microorganisms. Nitrite is supplemented among 80–240 mg/kg for antibacterial, color, and antioxidant purposes. Nitrate and nitrite are often used in combination, but nitrate is usually not necessary, except as a reservoir for nitrite, which can be useful in long-term processing. Simple sugars, such as glucose or dextrose (0.5% total, a minimum of 0.75% is recommended), can be readily utilized as a fermentation substrate. The quantity of sugar influences the rate and extent of acidulation and also contributes favorably to flavor, texture, and yield. The addition of dextrose will influence the final pH value of the product, and additional sugar will not decrease pH further since bacterial cultures cannot grow in excess acid. Spices (e.g., pepper, cardamom, allspice, paprika, ginger, mace, cinnamon, garlic) are often supplemented in the fermented meat, which are used for flavor and to impart antioxidant properties and in order to stimulate the growth of lactic bacteria.

6.2 The Method

Formulations are numerous even for products with the same name. As for the fermented sausage, the general method includes fermentation, casing, smoking, drying, and packaging (*see* **Note 2**). During processing, the time, temperature, humidity, and smoke all

affect the quality of the final product. Here the fermented sausage was listed as an example to illustrate the production process of fermented meat products.

6.2.1 Fermentation

In general, a higher temperature and a greater water activity result in faster speed for the production of lactic acid. In Europe, fermentation temperatures range from 5 °C to 26 °C, whereas the lower temperatures are applied in the Mediterranean area and the higher temperatures in Northern Europe. Thermostatic fermentation is widely applied in industrial production process; the temperature is 21–24 °C, relative humidity is 75–90%, and fermentation time is 1–3 d. For semidry fermented sausage, the temperature is controlled at 30–37 °C, the relative humidity is 75–90%, and the fermentation time is 8–20 h. The semidry products are usually fermented at temperatures that increase to over 35 °C, which has a good effect to shorten the times within 12 h. Half-dried sausage is usually fermented for 3 days at 7 °C, 3 days at 27–41 °C, and 2 days at 10 °C and then heated to 58 °C for 4–8 h. Depending on the tradition and product type, the smoking process is mainly used to improve the flavor of sausage. Here, the sausage needs to be wrapped in casing before being smoked, and the smoking temperature is 15–20 °C.

6.2.2 Casing

Casing types include natural casings, collagen casings, artificial casings, and fibrous casings, as well as cloth bags. The casing material has the property of allowing penetration and elimination of air or the smoking gas. During casing, the temperature should be controlled under 4 °C.

6.2.3 Drying

The degree of drying affects the physical and chemical properties, edible quality, and shelf life of fermented meat products. The temperature of the drying is generally controlled at 7–13 °C, and the relative humidity is controlled at 70–72%. The drying time depends on the diameter of the final product. Generally, the ripening time of the dry fermented sausage is generally 10–90 d.

6.2.4 Packaging

In order to facilitate transportation and storage, preserve the color, and avoid the oxidation of meat, the fermented sausages are usually packaged after drying. At present, vacuum packaging is the most widely used packaging method (*see* **Note 3**).

6.3 Notes

1. During the processing of fermented meat, the property of raw meat will also affect the final quality of the products. Here, the temperature of raw meat is generally controlled at 0–4 °C, and the temperature of fat is controlled at −8 °C. The pH of raw meat should be controlled at 5.6–5.8.

2. In the preparation of raw materials, it is necessary to mix the lean meat first and then add the fat.
3. In order to prevent the mixing of air, a vacuum chopping machine is recommended during chopping as it has a good effect to improve the homogeneity of minced meat.

7 In Conclusion

With the enhancement of consumer awareness, the addition of probiotics, prebiotics, or synbiotic components in fermented meat products is gaining popularity. This chapter reviewed the types of probiotics and prebiotics and the functions and flavor characteristics of synbiotic fermented meat products, such as salami, fermented sausages, and dry-cured pork loins. In general, the supplement of synbiotics pointed to the success of fermented meat as they can act to be the starter cultures, fat substitutes, or functional components to improve the healthy attribute of meat products. However, few in vivo studies were conducted with the consumption of probiotics, prebiotics, or synbiotic meat products of the human host, which is necessary to demonstrate the health property of synbiotic components in the meat matrix. In the future, the development of prebiotic types in meat products and their performance testing, as well as the synergistic effects of prebiotics and probiotics, also need further investigation.

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Chapter 5

Fermented and Structured Meat Products with Dietary Fibers for Reducing Fat Content

Paulo Eduardo Sichetti Munekata, Rubén Domínguez, Mirian Pateiro, Eva Maria Santos, Alfredo Teixeira, Marcelo Rosmini, and José Manuel Lorenzo

Abstract

Dietary fibers consist of food components that are affected by the digestion process. Alginate is a polysaccharide naturally found in brown seaweed that has been traditionally used in food industry as thickener, gelling, and stabilizing additive. The studies with dietary fiber (such as alginate) have advanced beyond the traditional incorporation as ingredient to improve nutritional value to be used as structured animal fat replacers in meat products. One key example is the Prosella® gel (mainly composed of alginate), which forms a gel-like structure and can also emulsify an oil and improve the general nutritional value and functional properties of meat products. Current scientific evidence indicates Prosella® gel as a relevant ingredient to replace animal fat in fermented sausages and burgers. This fat replacer can provide a similar development of texture and sensory properties than its counterparts produced with animal fat. This chapter describes the preparation and incorporation of Prosella® gel in a traditional fermented sausage (Spanish *Salchichón*) and burgers.

Key words Alginate, Prosella®, Fermented sausage, Burger, Texture

1 Introduction

The modernization and the increased availability of food products have led to changes in lifestyle, especially in food habits [1]. Consumption of food gained a more complex and individualized perspective, which also progressed to consider food products as elements associated with the preservation or enhancement of health status [1]. Consequently, food products with healthier or functional ingredients gained the spotlight, especially in times of global crisis and increasing awareness of human health [2].

Dietary fibers are food components that are not affected by the human digestion process; have specific effects on digestion by improving consistency, bulk, and transit time of stool; have also

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been associated with enhanced health status (e.g., laxative effect and reducing postprandial glucose levels, lowering blood cholesterol, and promoting the growth of beneficial gut microorganisms); and induce satiety for better weight management [3, 4]. However, a comprehensive and definitive definition for dietary fibers has not been established yet, but the main concept involves the importance of chemical structure and the range of biological effects associated with health benefits [3]. Currently, there are several natural polymers considered as dietary fibers such as pectins, fructooligosaccharides, cellulose, alginate, and resistant starch [4, 5].

Alginate is a natural polymer found in brown seaweeds composed of β-D-mannuronic acid and α-L-guluronic acid linked in a homopolymeric (only one of the two compounds) or heteropolymeric (both compounds) way. It is naturally found as salt form with calcium, magnesium, or sodium cations in the cell walls of these seaweeds. As structural component, this hydrocolloid confers flexibility and strength to the cell wall [6]. In terms of health benefits, alginate has been associated with beneficial effect such as the reduction of fasting blood sugar, induction of insulin expression, downregulation of inflammatory markers, modulation of gut microbiota (composition and activity), reduction of serum cholesterol, and improvement of body weight control [7, 8]. It is also important to remember that alginate has been traditionally used in food industry as gelling agent in desserts (e.g., ice cream and pastry filling cream), stabilizing additive in beverages (such as suspending solids and improving stability of foam), and thickening ingredient in viscous foods (e.g., soups, creams, and toppings), which highlights its versatility for foods with a wide range of temperatures during processing or preparation for consumption at households [9].

Among the many options of promoting the inclusion of dietary fibers (such as alginate) in food products, many studies have been carried out in the last decades to include them into meat products [10–12]. Moreover, an important advance in the incorporation of dietary fibers in meat products is their use as fat replacer [13]. This advance is in line with the growing interest in improving the quality of food products, especially meat products.

It is well known that the continuous and excessive consumption of fat (especially saturated fat) can be deleterious to human health. Scientific evidence supports the link between the regular consumption of fat with increased risk of developing cardiovascular diseases, diabetes, and cancer [14]. However, the human body still needs dietary fat (especially unsaturated fats that are associated with a lower risk of developing diseases) [15]. Consequently, health-related authorities have been promoting actions to advise consumers to limit the consumption of fat and also choose foods with healthier fats (rich in unsaturated fatty acids) [15] such as vegetable oils (e.g., olive, canola, and algae [16, 17]).

This important progression towards healthier and functional meat products has been stimulating efforts to replace animal fat (a traditional ingredient in meat products processing) by other healthier fat sources and combine the strategy with the incorporation of dietary fibers. However, satisfactorily replacing animal fat is still an important challenge due to its central role in the texture and sensory properties of meat products [18]. This challenge has been gradually overcame due to advances in the utilization of dietary fibers as structured fat replacers [13]. In this strategy, a gel or emulsion is formed using the gelification properties of dietary fiber to mimic animal fat and replace it partially or entirely [19]. Another key aspect is the possibility to improve the fatty acid profile of meat products by using an edible oil that will be retained in a solid-like ingredient formed with dietary fiber such as alginate [17]. Due to the importance of this advance, this chapter aims to give a step-by-step explanation and also additional comments about the use of dietary fiber (particularly alginate-based commercial product, named Prosella®) as fat replacer in meat products (fermented sausage and burger).

2 Materials

The use of dietary fibers as fat replacer involves a preparatory step that is intended to produce a structured raw material for meat products' processing. A key option for the production of structured raw materials is Prosella®, a commercial formulation mainly composed of sodium alginate and calcium sulfate as gellifying agents, wheat glucose syrup, disodium diphosphate, and sodium ascorbate [19]. The commercial Prosella® is a white powder that can be homogenized with water and other ingredients (particularly oils [16, 17, 20, 21]). The formation of Prosella® gel is simple and does not require additional heat treatment. Moreover, the mixture turns into a gel in few hours at refrigeration temperature [22].

The *Salchichón* is a traditional meat product from Spanish culture. This meat product is regulated by the Spanish Royal Decree 474/2014 which establishes the quality standard, ingredients, processing conditions, label information, and characteristic properties [23]. The Spanish *Salchichón* can be defined as raw, cured (sodium chloride, nitrate and/or nitrite salts, and sodium ascorbate), and dry-ripened sausage usually produced with minced meat and fat from pigs, added with pepper as characterizing ingredient and ripened. Fermentation and smoking are also indicated as optional stages, and other seasonings are also allowed [23]. Burgers/patties are well-known meat products consumed around the world [24]. It is the meat product obtained from the homogenization of minced meat, sodium chloride, seasoning, additives, and other ingredients [23]. The products usually do not receive any

treatment (e.g., salting or ripening), are preserved at a low temperature (refrigerated or frozen storage), and are labeled in accordance with animal species (single or more than one) from which the meat was used [23].

Fresh pork and fat must comply with sanitary and technological requirements for its production, transport, handling, storage, processing, and commercialization [25, 26]. Ingredients also have an important role in this traditional meat product. The use of commercial ingredient preparations is an interesting option to produce specific sausages such as the Spanish *Salchichón*. These preparations have well-defined proportion of ingredients that will give the expected sensory attributes. In the case of *Salchichón*, the composition may include (proportions not indicated on label) lactose, sucrose, sodium chloride, characteristic seasoning (such as black and white pepper and nutmeg), and additives monosodium glutamate (E621), phosphates (E450 and E451), sodium erythorbate (E316), potassium nitrate (E252), and cochineal coloring (E120) [16, 17]. Moreover, the commercial ingredient preparations are developed to comply with the legislation of controlled ingredients and improve the standardization of characteristics among produced batches. Other key ingredients are nitrate and nitrite salts, which have a maximum residual level to be included in cured meat products (e.g., 250 mg nitrite/kg for dry-cured sausages) [27, 28].

3 Methods

3.1 Prosella® Gel Preparation

The preparation of structured gel involves Prosella® powder and water as main components. An unsaturated oil can also be included (*see* **Note 1**).

1. The Prosella® powder and water are weighed in the following proportion: 1:9 w/w. Then, ingredients are mixed for 3 min until a homogeneous mass is obtained (Fig. 1).
2. The mixture rests in trays for 2 h at 4 °C for gelification (*see* **Note 2**).
3. The gel is vacuum-packaged and kept at 4 °C until further use [22]. Once the gel is formed, its texture and visual aspect resemble, to some extent, animal fat (Fig. 1). At this point, the Prosella® gel can be minced and added to meat products in a similar fashion to animal fat.

3.2 Fermented Sausage Preparation (Spanish Salchichón*)*

The ingredients used in the production of *Salchichón* are pork (74 g/100 g), fat/Prosella® gel (18 g/100 g), water (3.4 g/100 g), and seasoning (containing pepper, nitrite salt, and other ingredients; 4.6 g/100 g).

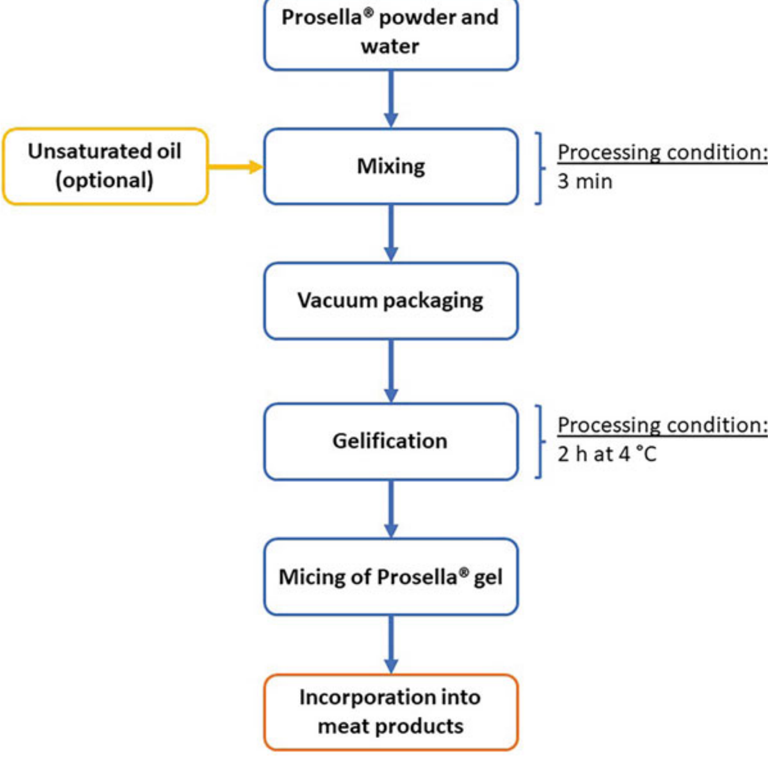

Fig. 1 Preparation of Prosella® gel

1. Lean meat, fat, and Prosella® gel are cut into chucks and minced (TOP-114, Talleres Ramon, S.L., Spain) in 6–8 mM sieve plates (*see* **Note 3**). The description of processing of *Salchichón* is indicated in Fig. 2.
2. Once the meat and Prosella® gel are minced, the homogenization with other ingredients can be carried out in a vacuum bow (AO-85, Fuerpla, Spain) for 5 min.
3. After complete homogenization of ingredients, the meat mass is kept for 24 h at 4 °C (*see* **Note 4**).
4. The meat mass can be stuffed into natural casings (50–55 mM diameter) using a vacuum stuffer (Sia Junior, Plegamans, Barcelona, Spain).
5. Sausages are hanged in carts and taken to the fermentation and ripening chambers to stimulate and promote the growth of fermentative bacteria, biochemical reactions associated with texture and sensory properties, and dehydration. The specific conditions of fermentation and ripening are characterized by a short period with relatively high relative humidity (RH) and temperature followed by a longer period at low RH and temperature, respectively [17, 29]. One possible condition is

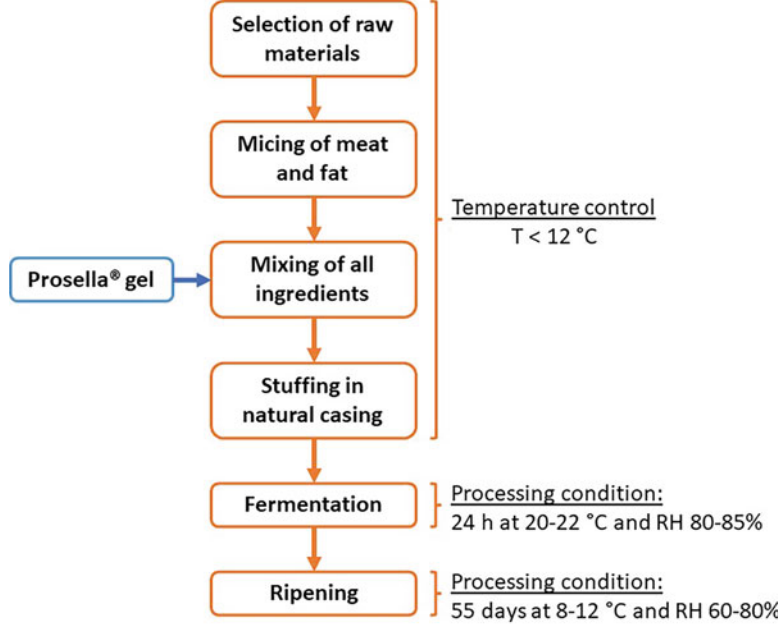

Fig. 2 Schematic representation of Spanish *Salchichón* processing with Prosella® gel

carrying out the fermentation for 24 h, 80–85% RH, and 20–22 °C followed by a ripening period of 55 days at 65–80% RH and 8–12 °C [17]. The final product is characterized by a dark red color with dry and hard aspect and the presence of characteristic white mold (*see* **Note 5**).

3.3 Burger Preparation

The ingredients used in the production of *burger* are meat (82 g/100 g), fat/Prosella® gel (10 g/100 g), water (7 g/100 g), and salt (1.0 g/100 g).

1. Lean meat and Prosella® gel are cut into chucks and minced in 6–18 mM sieve plates (*see* **Note 3**). The description of burger processing is indicated in Fig. 3.
2. Once the meat and Prosella® gel are minced, the homogenization with other ingredients can be carried out in a vacuum bow (AO-85, Fuerpla, Spain) for 5 min.
3. The meat mass is formatted into patties with a patty-maker (A-2000, Gaser, Girona, Spain). Patties can be placed in sealed trays for refrigerated/frozen preservation.
4. Cooking of patties can be performed using the same conditions applied for conventional patties: heating until reaching core temperature of 70 °C.

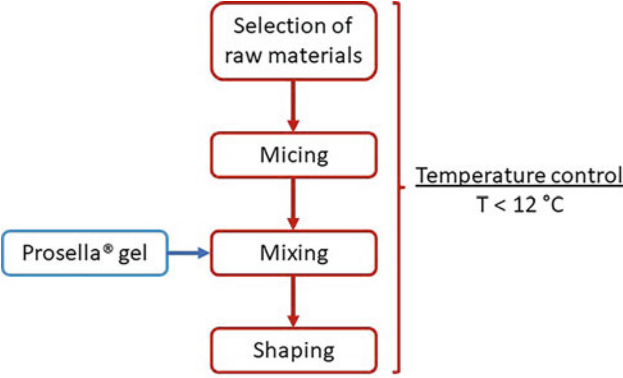

Fig. 3 Schematic representation of burger processing with Prosella® gel

Some examples of the incorporation of Prosella® gel into meat products as fat replacer are presented in Table 1. The influence of animal fat replacement by Prosella® gel is more noticeable into sausages than other meat products with animal fat comprising around 20% of total formulation total [16].

In the case of burgers with up to 15% of animal fat in formulation, the impact of partial or total replacement of animal fat is minimal in terms of texture and sensory analysis of meat products [19, 22, 30–33].

The incorporation of dietary fiber into meat products has been traditionally made with direct addition in powder form [34]. This conventional strategy may require minimal preparation in relation to any other ingredient used in the production of meat products. Therefore, the incorporation of Prosella® gel would be similar to that presented in Figs. 2 and 3. However the advantage of having an additional benefit (particularly the improvement of fatty acid profile) cannot be achieved with the conventional addition of dietary fibers as ingredient without forming a gel.

The incorporation of Prosella® gel into meat products has an underlying aspect: consumers expected to see pieces of animal fat. This characteristic is mainly observed in fermented and dry-cured sausages where fat and meat portions can be easily distinguished. This condition is an important aspect to strengthen the use of structured fat replacers containing dietary fibers in the development of healthier and functional meat products.

4 Notes

1. The Prosella® gel can also be formed to produce an emulsion to mimic animal fat and modify the fatty acid profile of the final meat product with in this case oils rich in unsaturated fatty acids (monounsaturated and polyunsaturated). In this case, the

Table 1
Effect of Prosella® gel with unsaturated fatty acids on the texture and sensory properties of meat products

Meat products with Prosella® gel containing healthier oils	AFR (I%)	Texture					Sensory analysis	References
		Hardness (N)	Springiness (mm)	Cohesiveness	Gumminess (N)	Chewiness (N•mm)		
Dry-cured sausages								
Olive, canola, or soy oils	50% (19%)	271.6–316.2	0.52–0.54	0.34–0.35	92.8–109.5	50.2–56.5	Differences were dependent on oil source	[16]
Tigernut+algae or sesame +algae oils	50% and 100% (18%)	283.2–317.3	0.53–0.55	0.36	104.0–110.5	49.6–56.2	Similar to control (meat color, fat color, odor, taste, black pepper flavor, and global flavor)	[17]
Burgers								
Chia oil	100% (4%)	n.e.	n.e.	n.e.	n.e.	n.e.	Similar to control (taste and odor)	[22]
Tigernut, chia, or linseed oils	100% (3%)	80.1–90.3	0.72–0.76	0.59–0.61	48.1–53.4	35.9–39.8	Similar to control (visual aspect, odor, fibrousness, flavor, greasy character, and juiciness)	[30]
Algae and wheat germ oils	50 and 100% (5%)	177.0–185.5	0.80–0.85	0.62	109.3–115.8	92.7–93.3	Similar to control (color, odor, texture, flavor, fatty flavor, and overall acceptance)	[19]
Tigernut oil	100% (15%)	127.4–139.6	0.75–0.78	0.55–0.59	72.6–81.3	56.1–62.9	Similar to control (odor, texture, flavor, fatty flavor, and overall acceptance)	[31]
Avocado+algae or pumpkin seed+algae oils	100% (10%)	77.6–80.6	0.75–0.76	0.56–0.58	41.9–52.5	32.7–43.4	Similar to control (visual aspect, raw and cooked odor, firmness, juiciness, greasy character, flavor, and overall acceptability)	[32]
Olive or sunflower oils	100% (4%)	108.8–113.3	0.59	0.37	n.e.	23.8–24.5	n.e.	[33]

AFR animal fat replacement; *I%* incorporation into meat product formulation (%)
n.e. not evaluated

proportion of ingredients can be modified: 560 g/kg water, 372 g/kg oil, and 67 g/kg Prosella®.

2. The gel acquires a white opaque color and texture similar to animal fat.
3. Due to the formation of a solid state of Prosella® gel, preliminary cutting into chucks and posterior mincing can be done in a similar fashion that is usually carried out with animal fat.
4. Refrigerated rest is a necessary step to ensure that curing process initiates and promotes the necessary changes in the meat mass.
5. The presence of characteristic white mold in *Salchichón* is a visual sign, *but not conclusive*, of the growth of expected molds in the product. The development of spoilage and pathogenic microorganisms can take place during ripening and lead to quality deterioration (e.g., off odor and putrefaction odor) and accumulation of toxic compounds (such as mycotoxins). Prevention of microbial contamination can be achieved using raw materials with satisfactory sanitation, proper cleaning of facilities and equipment, control of all processing stages, and other regulated aspects for the processing of meat products.

Acknowledgments

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Chapter 6

Emulsified Meat Product with Oleogels for Reducing Saturated Fat Content

Camila de Souza Paglarini, Ana Karoline Ferreira Ignácio Câmara, Thais Lomonaco Teodoro da Silva, and Marise Aparecida Rodrigues Pollonio

Abstract

Emulsified meat products are widely consumed around the world mainly due to their sensory properties and convenience in preparation. The most popular meat emulsions are frankfurters/hot dogs and mortadella/bologna sausage, and its main ingredients are pork meat, pork backfat, salt, nitrite, phosphate, erythorbate, and spices. However, these products are recognized as unhealthy mainly due to their considerable amount of saturated fat; therefore, reformulation is a strategy to make them healthier. Oleogels are structured oils, rich in unsaturated fatty acids, with potential use as an animal fat replacer in meat emulsion. Several oil sources can be used to elaborate oleogels where the most common are vegetable oils such as soybean, sunflower, and canola. The effect of oleogel addition in the making process, formulation, and quality of meat emulsion is approached in this chapter considering the information from literature, industry, regulations, and standards.

Key words Meat emulsion, Healthier meat products, Structured oil

1 Introduction

Meat emulsions are multiphase systems in which the continuous phase is a complex hydrophilic colloidal aqueous solution of salts and soluble proteins and the discontinuous phase consists of solid compounds such as insoluble proteins, fat particles, spices, and other insoluble components of muscle tissue [1]. The most popular meat emulsions, also known as comminuted products, are frankfurters and mortadella, usually made from pork meat and pork backfat with characteristic flavor resulting from the addition of garlic, onion, and black pepper. Products with other meat sources (beef, chicken) also can be found in the market.

Products are prepared in a cutter or emulsifier, stuffed in artificial cases, and cooked in water or hot air. In the frankfurter

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processing, the cases are removed before packaging, and in the processing of mortadella, the cases remain in the final product. A peculiar characteristic of Brazilian frankfurter is a dyeing process, before packaging, with a solution of annatto dye [2].

The consumer's requirements have led the industry to think new strategies to make these products healthier since they contain a considerable amount of saturated fat, sodium, and other additives. Partial or total replacement of animal fat with oleogel are strategies of reformulation to make meat emulsions healthier. It should be noted here that we still do not have meat products with added oleogel on the market, but studies have demonstrated its potential in the reformulation of comminuted meat products [3–8].

Oleogels are liquid oil transformed into a "gel-like" viscoelastic structure by adding some structuring agents that mimic high-saturated-fat behavior on many food applications [9]. These structuring agents should be able to form the oleogel in lower concentrations (≤10%) [10]. In recent years, several structuring agents have been explored for edible oil structuring, such as vegetable waxes [11], monoglycerides [12], phytosterols [13], lecithin [14], and cellulose derivatives [15, 16], among others. The structuration mechanism of these different molecules can be categorized into four main groups: (1) crystalline particles; (2) self-assembled structures of low-molecular-weight compounds; (3) self-assembled structures of polymers or polymeric strands; and (4) miscellaneous structures like colloidal particles and emulsion droplets [17].

The very distinct chemical composition and structure of the diverse structuring agents found require different strategies to form oleogels; these strategies are divided into two categories: direct dispersion method and indirect dispersion methods [17]. Direct approaches refer to a simple dispersion of the melted oleogelators into the oil phase, followed by a cooling step which makes possible the formation of a network that entraps liquid oil within a solid structure, leading to a self-supporting gel [14, 18]. Indirect dispersion is needed especially for hydrophilic polymers, which cannot be directly dispersed in oil, where another solvent is used to dissolute the gelator, mostly water, and after stripping off the water from hydrated polymer solutions, a direct dispersion or oleogel is formed [17, 19]. This category of methods includes the emulsion-templated method [19], foam-templated method [20], and solvent exchange [21]. Emulsified meat products has been done using direct dispersion [3, 7, 8, 22]. Nonetheless, recently the indirect method has also been used [23].

This chapter aims at describing in detail the formulation and the process of making reformulated meat emulsion by adding oleogels, considering the information from literature, industry, regulations, and standards regarding emulsified meat products quality.

2 Materials

2.1 Oleogel

Vegetable liquid oils: Soybean oil, sunflower oil, canola oil, rapeseed oil, corn oil, olive oil, avocado oil, linseed oil, hemp seed oil, rice bran oil, chia oil, high-oleic oils, etc.

Oleogelators: Candelilla wax, bees wax, rice bran wax, carnauba wax, sunflower wax, monoglycerides, diglycerides, phytosterols (β--sitosterol and sterol blends), γ-oryzanol, lecithin, ethylcellulose, and sorbitan monostearate, among others.

Gelators: Hydroxypropyl methylcellulose (HMPC), methylcellulose, vegetable proteins (soy, pulses, etc.), and so on.

2.2 Meat Emulsion

To avoid possible fraud to the consumer, frankfurter and bologna sausage are regulated by legislation. Each country has established its rules that in general are similar. The standard requires that they be comminuted, semisolid products made from one or more kinds of raw skeletal muscle from livestock (beef, chicken, or pork). Smoking and curing ingredients contribute to the flavor, color, and preservation of the product. They are link-shaped and come in all sizes—short, long, thin, and chubby.

Brazilian law establishes that the finished products may not contain more than 30% fat, 2–5% starch or no more than 65% of moisture, and a minimum of 12% meat protein. Up to 4.0% of nonmeat protein may be used [24]. In American law, the final product's composition is limited to a maximum of 30% fat or no more than 10% water or a combination of 40% fat and added water. Up to 3.5% nonmeat binders and extenders (such as nonfat dry milk, cereal, or dried whole milk) or 2% isolated soy protein may be used [25].

Meat: Lean meat (beef, chicken, pork, etc.) and/or mechanically separated poultry meat (MSPM). Meat raw materials must be previously cleaned to remove apparent fat and aponeuroses. The amount of MSPM is also limited by the legislation of each country. In Brazil, up to 60% of MSPM may be used in frankfurters and bologna, whereas the USDA and the European Commission allow any amount of MSPM as long as it is declared on the label.

Fat: Traditionally, pork fat from the dorsal region of the carcass is used as a lipid source in meat emulsions. The addition of oleogel in meat emulsion is a good option to improve the lipid profile of the products, as presented in Table 1. A combination of animal fat and oleogel can result in products similar to traditional ones [3].

Table 1
Effect of oleogel on the lipid profile of emulsified meat products

	Bologna	Bologna	Frankfurter	Frankfurter	Frankfurter	Frankfurter	Bologna with MSPM	Bologna with MSPM
Lipid source	Pork backfat	High oleic sunflower oleogel	Pork backfat	50% pork backfat, 50% linseed oleogel	Pork backfat	Soybean oleogel	Pork backfat	High oleic soybean oleogel
Fat content (%)	18.30	19.70	18.35	18.95	21.27	20.48	25.80	26.10
SFA (g/100 g product)	7.11	2.35	6.45	6.13	6.55	3.80	8.42	6.19
MUFA (g/100 g product)	8.51	16.82	8.82	7.99	8.68	4.65	11.73	15.35
PUFA (g/100 g product)	2.61	2.26	3.08	4.82	4.32	11.47	5.06	4.26
n-6/n-3 ratio	20.75	44.20	14.92	1.61	22.85	6.71	13.33	7.31
Reference	[3]	[3]	[4]	[4]	[8]	[8]	[7]	[7]

MSPM mechanically separated poultry meat

Water: Water or ice, or both, may be used to facilitate chopping, mixing, and stuffing, dissolve curing ingredients, and improve texture and yield. The amount added has to respect the legislation of each country.

Salt: Sodium chloride is added in a fraction of 1.5–2.2% of the formulation and has several functions in meat emulsion. It solubilizes and extracts the myofibrillar protein, important in sausage making because they retain water and encapsulate fats being responsible for stable emulsion and gel structures along the processing steps. Salt also contributes to flavor and has bacteriostatic properties.

Additives: Sodium nitrite (up to 0.02%), sodium tripolyphosphate (up to 0.5%), and sodium erythorbate/ascorbate (up to 0.055%) are commonly used to make emulsified meat products. Sodium nitrite, also known as a curing agent, has the main function of being antimicrobial, in addition to color and flavor development and antioxidant action. Ascorbates and erythorbates are cure accelerators because they accelerate color development and also stabilize the cure color in the final product besides increasing the bacteriostatic efficiency of nitrite. Phosphates are important additives in meat emulsion because they increase the water-binding capacity of the meat proteins. They also contribute to sensorial properties, improve the stability and uniformity of the cure color, and act as antioxidants.

Spices, seasonings, and flavorings: These ingredients are used to add flavor to the products, but they also may act as antimicrobials, antioxidants, and/or color enhancers. The wide range of these ingredients is the main reason for the diversity of emulsified products in the market. The most common spices added in meat emulsion are garlic, onion, paprika, and black pepper. Spices can contribute to improving the sensorial properties of meat emulsion with oleogel when the oil source has a strong or undesirable flavor, such as linseed oil, and some structuring agents (e.g., candelilla wax).

Nonmeat protein: Vegetable proteins (soy is the most common) are added in meat emulsion as a binder because they improve the sensory characteristics of the product (texture, juiciness, and flavor), sliceability, and yield. Furthermore, vegetable proteins, like isolated soy protein, have functional properties such as emulsification, water absorption, elasticity, cohesion, and adhesion to the meat matrix.

Casings: The types of casings used may differ depending on the type of heat treatment and whether or not the smoking procedure is carried out. Permeable collagen and cellulose casings are recommended for products that will undergo the smoking process, and impermeable plastic casings are used in emulsified meat products cooked in immersion cooking tanks [26].

3 Methods

3.1 Oleogel

Direct dispersion (Fig. 1b)

1. Warm the oil phase in a glass beaker, water jacket cell, or heating tank until solid–gel transition temperature of the oleogelator is achieved (*see* **Note 1**). Keep the oil under agitation (100–500 rpm; *see* **Note 2**) during the process.
2. Add the oleogelator at a predetermined concentration, and keep the agitation on.
3. Set the time, and keep the mixture over the solid–gel transition temperature from 7 to 60 min (*see* **Note 1**).
4. After complete dissolution samples are statically cooled to 4 ± 1 °C (fridge temperature) for further application. At least 12h is needed to stabilize the oleogel before processing the meat emulsion.

3.2 Batter Preparation

The batter preparation stages are shown in Fig. 1a and can be summarized as:

1. *Grinding*: In this process, meat is forced through a grinding plate preceded by a rotating blade [27] (*see* **Note 3**). All lean and fat cuts are milled in plates which have different size openings (2–10 mm) and shapes (Fig. 2) according to each emulsified meat product's specifications.
2. *Comminution/chopping*: This step occurs when the meat is passed through a set of cutting blades. The reducing particle size is controlled by the number of passes through the rotating knives and the distance of the knives from the bowl. The degree of comminution is controlled by the overall comminution time, the number of blades, and their speed [27]. Two pieces of equipment are used in the chopping process, the bowl chopper/cutter and the emulsion mill (emulsifier) (Fig. 2), and the last one has been more used by bigger companies because it combines the principles of grinding and chopping and provides a mass with very fine particles and a homogeneous mixture of the ingredients. In the chopping process using a cutter, meat raw materials, partially defrosted and previously ground, and MSPM are added to the cutter at a low speed along with the salt, phosphates, sodium nitrite, and half ice to provide solubilization of myofibrillar proteins (*see* **Note 4**). Lastly, the rest of the ice and the other ingredients must be added with constant monitoring of the temperature of the meat batter to ensure that you have a stable meat emulsion (*see* **Note 5**). Generally, the lipid sources are the last ones added in the comminution stage (*see* **Note 6**), which continues until the batter temperature reaches between 12 and 13 °C. When the emulsifier is

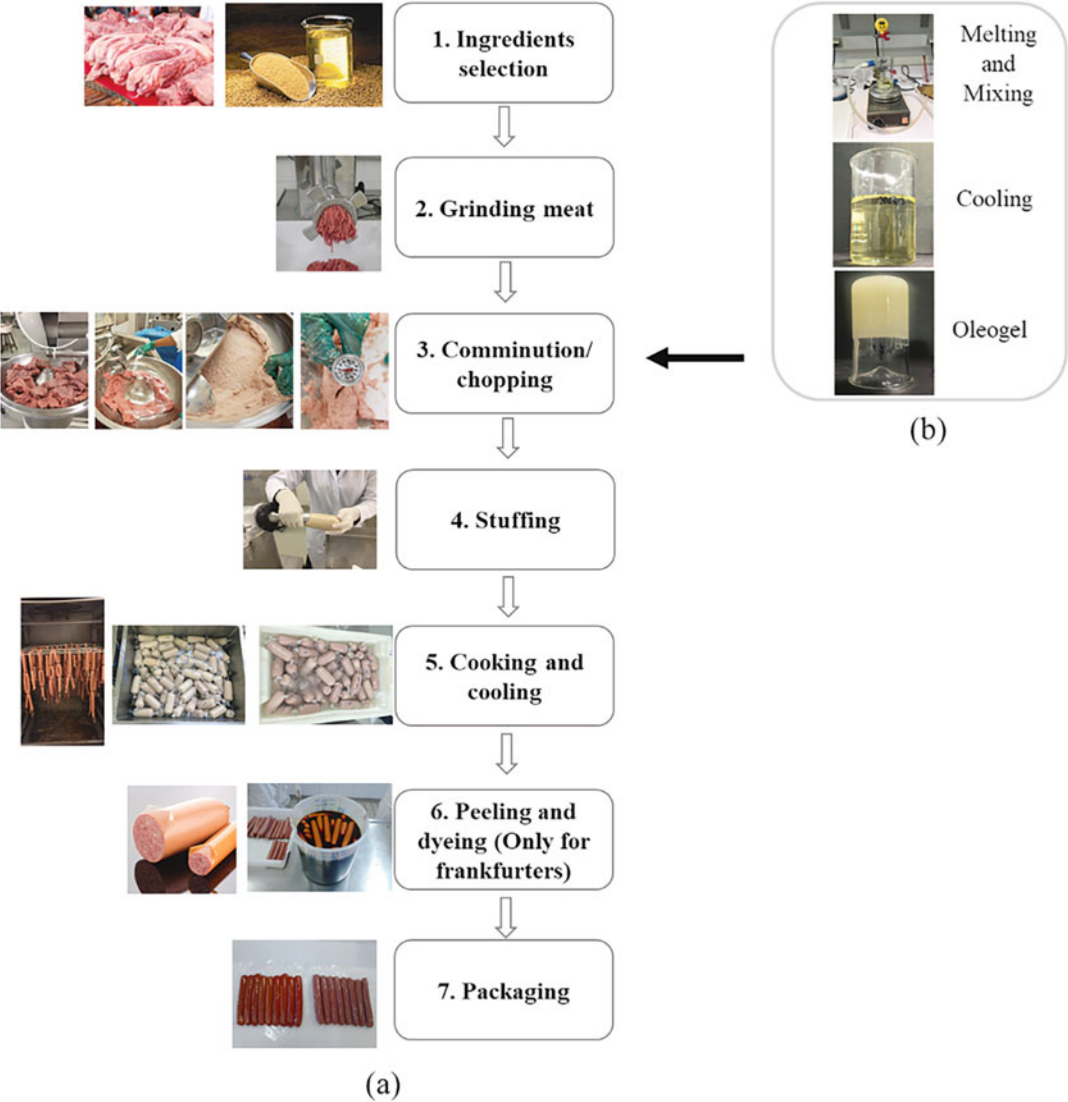

Fig. 1 (**a**) Processing steps of the production of meat emulsion and (**b**) oleogel

used, the process takes place at a very high speed which is ideal for an automatic process line as a large volume of batter can be produced in a continuous process.

3.3 Batter Stuffing

After the comminution stage, the meat batter is placed in casings that provide a distinct shape and appearance and must follow the identity and quality standard determinations for each product type. Stuffing also minimizes fat separation and breakage of emulsified membranes before heating. In general, equipment called stuffers is used, with pneumatic or vacuum operation (Fig. 2) and with an automated portioning system to guarantee standardized weights of the sausage products [26]. The oleogel addition doesn't affect this step of meat emulsion processing.

Fig. 2 Equipment used in the processing of emulsified meat products. (Photos by Pagliarini (Meat Laboratory of School of Food Engineering, State University of Campinas))

Vacuum stuffers are more expensive; however, a product with an improved appearance and extended shelf life is obtained (*see* **Note** 7). For this reason, the large meat industry uses vacuum stuffing [27]. In a continuous operation, fully automated co-extrusion systems are an attractive technology, where the meat

batter coming out of the stuffer's horn is covered with a semiliquid casing that can later be cross-linked, instead of using pre-made casings. The casings can be made of collagen, alginate, or alginate–collagen hybrids [28].

After stuffing the product is tied at the ends or segmented into individual links. In the frankfurter processing, twisting links of small sausages by hand or using special equipment is performed. Products with larger diameters like bologna are tied at the ends with metal clips.

3.4 Batter Cooking

Most equipment used for the heat treatment of meat products reaches moderate final temperatures at the geometric center of the products (between 60 and 80 °C), eliminating vegetative microorganisms but not spores. Thus, the cooking processes that meat products are subjected to are often called pasteurization. Although cooking equipment varies widely depending on the scale of production and product types, three heating methods are most common [29].

- Hot air (free and forced convection system)
- Steam
- Hot water

Forced air convection ovens, steam ovens, and hot water tanks are the most common types of equipment in the meat industry (Fig. 1a). Frequently, in cooking ovens that operate in continuous processes or batches, at the end of the cooking process, there is a cooling system for the equipment itself with sprinkler showers with cold water that perform a thermal shock on the cooked products, interrupting the heat treatment process [29].

Using oleogel as an animal fat replacer may help reduce the cooking loss in meat emulsion [30].

3.5 Peeling, Dyeing, Slicing, and Packaging

The casing can be peeled at the plant or by the consumer. In the case of small-diameter products such as frankfurters, peeling is often done at the plant by automated equipment (*see* **Note 8**). The products are passed through a short steam tunnel to help loosen the casing, and then a small blade is used to cut open the casings along the moving product. The machine can strip off hundreds of casing links per minute. In such products, the so-called easy-to-peel cellulose casings are used to prevent excessive adherence. When large-diameter products are prepared for slicing (e.g., bologna), the thick cellulose/plastic casing is removed by hand or semiautomated equipment.

To promote an attractive color, the Brazilian industry dyes the frankfurters with annatto dye after peeling (*see* **Note 9**). The products are dyed by immersion in a solution of annatto dye (*urucum*)

for approximately 1 min followed by dipping in a tank containing a solution of phosphoric acid for 5–6 s to fix the color. It is an important stage when oleogels are added to the meat emulsion since lighter and less red samples are obtained [3–5].

Cooked sausages are often portioned or sliced and packaged in processing plants, which offers convenience to the consumer. The industry has developed high-speed, automated slicing equipment with precise portion control. The introduction of computerized weighing equipment has had a significant contribution to the development of modern slicing equipment [27].

In general, emulsified meat products are vacuum packaged before commercialization. The function of the package is to protect the product from physical damage and recontamination, guiding the consumer about the constituents of foods, and can also serve as a marketing tool.

4 Notes

1. The time *versus* temperature binomial is very important in the direct dispersion to ensure the full dissolution of the oleogelator. The temperatures usually vary from 70 °C (low-melting-point waxes, such as beeswax) to 140 °C (e.g., for ethylcellulose). The time counting of 7 min should start after the mixture of oil and oleogelator achieve the desired temperature. Short times as 7 min are applied to dissolve oleogelators as monoglycerides and vegetable waxes. But a long time is needed for phytosterols, lecithin, and ethylcellulose (around 40–60 min).
2. The agitation speed is important too; nevertheless, there is not exactly a number for this parameter. It is important to assume that the sample is homogeneously sheared. The agitation can be performed with magnetics, overhead agitators, or stirring blades.
3. If an excessive amount of meat is placed on the plate, backup can occur and causes an ineffective operation, overheating of the meat mass, and fat smearing. In a small manual process, this is controlled by the operator, but on a large-scale line, automatic controls should be set to avoid this problem.
4. Myofibrillar proteins require a minimum ionic strength of 0.5 M for their solubilization, an essential physical–chemical process for the elaboration of emulsified meat products.
5. During the grinding process, the temperature of the meat mass increases due to the friction of the equipment blades, and excessive comminution negatively interferes with the stability of the meat emulsion.

6. The oleogels, when present in the treatments of emulsified meat products, must be added at the end of the process to reduce the amount of mechanical shearing applied and guarantee greater stability to the meat batter.
7. The air incorporated in the batter looks like empty holes in the cooked product. The holes can also be filled with melted collagen (especially in products with mechanically separated meat) or melted fat during the cooking process which is unattractive to consumers. Additionally, evacuation of air/oxygen also reduces lipid oxidation and bacterial action and prevents proteolysis.
8. The hygienic condition of the skin peeler is crucial since peeling involves extra amounts of handling of the product. Additionally, every single unit of product has to run through the peeler.
9. The concentration of annatto dye and phosphoric acid solutions is specified by the supplier. In addition to color, annatto dye also acts as an antimicrobial and/or antioxidant in sausages, mainly due to the presence of bixin and norbixin carotenoids.

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Chapter 7

Emulsified Meat Product with Fibers for Reducing Fat Content

Vivian Feddern, Eduardo Huber, Vanessa Biasi, and Vicky Lilge Kawski

Abstract

Meat products are rich in almost every macro- and micronutrient, with the exception of carbohydrates and fibers. Despite all the benefits provided by meat intake, consumers' perception is towards reduction of fat in their diets. Therefore, one of the alternatives to overcome this desire is to reformulate meat products to meet this demand. Fiber is being used as fat replacer in emulsified products, and depending on the concentration in the final product, it may pose nutraceutical appeal. In order to collaborate to better understand the mortadella production, this chapter brings a protocol containing the steps of mortadella development added with fibers and the necessary determinations once the final product is achieved.

Key words Mortadella, Sausage, Fiber, Meat, Functional

1 Introduction

Currently, the society is concerned with health and pursues alternatives to change its diet, enriching it with ingredients that, in addition to nourishing, prevent diseases [1]. In this sense, functional ingredients are fibers, antioxidants, and omega-3, among others. Meat consumption has been increasing worldwide, which leads industries in the sector to diversify the supply of products, committing to nutritional quality and an affordable price.

Mortadella is a cooked emulsified meat product consumed all over the world and plays an important role in the diet, as it is a source of protein; however, it contains between 20% and 30% fat, which may drive consumers to lower their purchase. It is popular among different age groups, with great economic importance [2]. Its composition may vary according to the type [bologna, Italian, among others], manufacturing techniques, and characteristics related to the formulation, such as types of meat cuts and the amount of mechanically separated meat. All these characteristics

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Table 1
Requirements for mortadella preparation

Physicochemical parameters	Amount
Protein	Minimum 12%
Lipids	Maximum 30%
Mechanically separated meat (MSM)	Maximum 60%
Skin and tendons	Maximum 10%
Nonmeat proteins	Maximum 4%
Starch[a]	Maximum 1–5%
Total carbohydrates[a]	Maximum 1–10%
Calcium (dry basis)	0.9%
Moisture	Maximum 65%

Source: [a]the sum of starch + total sugars < 10%. Source: Ministry of Agriculture and Livestock. Instrução Normativa n.°4, de 31 de março de 2000. In Aprovar os Regulamentos técnicos de identidade e qualidade de carne mecanicamente separada, de mortadela, de linguiça e de salsicha, em conformidade com os Anexos desta Instrução Normativa. Anexo II. Brasília, DF: Regulamento técnico de identidade e qualidade de mortadela. https://pesquisa.in.gov.br/imprensa/jsp/visualiza/index.jsp?data=05/04/2000&jornal=1&pagina=54&totalArquivos=73

may be different according to each country. However, the main ingredients present in mortadella formulation are depicted in Table 1.

As people are concerned to reduce the consumption of processed and high-fat foods (*see* **Note 1**), especially present in meat products, it becomes important to adapt their formulations, making these products more attractive to consumers by incorporating bioactive and functional ingredients, with a greater appeal to health. Among some ingredients and nutrients, antioxidants, dietary fibers, phytochemicals, and vegetable proteins can be used to improve the nutritional characteristics of meat products. The use of fibers in meat products for technical (yield improvement) and health (fat reduction) purposes can show different effects on the textural properties of these products depending upon the type of fiber used [3–5]. These modifications will imply acceptance or not of the product by the consumer. Interesting dietary fibers can be used in the preparation of meat products (*see* **Note 2**), coming either from grains (wheat, oat, barley), vegetables, fruits (pectin from citrus peels, apple pomace), or other sources such as alginates from algae, preparations of cellulose from bamboo, and other possibilities [6].

The recommended acceptable daily intakes (ADI) of dietary fiber are 28–36 g, from which 70–80% comprise the insoluble fraction [7]. As sources of fibers, some alternatives have been

proposed as potential ingredients to be added in emulsified products. For instance, barley malt pomace was evaluated in sausages, and the formulation containing 3% of pomace appeared to be the best among other percentages added (3, 6, 9%) in terms of nutritive value (2% fiber barley malt formulation × 0.77% fiber in control), texture, and acceptability by the consumers [8]. More recently, Biasi et al. [9] developed bologna-type mortadella added by goldenberry flour as a natural antioxidant containing polyphenols, carotenoids, and vitamin C. The authors concluded that this fruit flour might be added without compromising physicochemical parameters. Also, blueberry formulations were evaluated by the same group of researchers [10]. The authors recommended 0.5% addition of blueberry flour to replace synthetic antioxidant in bologna-type mortadella while maintaining physicochemical characteristics, texture, color, and reduced lipid oxidation.

As a means of collaborating with the lack of details presented in most publications, this chapter will provide mortadella formulation with added fibers discussing the important parameters that must be taken into account when formulating new emulsified products with nutraceutical appeal.

2 Materials

1. 5 L cutter.
2. Cold chamber.
3. Polyethylene casings.
4. Lean pork (5% fat content) 60.0% (*see* **Note 1**).
5. Pork back fat (80% fat content) 10.0%.
6. Ice 14.5%.
7. Salt 1.8%.
8. Water 20.6%.
9. Polyphosphate-based stabilizer 0.5%.
10. Nitrite- and nitrate-based preservative 0.3%.
11. Sugar 0.5%.
12. Seasoning 0.8%.
13. Cassava starch 1.2%.
14. Garlic 0.1%.
15. Erythorbate-based antioxidant 0.25%.
16. Blueberry flour 0.04%.
17. Physalis flour 0.40%.
18. Vegetable-source fibers (1.6% wheat fiber, 1.6% pea fiber, and 0.4% bamboo fiber). For more details, *see* **Notes 2–5**.

3 Methods

3.1 Mortadella Development

Mortadella production may be carried out according to the flowchart depicted in Fig. 1, which was based on the methodology described by Pires et al. [11]. This procedure is performed in triplicate, on different days. The first step is to grind the meat in a cutter together with salt and half of the water (ice). After complete extraction of myofibrillar proteins, the other ingredients (according to each formulation) and the fat to be emulsified may be added. During processing, the temperature must be controlled and kept below 12 °C to ensure emulsion stability. After the dough reaches the desired appearance, it may be wrapped, in the absence of vacuum, in previously clipped polyethylene casings suitable for mortadella, and taken to a cooking tank. The application of heat is gradual, starting at 60 °C for a period of 30 min, being later increased to 80 °C, continuing cooking until the geometric center of the product reaches 73 °C. Soon after, the mortadella is cooled in running water for 30 min and stored in a cold chamber at 2 °C.

See **Note 6** for considerations on the type of ingredients and **Note 7** for costs.

3.2 Determination of Parameters Demanded in the Final Emulsified Product

3.2.1 Assessment of Mortadella Proximal Composition

Moisture content is measured by the weight difference before and after oven drying at 105 °C for 16 h. Crude lipid content is measured by drying the sample in a 105 °C oven for 6 h and then extracting the lipid with ether in a Soxhlet extractor for 4 h. Crude protein content can be measured either by the combustion method of AOAC number 992.15 [12] or by block digestion method according to AOAC number 981.10 [13]. The crude fiber is determined according to the ANKOM Technology Method number Ba 6a-05 [14]. Initially, 1 g (±0.0001) of the sample is weighed into an Ankom F57 filter bag (25 μm porosity) previously dried and weighed.

3.2.2 Evaluation of pH and Color

The pH values are read using a pH meter with the insertion of an electrode probe and another using automatic temperature compensation in animal carcasses. The objective color parameters are evaluated using a portable colorimeter with an opening diameter of 25 mm, a D65 illuminant, and 0 viewing angle. L* lightness, a* redness, and b* yellowness parameters are then registered and the data analyzed. L* varies from darkness to lightness; thus, a greater L* value indicates lighter colors, while a* and b* values indicate the tendency to redness and yellowness, respectively [15, 16]. Based on the a* and b* values, it is possible to calculate the hue and saturation (chroma), which indicate the intensity of the color:

$$\text{Chroma} = \sqrt{a^{*2} + b^{*2}}$$

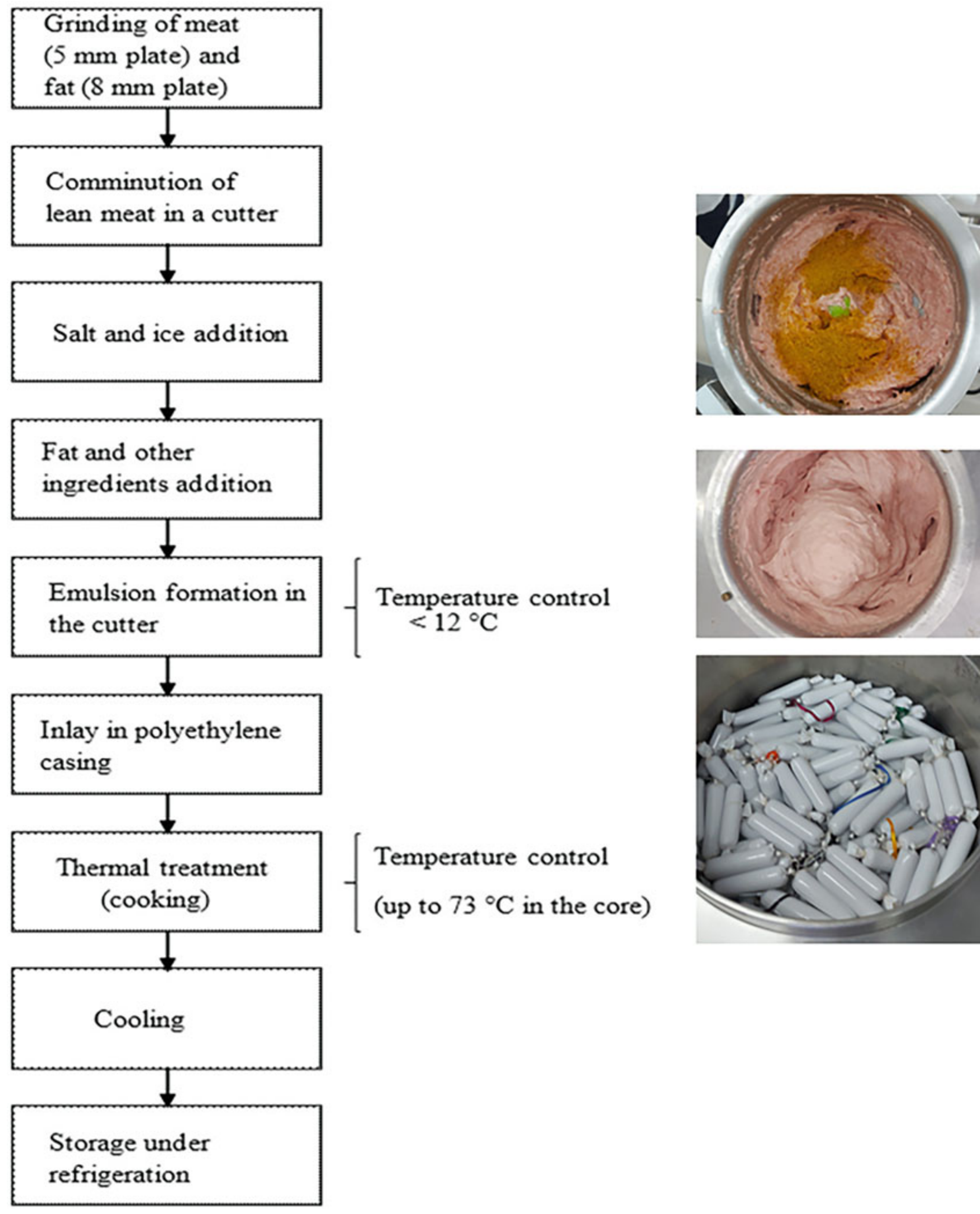

Fig. 1 Mortadella production flowchart. (Source: Biasi et al. [9])

3.2.3 Evaluation of TBARS

Samples are stored at −20 °C until the moment of analysis in the laboratory. To evaluate oxidative stability, the raw samples undergo the 2-thiobarbituric acid reactive substances (TBARS) assay, which quantifies malondialdehyde (MDA), one of the main decomposition secondary products of polyunsaturated fatty acid

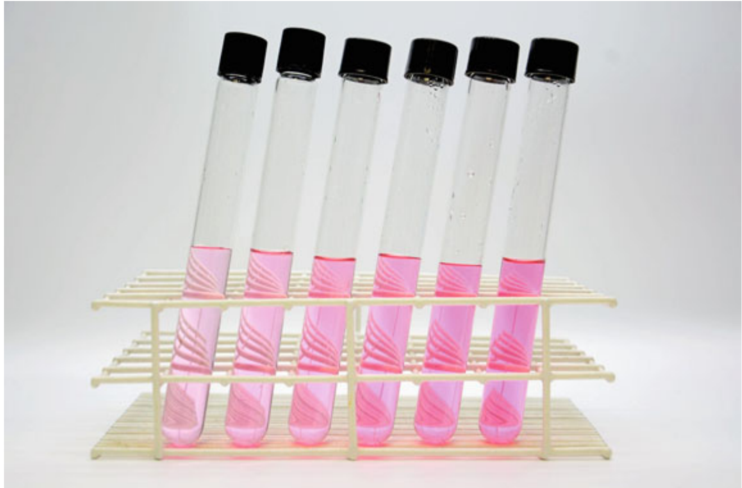

Fig. 2 Colorful compound measured after TBA + MDA reaction

hydroperoxides, formed during the oxidative process. After the reaction of TBA with MDA, a colorful compound is formed (Fig. 2) and can be measured at a spectrophotometer at 532 nm absorbance [17–21]. A calibration curve is constructed from an external standard solution with different known concentrations for quantifying MDA. The external calibration method enabled comparison of the absorbance of the samples with that obtained in the analysis of standard solutions with known concentrations. The concentration range of MDA on the curve follows the Lambert–Beer law. The results are expressed as mg MDA/kg sample.

3.2.4 Texture Analysis

The texture profile of mortadella samples may be assessed according to Silva et al. [22], with some modifications. The temperature of the samples is adjusted to room temperature. The samples are then cut in cylindrical shape, with sizes of 2 cm height × 2 cm diameter, and obtained from the central region of the samples. The probe consists of an acrylic cylinder, 3.8 cm in diameter × 2 cm in height (Fig. 3). The analysis parameters are compression speed of 0.2 cm/s, percentage of compression of 70% in relation to the height of the sample, and time between cycles of 5 s. The texture parameters to be evaluated are hardness, cohesiveness, adhesiveness, elasticity, and chewiness.

3.2.5 Determination of Water Activity (Aw)

To determine the water activity, a water activity analyzer is used with direct reading of the sample, after calibration with distilled water and saturated saline solution, as specified in the manufacturer's manual (*see* **Note 8**).

Fig. 3 Texture analysis of mortadella

3.2.6 Microbiological Assay

According to the Brazilian Normative Instruction (IN) 161:2022 [23] which is the current microbiological regulation adopted in Brazil, the following analyses are required for mortadella: *Salmonella*/25 g, *Clostridium perfringens*, *Staphylococcus* coagulase positive, and *Escherichia coli*. The determination of coagulase-positive staphylococci is performed according to the ISO 6888-1:2021 method [24], where sample dilutions in 0.1% peptone water are inoculated in Petri dishes, with previously prepared Baird–Parker Agar, and incubation at 35 °C/48 h for later reading. Some colonies are transferred to tubes with brain heart infusion (BHI) broth incubated at 35 °C for 24 h and fractions mixed with plasma coagulase EDTA. After incubation in a water bath at 37 °C for 2–6 h, clot formation needs to be checked. The determination of *Salmonella* sp. is done according to the *Bacteriological Analytical Manual* [25]. The 25 g samples are homogenized and pre-enriched with 225 mL of 1% buffered peptone water and incubated for 16–20 h at 36 °C. Afterwards, 1 mL aliquots of sample are transferred to a tube with 10 mL of Rappaport Vassiliadis broth and to another tube with 10 mL of selenite–cystine broth, which are incubated for 24–30 h at 41 °C, for selective enrichment. After incubation, the tubes are shaken, and two loops are collected from

each of them, one streaked on a plate with xylose lysine deoxycholate agar (XLD) and the other on a plate with brilliant green red phenol lactose sucrose agar (BPLS). After incubation for 18–24 h at 35 °C, the presence or absence of typical *Salmonella* colonies is checked. Biochemical and serological tests are carried out to verify that the colonies obtained are really *Salmonella* (urease production, reactions on TSI Agar or Kligler Agar (KIA), lysine decarboxylation, SIM medium, and oxidase test). For *Clostridium perfringens* determination, the Bacteriological Analytical Manual [26] is followed. Using aseptic technique, place 25 g food sample in sterile blender jar. Add 225 mL peptone dilution fluid (1:10 dilution). Homogenize 1–2 min at low speed. Obtain uniform homogenate with as little aeration as possible. Using 1:10 dilution prepared above, make serial dilutions from 10–1 to 10–6 by transferring 10–90 ml peptone dilution fluid blanks. Mix each dilution thoroughly by gently shaking before each transfer. Pour 6–7 ml TSC agar without egg yolk into each of ten 100 × 15 mm petri dishes and spread evenly on bottom by rapidly rotating dish. When agar has solidified, label plates, and aseptically transfer 1 mL of each dilution of homogenate to the center of duplicate agar plates. Pour additional 15 mL TSC agar without egg yolk into dish and mix with inoculum by gently rotating dish. After incubation, remove plates from anaerobic jar and select those containing 20–200 black colonies for counting. *C. perfringens* colonies in egg yolk medium with a 2–4 mm opaque white zone surrounding the colony as a result of lecithinase activity. Using Quebec colony counter with white tissue paper over counting area, count black colonies and calculate number of clostridia cells/g food. In order to perform *E. coli* assay, the Bacteriological Analytical Manual [27] is recommended. Weigh 50 g of sample into sterile high-speed blender jar. Add 450 mL of Butterfield's phosphate-buffered water and blend for 2 min. If $<$ 50 g of sample are available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades. Prepare decimal dilutions with sterile Butterfield's phosphate diluent or equivalent. Number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25 times in 30 cm arc or vortex mix for 7 s. Using at least 3 consecutive dilutions, inoculate 1 mL aliquots from each dilution into 3 LST tubes for a 3 tube MPN. Incubate LST tubes at 35 °C ± 0.5 °C. Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 3 h. From each gassing LST or Lactose broth tube, transfer a loopful of each suspension to a tube of EC broth.

Incubate EC tubes 24 ± 2 h at 44.5 °C and examine for gas production. If negative, reincubate and examine again at 48 ± 2 h. Gently agitate each gassing EC tube, remove a loopful of broth and streak for isolation on a L-EMB agar plate and incubate for 18–24 h at 35 °C ± 0.5 °C. Examine plates for suspicious E. coli colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to 5 suspicious colonies from each L-EMB plate to PCA slants, incubate them for 18–24 h at 35 °C ± 0.5 °C and use for further testing.

3.2.7 Sensory Analysis

The sensory analysis is evaluated by at least 20 untrained assessors selected according to their habits. Samples are cut into slices of about 5 mm thickness. Samples are then labeled with three-digit random numbers and served in random order to assessors in individual booths. Assessors are instructed to cleanse their palates with water between samples. A hedonic test is carried out using 9-point scales such as the one displayed in Scheme 1 (9 = like extremely and 1 = dislike extremely) in which the assessors evaluate different attributes: appearance, taste, texture, flavor, and overall acceptability [28].

Acceptance Test - Structured verbal hedonic scale

Rate the samples and use the scale below to indicate how much you liked or disliked them.

Sample code:______ Name:________________________ Gender:____ Age:_______

(9) I liked it extremely

(8) I liked it a lot

(7) I like it moderately

(6) I liked it slightly

(5) Indifferent

(4) Slightly disliked

(3) Disliked moderately

(2) I disliked it very much

(1) Extremely disliked

Scheme 1 Example of a structured verbal hedonic scale. (Source: Adapted from Meilgaard [28])

4 Notes

1. The main challenge in the development of fat-reduced foods is the maintenance of quality compared to the traditional product. For most food products, fat reduction is associated with increased water content. Therefore, the first need to match the quality of the standard product is to structure the water phase, by using functional ingredients such as proteins, gums, stabilizers, gelling agents, and other thickeners, or by increasing the amount of emulsifying agents and of the fibers [5]. For instance, improvements in texture (softer and smoother), elasticity, and adhesiveness should be very similar to conventional sausages. As an example, inulin can be used as a fat substitute in fermented sausages (50% and 25% of the original fat content) at 7.5% and 12.5%. A low-calorie product (30% of the original) can be obtained with approximately 10% inulin according to Mendoza et al. [29]. Li et al. [30] developed a hybrid hydrogel comprised of long-chain inulin, microcrystalline cellulose, and glucolactone which were incorporated to reduced-fat emulsifying pork sausage. The findings demonstrated that the formed gel acted as a fat replacer, offering potential advantages in upscaling healthier reduced-fat sausage quality.
2. Dietary fibers have been studied in a variety of researches involving meat products [31, 32]. Options of different plant sources, different granulometry ranges, diverse functionalities, and a relatively lower cost than some fat substitutes make fibers an interesting alternative for replacing fat in industrialized foods. Fiber is the most used ingredient in the preparation of functional food preparation accounting for >50% of the total ingredients on the market [33]. Oat fiber is an interesting ingredient to replace fat, as this cereal has a high water absorption capacity, being beneficial for low-fat sausages and mortadella. Increases in yield percentages were observed, but with a less pronounced red color and a firmer texture in the quality characteristics of light mortadella enriched with oat fiber [34, 35].
3. Cereal fibers are commonly used compared to fruit fibers; however, the latter have better quality due to increased amounts of total and soluble fibers, besides presenting better water and fat retention capacities, colonic fermentability, and lower calorific values [36–38]. By-products (peel and albedo) of citrus fruits such as lemon and orange are promising ingredients. The addition of dietary fiber from lemon albedo (raw and cooked) in a mortadella formulation (bologna type) resulted in a nutritional improvement of the product when compared to a control. There was indication of nitrite level

reduction, most likely due to the presence of active biocompounds in the albedo [39].

4. Different fibers tested alone or combined in mortadella, such as wheat (0–5%), oats (0–5%), and inulin (0–10%) fibers, contributed to increase firmness and chewiness and to decrease elasticity and cohesiveness in low-fat content mortadella. The sensory evaluation did not show significant differences when the test samples were compared with a control sample containing 20% fat and no added fiber [2]. Pea fiber can be added in mortadella to improve texture. Other promising fiber sources that have been tested are peach fiber in sausages [36], peach, apple and orange fibers in salamis [40], rice bran in emulsified meat products [41], dietary carrot fiber in fermented sausages [42], and bamboo fiber in meat emulsions [43].
5. Regardless of dietary fiber, fruits and vegetables contain beneficial organic micronutrients such as carotenoids, polyphenols, tocopherols, vitamin C, and others, thereby reducing the risk of coronary heart disease, stroke, and certain types of cancer [44].
6. The choice of ingredients depends on the type of product and the desired level of fat reduction; however, this choice needs to be carefully balanced against their effects on the variety of product characteristics. The strategy requires complete knowledge of available ingredients and understanding of their relationships in the product matrix structure [45].
7. The cost of ingredients used to replace fat is another important factor in the development of low-fat products. Although the initial costs of fat substitutes have been considered high, competitiveness and large-scale production have contributed to their reduction. However, to survive in the marketplace, an ingredient needs to have a clear performance advantage over existing alternatives. The isolated cost assessment should not be considered as a decision parameter. Often, the use of a low-cost substitute implies changing some other ingredients in the formulation that may negatively affect the final cost of the product.
8. Increasing water content can affect microbiological stability. Thus, the evaluation of water activity remains a basic method for determining microbiological stability [46].

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Chapter 8

Analysis of Thiamine, Riboflavin and Nicotinic Acid in Meat

Ceres Mattos Della Lucia and Lívya Alves Oliveira

Abstract

Vitamins play a crucial role in ensuring food quality. When analyzing B complex vitamins in food samples, a variety of methods are employed, including extraction, purification, derivatization, analysis, and detection. Among these techniques, high-performance liquid chromatography (HPLC) is the most widely used for accurate quantification of water-soluble vitamins. Here, we describe the extraction methods and chromatographic conditions to analyze thiamine, riboflavin, and nicotinic acid in meat samples. The extraction process involves acid hydrolysis and enzymatic hydrolysis, followed by subsequent filtration and centrifugation steps. For chromatographic analysis, HPLC is used with specific column types, flow rates, running times, injection volumes, and wavelength settings to achieve optimal results.

Key words Thiamine, Riboflavin, Niacin

1 Introduction

The need for information on the nutritional aspects of food has increased since professionals in the area and consumers have become more aware of this issue. So it is of great importance to analyze the nutritional content of the large number of foods existing in nature, added to several others developed by food industries and research institutions.

One of the most important factors in determining the quality of food refers to its vitamin content. Vitamins are essential micronutrients of great importance in the normal growth and maintenance of health [1]. Additionally, they are essential in several and varied metabolic reactions in the organism and act as essential active agents for the maintenance of biological functions [2]. Insufficient intake or poor absorption of vitamins often induces disease with characteristic symptoms [3–6].

The study of the literature published in recent years suggests that the chromatographic methods have become an indispensable

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tool for rapid determination of vitamins in foods [7]. In particular, high-performance liquid chromatography (HPLC) has been lately the most applied technique, and it is becoming also a standard method in the assessment of vitamins, especially in routine work, as it is an accurate and fast method of analysis [8–10]. In the case of analysis of B complex vitamins in meat products, fluorometric detection has been widely used because it is more selective and detects a smaller number of interfering compounds that prevail in these foods [11].

2 Methodology for Thiamine, Riboflavin, and Niacin Analysis

Vitamin assays typically necessitate the extraction of vitamins from their biological matrices for subsequent analysis. This extraction process often involves a combination of treatments, such as heating, acidification, alkalization, solvent extraction, and enzymatic digestion. Specific extraction procedures are employed for each vitamin, tailored to ensure the stability and preservation of the target vitamin during the extraction process [12].

The analytical techniques that have been most applied in the quantitative determination of vitamins in food can be classified as bioassays, microbiological assays, and chemical assays [12]. Biological methods use animals, mainly rats, where the vitamin utilized by the organism is measured. Vitamin doses are given to certain groups to cause deficiency or recovery. These methods can be useful to establish the biological activity or bioavailability of a new preparation. However, they can be susceptible to large variabilities and require large amounts of material and long periods to obtain results [13].

Microbiological methods are based on the absolute need of certain microorganisms for specific vitamins. In this way, these microorganisms can only multiply when vitamins are present in the culture medium. The microorganisms grow proportionally to the amount of vitamins present. Cell growth or multiplication is determined by turbidimetric measurement. These methods are still widely used in the determination of nicotinic acid in foods. Lactic acid bacteria are the most commonly used in microbiological assays, but yeasts and protozoa can also be used. Assays with lactic acid bacteria are preferred because their nutritional needs are specific, they grow rapidly in synthetic and semisynthetic culture media, and they are not pathogenic. *Lactobacillus plantarum* is used for determination of total niacin, *Leuconostoc mesenteroides* is used for added nicotinic acid, and historically, thiamine tests have relied on *Lactobacillus viridescens* or *Lactobacillus fermenti* [14].

The general analytical procedures for determining B complex vitamins in food can be divided into six stages: sampling, extraction, purification, measurement, calculation of results, and interpretation

of data. Other factors to be considered are the maintenance of sample integrity, sample storage, and sample preparation for analysis [14].

Physicochemical methods are more applicable to routine vitamin determinations because they are generally more precise, faster, and economical. This category includes spectrophotometric, colorimetric, fluorometric, and chromatographic methods.

Spectrophotometric methods are more commonly used for pharmaceutical preparations and are not satisfactorily used for food analysis due to their low sensitivity and the presence of interfering substances [14]. Fluorometric methods are highly sensitive, being quite used for analysis of thiamine and riboflavin in foods. The fluorometric method for thiamine determination [15] is based on the conversion of thiamine to its fluorescent oxidation product, thiochrome, by reaction with alkaline potassium ferricyanide. Mercury and cyanogen bromide can also be used, but they are toxic and, therefore, dangerous for health. Under standardized conditions, the method is sensitive and specific. Specificity is achieved by the use of chromatography and extraction in isobutanol, both acting as purification steps. The last step contributes to sensitivity, as thiochrome is more fluorescent in isobutanol than in aqueous solutions [14].

The native fluorescence displayed by riboflavin allows this vitamin to be evaluated fluorometrically without the need for chemical derivatization. The fluorescence method has been adopted by the AOAC [16] for determination of total riboflavin in food.

Colorimetric methods can be used to determine niacin in food. However, they have a low specificity, the colored complexes formed for measurement have a low stability, and certain reagents used, such as cyanogen bromide, are toxic and difficult to handle [14]. Despite this, it is still an official method, recommended by the AOAC [17].

Chromatographic methods have been an indispensable tool for rapid and specific determinations of vitamins in food. The development of simultaneous determination of multiple vitamins is mainly attributed to the application of chromatographic techniques. However, there are challenges in standardizing extraction conditions, physical–chemical characteristics, and stability of each vitamin. Estimating the content of B vitamin complexes is particularly challenging due to variations in matrices and specific factors in different food samples [18, 19]. Additionally, the presence of interfering substances from the matrix and significant concentration variations of the analytes require highly selective HPLC detectors [18]. The simultaneous analysis of vitamins is further complicated by the existence of multiple biologically active forms, as well as their chemical instability and heterogeneity [19].

It is noteworthy that the integration of liquid chromatography with mass spectrometry (MS) has revolutionized the field of vitamin analysis. Typically, both fat-soluble and water-soluble vitamins

can be accurately analyzed using either liquid chromatography coupled to a mass spectrometer or electrospray ionization methods. The utilization of MS for detection offers enhanced sensitivity, allowing for precise identification and thorough characterization of the vitamins [12].

2.1 High-Performance Liquid Chromatography (HPLC)

HPLC is a standard technique for qualitative and quantitative determination of water-soluble and fat-soluble vitamins. Lately, this method has been the most applied method for the analysis of vitamins in food. The determination of B complex vitamins in pharmaceutical multivitamin preparations or premixes has been easily performed by HPLC due to the simplicity of the matrix and the high content of the formulas, not requiring extraction and purification procedures [20, 21]. The HPLC analysis method is also applied to determine the B complex vitamins in a wide variety of foods such as vegetables, leafy vegetables, cereals, grains, and foods of animal origin [9, 10, 22–25].

The analysis of HPLC vitamins in foods, especially the natural content, is still being researched due to difficulties in extracting the vitamins and chromatographic analysis. Quantifying vitamin levels in food presents a multifaceted analytical challenge due to several factors: the diverse chemical structures and properties of vitamins make it exceedingly difficult to devise a universal method for simultaneous determination of multiple vitamins in the food; the food matrices are inherently complex; vitamins constitute only a minute fraction of the food matrix, even and for fortified foods; and precise knowledge of natural vitamin content is essential to ensure compliance with legal requirements [26]. Various liquid chromatography techniques have been employed to separate water-soluble vitamins; the methods presented in this chapter refer to the extraction and analysis of B complex vitamins (thiamine, riboflavin, and niacin) in meat.

2.1.1 Extraction Procedures

The extraction stage serves to isolate the vitamin from the molecule coenzyme and release it from its association with proteins or carbohydrates. The extraction procedure must be able to release the linked shapes from the vitamin for subsequent quantification. Research has shown that there is no universal extraction process for vitamins. The problem is also compounded by the complex and variable nature of the food matrix. The analytical results must therefore be interpreted on the basis of knowledge of the sample and the extraction process employed [14].

When the objective is to determine added vitamins in fortified foods, extraction is relatively easy. Under these circumstances, it is possible to use a procedure that can extract two or more vitamins simultaneously [14, 27].

For the determination of total thiamine, the extraction procedure usually involves an acid hydrolysis and an enzymatic hydrolysis. The hot acid hydrolysis aims to liberate the thiamine and

thiamine phosphate esters of its association with proteins [14]. In this step, the sample is autoclaved at 121 °C for 30 min with 0.1 N HCl [15]. Thiamine ligands present in foods of animal origin are stable under these conditions. Enzymatic hydrolysis aims to transform thiamine esters phosphate to free thiamine.

The technique used to determine total riboflavin is dictated by the intense fluorescence of three flavins: flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and free riboflavin. FMN and free riboflavin exhibit equal fluorescence intensity under the same molar basis, whereas FAD fluorescence is much less intense. Therefore, it is necessary to release flavins from their intimate association with proteins and completely convert FAD to FMN and this to free riboflavin. This step is achieved by autoclaving the sample at 121 °C for 30 min with dilute mineral acid (usually 0.1 N HCl) at pH less than 3 [16]. Bound flavins are released by denaturing the proteins, and any enzymes that might be present are inactivated.

With the use of HPLC, FMN and riboflavin released during acid treatment can be chromatographically separated. So there are two ways of extraction in the determination of total riboflavin by HPLC. The first way is to convert all FMN to free riboflavin and then calculate the total riboflavin based on riboflavin peak. The second way is to calculate FMN and riboflavin separately and report results summed as total riboflavin. In the first way, the conversion of FMN to riboflavin can only be achieved by enzymatic hydrolysis. It is essential to check whether there has been a complete conversion by checking for the presence of a peak corresponding to FMN. In the second way, enzymatic hydrolysis is omitted [14].

The terms "total niacin" and "free niacin" are defined by the methods of extraction used in the analysis. Total niacin generally refers to niacin which is extractable by autoclaving the sample with 1N alkali or acid; free niacin is defined as niacin extractable by acid autoclaving [14]. Autoclaving with H_2SO_4 0.1 N for 30 min at 121 °C [17] releases nicotinamide from its coenzymatic form. The treatment is also sufficient to release bound nicotinic acid which may be present. More commonly, the extraction of B vitamins is done with the use of diluted HCI.

2.1.2 Extract Purification

The purification or cleaning of the extract is important to remove the interfering compounds and thus facilitate the separation and adequate quantification of the vitamin under analysis by HPLC. In the determination of thiamine derivatized to thiochrome by fluorescence or HPLC, the use of isobutanol works as an efficient purification step [14].

Sep-Pak C18 cartridges have been widely used for purification and concentration of various food extracts for the determination of vitamins. Before use, cartridges are conditioned with a buffer

solution or other reagents, the vitamins being eluted with methanol or ethanol [28, 29].

Ion exchange columns are indicated for purifying extracts for the determination of thiamine and nicotinic acid [30, 31]. In addition to the methods of aforementioned purification systems, the use of column exchangers has been a quick and simple alternative in the preparation of samples, preventing oxidation of some vitamins. This procedure has become important in the work of routine food control laboratories where a simple sample preparation is necessary, with a fast and safe determination.

2.1.3 Derivatization

Derivatization of some vitamins may be necessary to facilitate the use of a more adequate detection (fluorometric) and/or a type of most suitable chromatography. The pre-column and post-column derivatization can be employed [14]. For thiamine determination, the derivatization with alkaline potassium ferricyanide has been the most used [32, 33].

2.1.4 Chromatographic Analysis

The main types of chromatography that have been used in the analysis of B complex vitamins are reversed-phase chromatography and ionic interaction chromatography [34]. Most separations involving reversed-phase chromatography have used packaging based on silica microparticles, in which the nonpolar stationary phase, usually octadecylsilane (OOS), is chemically bonded to the silica surface through siloxane bridges [35]. The most used analytical columns for vitamin analysis in HPLC are the conventional ones (150 or 250 mm in length × 4.6 mm in diameter) stuffed with nonpolar stationary phase.

2.1.5 Detection

Two types of detectors are most often used to monitor the column effluent in HPLC systems for the determination of thiamine, riboflavin, and niacin in food samples. They are the detector of absorbance (simple or photodiode arrays) and the detector of fluorescence. Absorption monitoring is indicated for the detection of niacin. Fluorometric detection provides a greater sensitivity and selectivity for the detection of thiamine (thiochrome) and riboflavin [14]. Fluorescence detection provides the advantage of minimal interference from other compounds, whereas ultraviolet detection techniques often encounter interference from numerous compounds in complex matrices, especially in food samples. The fluorescence detector is known for its exceptional sensitivity in high-performance liquid chromatography (HPLC), surpassing the sensitivity of UV absorption by a factor of 100 in many cases. This heightened sensitivity makes the fluorescence detector particularly advantageous for applications involving trace analysis, small sample sizes, or extremely low solute concentrations, such as the analysis of vitamins [36].

Vitamin determinations in foods face challenges due to the complex nature of the food matrix, the low vitamin levels, and the presence of vitamins in various bound forms. These challenges can introduce potential sources of error, underscoring the need for validated analysis methods. When selecting a validation method, it is important to consider performance characteristics such as precision, accuracy, and detection limit. Furthermore, the chosen method should be robust, capable of withstanding interference or minor variations under routine conditions [14].

The methods described in this chapter have been adapted and validated by Pinheiro-Sant'Ana [37–39] and are based on AOAC [16, 17, 40] and HÄGG [41]. In her study, Pinheiro-Sant'Ana adapted and optimized the extraction and chromatographic conditions for the analysis of thiamine, riboflavin, and nicotinic acid in beef, pork, and chicken meat. Her objective was to evaluate the retention of these vitamins in meat prepared in food services. Through the linear range, detection limits, and recovery percentages, the sample preparation conditions and optimized chromatographic conditions for the analysis of thiamine, riboflavin, and nicotinic acid in beef, pork, and chicken meat were ensured.

3 Materials

3.1 Determination of Nicotinic Acid

1. *Sulfuric acid solution (H_2SO_4) 0.1 N*: To prepare 100 mL, add distilled water in a volumetric flask (100 mL) up to the middle of the flask; add 9.8 mL of H_2SO_4, slowly concentrated to the volumetric flask. Complete the volume to 100 mL of solution.
2. *Mobile phase*: Mobile phase must be composed of 5% acetonitrile, 95% ultrapure water, 0.15% triethylamine, and 0.005 M heptanesulfonic acid and pH adjusted to 2.8 with H_2SO_4. To prepare 100 mL of mobile phase, measure 5 mL of methanol HPLC grade in a 100 mL test tube. Add 95 mL of ultrapure water to the test tube and 0.15 mL of triethylamine. Dissolve 0.1011 g of heptanesulfonic acid in the mobile phase, and adjust the pH to 2.8 with H_2SO_4.

3.2 Determination of Thiamine

1. *Trichloroacetic acid solution (TCA) 50%*: To prepare 100 mL, weigh in a beaker 50 g of TCA. Measure 100 mL of ultrapure water in a beaker, and add it slowly to the beaker containing TCA. Mix the solution until the TCA dissolves completely, and store the solution in an amber glass bottle.
2. *Hydrochloric acid solution (HCI) 0.1 N*: To prepare 100 mL, add distilled water in a volumetric flask (100 mL) up to half the balloon. Add 1.0 mL of concentrated HCl slowly to the volumetric flask. Complete the volume to 100 mL of solution. Transfer the solution to a properly identified bottle.

3. *2 M sodium acetate solution*: To prepare 100 mL, weigh 16.4 g of sodium acetate, and dissolve in 100 ml of distilled water. Transfer the solution to a properly identified bottle.
4. *Potassium ferricyanide alkaline solution*: To prepare 100 mL, pipette 4 mL of a 1% potassium ferricyanide solution into a 100 mL volumetric flask. Add 48 mL of a 15% NaOH solution, and complete the volume with distilled water.
5. *Mobile phase*: Composition – 40% methanol, methanol: potassium chloride 0.25% (40:60). To prepare 100 mL, dissolve 0.25 g of potassium chloride in 100 mL of ultrapure water. Measure 40 mL of HPLC methanol in a 100 mL beaker, and complete the volume of the test tube (60 mL) with the potassium chloride solution at 0.25%.

3.3 Determination of Riboflavin

1. *Trichloroacetic acid solution (TCA) 50%*: To prepare 100 mL, weigh 50 g of TCA in a beaker. Measure 100 mL of ultrapure water in a beaker, and add it slowly to the beaker containing the TCA. Mix the solution until the TCA dissolves completely, and store the solution in an amber glass bottle, duly identified.
2. *Hydrochloric acid solution (HCl) 0.1 N*: To prepare 100 mL, add distilled water in a volumetric flask (100 mL) up to half the balloon. Add 1.0 mL of concentrated HCl slowly to the volumetric flask, and complete the volume to 100 mL of solution. Transfer the solution to a properly identified bottle.
3. *2 M sodium acetate solution*: To prepare 100 mL, weigh 16.4 g of sodium acetate, and dissolve in 100 mL of distilled water. Transfer the solution to a properly identified bottle.
4. *Mobile phase*: Composition – 34% methanol, 65% ultrapure water, 1% acetic acid, and 0.005 M of heptanesulfonic acid. To prepare 100 mL, measure 34 mL of HPLC methanol in a 100 mL beaker. Add 65 mL of ultrapure water and 1 mL of acetic acid to the test tube. Dissolve 0.1011 g of heptanesulfonic acid in the mobile phase.

4 Methods

4.1 Nicotinic Acid Extraction

The extraction of nicotinic acid is carried out based on AOAC [17] and optimized by Pinheiro-Sant'Ana [37] performed through acid hydrolysis. For this, the following steps must be performed:

- Weigh 8 g of sample (8 g of beef, 8 g of pork, and 8 g of chicken meat).
- Add about 75 mL of 1 N H_2SO_4. The samples are ground using a micro grinder and hydrolyzed in an autoclave at 121 °C for 60 min.

- Cool the mixture in an ice bath, and make up to 100 mL with distilled water.
- Centrifuge the samples at 4000 rpm (2800 g) for 25 min, and then filter through Whatman #40 filter paper.
- Filter the samples through Millipore membranes with a porosity of 0.45 μm, stored in amber glasses at 0–3 °C, for the shortest possible period (1–4 h), and inject them into the chromatographic column for analysis.

Chromatographic Analysis

- High-performance liquid chromatography, coupled to a fluorescence detector.
- RP-18 column (packed with 5 pm silica particles, coated with octadecylsilane), 250 mm long and 4 mm internal diameter.
- Flow rate: 0.8 mL/min.
- Running time: 20 min.
- Injection volume: 50 μL.
- Wavelength: 261 nm.

Data Analysis

- Determine the linear regression equation to be used for nicotinic acid.
- Calculate the nicotinic acid levels present in the samples, considering the weights and dilutions performed.

4.2 Thiamine Extraction

The described thiamine extraction procedure was optimized by Pinheiro-Sant'Ana [39] based on AOAC and HÄGG and involved acid hydrolysis and enzymatic hydrolysis.

- Weigh the sample (5 g of beef or chicken and 1 g of pork).
- Add approximately 50 mL of 0.1 N hydrochloric acid. Samples are ground using a micro grinder and hydrolyzed in an autoclave at 121 °C for 30 min.
- The mixture is cooled in an ice bath to room temperature and the pH adjusted in a pH meter with the aid of a magnetic stirrer to 4.0–4.5 with about 3 mL of 2 M sodium acetate.
- Add 5 mL of 6% clear diastasis, and incubate the mixture in a water bath for 3 h at 50 °C.
- Precipitate the proteins by adding 2 mL of 50% trichloroacetic acid and heating at 97 °C for 10 min.
- Cool the mixture, and complete the volume to 100 mL with distilled water. The samples are centrifuged at 4000 rpm (2800 g) for 25 min and then filtered through Whatman #40 filter paper.

Derivatization of Thiamine

After this step, carry out the oxidation of thiamine to thiochrome (fluorescent compound) as follows:

- To a tube containing 1.5 g of NaCl, add 10 mL of the sample, protecting it from light.
- Then, carefully add 5 mL of alkaline potassium ferricyanide without letting it drain from the walls of the tube, stirring vigorously in a tube shaker at constant speed for 1 min.
- Then, add 10 mL of isobutanol, stirring vigorously in a tube shaker at constant speed for 2 min.
- After separating the phases, carefully pipette the extract in isobutanol (formed thiochrome – upper layer).
- Filter the samples through Millipore membranes with a porosity of 0.45 μm, stored in amber glasses at 0–3 °C, for the shortest possible period (1–4 h), and inject them into the chromatographic column for analysis.

Chromatographic Analysis

- High-performance liquid chromatography coupled to a fluorescence detector.
- RP-18 column (packed with 5 pm silica particles, coated with octadecylsilane), 250 mm long and 4 mm internal diameter.
- Mobile phase flow: 0.8 mL/min.
- Running time: 8 min.
- Injection volume: 40 μL.
- Wavelength: 234 nm.

Data Analysis

- Determine the linear regression equation to be used for thiamine.
- Calculate the thiamine levels in the samples, considering the weights and dilutions performed.

4.3 Riboflavin Extraction

The method for extracting riboflavin in meat was based on AOAC [16] and HAGG [41] and optimized by Pinheiro-Sant'Ana [38]. This extraction procedure involves acid hydrolysis and enzymatic hydrolysis. For this, the following steps must be performed:

- Weigh the sample (5 g of beef, pork, and chicken meat).
- Add approximately 50 mL of 0.1 N hydrochloric acid.
- The samples are ground using a micro grinder and hydrolyzed in an autoclave at 121 °C for 30 min.

- The mixture is cooled in an ice bath to room temperature, and the pH is adjusted in a pH meter with the aid of a magnetic stirrer to 4.0–4.5 with approximately 3 mL of 2 M sodium acetate.
- Add 5 mL of 6% clear diastase, and incubate the mixture in a water bath for 3 h at 50 °C.
- Precipitate the proteins by adding 2 mL of 50% trichloroacetic acid and heating at 97 °C for 10 min.
- Cool the mixture, and complete the volume to 100 mL with distilled water. The samples are centrifuged at 4000 rpm (2800 g) for 25 min and then filtered through Whatman #40 filter paper.

Chromatographic Analysis

- High-performance liquid chromatography coupled to a fluorescence detector.
- RP-18 column (packed with 5 μm silica particles, coated with octadecylsilane), 250 mm long and 4 mm internal diameter.
- Mobile phase flow: 1.0 mL/min.
- Running time 10 min.
- Injection volume: 50 μL.
- Wavelength: 267 nm.

Data Analysis

- Determine the linear regression equation to be used for riboflavin.
- Calculate the levels of riboflavin present in the samples, considering weights and dilutions performed.

5 Notes

- Precautions to Prevent Vitamin Losses During Extraction and Analysis Processes

  The standard analytical protocol for vitamin determination in food needs careful attention to sample preservation, storage prior to analysis, and sample preparation. This is imperative due to the inherent high instability of vitamins, which undergo rapid degradation when exposed to diverse conditions including heat, oxygen, light, humidity, and specific pH levels [42].

  Therefore, it is important to protect samples and extracts from sunlight, artificial light, and oxygen throughout the entire extraction and analysis process by using glassware with lids, such

as amber-colored containers or aluminum foil, and blackout curtains. Also, samples and extracts must be kept outside the refrigerator/freezer for the shortest time possible.

- Representative Sampling

  Food analysis encounters a significant challenge in dealing with the heterogeneity of food samples. This heterogeneity can be classified into macroheterogeneity, which refers to variations among different units of a lot, and microheterogeneity, which pertains to variations within different parts of a unit [43]. In the context of vitamin assays, microheterogeneity becomes particularly important.

  The uneven distribution of fat in meat necessitates expressing analytical values on a fat-free basis [14]. In the case of vitamin-enriched foods, the distribution of added vitamins often lacks uniformity, further exacerbating the issue of heterogeneity. It is crucial to acknowledge that the concentration of a nutrient represents an average value with associated uncertainty. Consequently, it is essential to obtain a representative sample before conducting the food analysis, aiming to minimize this uncertainty [14].

  Ideally, immediate analysis of the food sample after selection is desirable, but not always feasible. Therefore, it is necessary to properly package and store the sample to minimize changes in its composition. This involves the use of appropriate containers and storage conditions at low temperatures to preserve the integrity of the food, preventing moisture loss, chemical deterioration, contamination, and undesirable microbial growth [14].

  To obtain a representative sample, prior to laboratory analysis, it is essential to remove inedible parts of the food. In meats, for example, bones and skins and other inedible tissues are discarded. Then it is necessary to homogenize approximately 200 g of the sample using a food processor or micro grinder before weighing (for meaningful sampling). For larger-sized samples, one should divide them into two opposing quarters before homogenization.

- Preparation of the Mobile Phase

  The mobile phase should be prepared using HPLC-grade liquid reagents or ultrapure water. The volume of mobile phase required for analysis should be calculated using the equation below:

$$(\text{Number of samples} + \text{Number of standard injections} + 2) \times \text{Flow rate of the mobile phase} \times \text{Run time} + 300\ \text{mL}).$$

- Identification

  The identification of B complex vitamins is carried out by comparing the absorption spectrum of the peaks provided by the samples in comparison with those provided by analytical standards. Comparison of retention times with standards is also a criterion for identifying vitamins.

6 Conclusions

The determination of nicotinic acid, thiamine, and riboflavin in food samples requires careful sample preparation and chromatographic analysis. The extraction methods involve acid hydrolysis and enzymatic hydrolysis, followed by protein precipitation and filtration. High-performance liquid chromatography (HPLC) coupled with fluorescence detection is used for the analysis of nicotinic acid and riboflavin, while thiamine is analyzed by HPLC with UV detection. The mobile phases for each analysis are carefully prepared using specific compositions and pH adjustments. It is crucial to handle the samples and extracts with precautions to prevent vitamin losses, including protection from light and oxygen. Representative sampling is essential to minimize heterogeneity and obtain accurate results. Proper packaging and storage conditions are necessary to preserve the sample's integrity. Overall, this methodology provides a reliable approach for the determination of vitamins in meat, facilitating nutritional assessment and compliance with quality standards.

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Chapter 9

Natural Additives in Meat Products as Antioxidants and Antimicrobials

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Abstract

The perishable nature of meat has led to the use of various additives in the development of meat products. Specifically, its susceptibility to oxidation reactions, together with the fact that it is an excellent substrate for microbial development, has made the employment of antioxidant and antimicrobial additives essential. In this way, the quality of meat products as well as their safety can be maintained for longer, extending the shelf life of the products. Nonetheless, in recent years the use of synthetic additives in the food industry has been in the spotlight of consumers since some of these compounds have been associated with adverse health effects. In light of this situation, the search and development of new natural antioxidant and antimicrobial additives are currently mandatory for the meat industry in order to obtain healthier meat products and to be in line with consumer demand.

Given that many antioxidant compounds in matrices are present in nature, this book chapter aims to propose a method for obtaining and producing bioactive antioxidant and/or antimicrobial compounds that can be used successfully in the meat industry to replace harmful synthetic additives. Thus, this document presents a detailed guide to the reader on how to extract antioxidant and/or antimicrobial compounds from natural sources through a solid–liquid extraction assisted by a pulsed electric field (PEF) and how to microencapsulate them to obtain the natural antioxidant and/or antimicrobial additive for food use. In addition, this book chapter also includes a clear and comprehensive procedure to develop a model meat product that can be easily adapted to other requirements.

Key words PEF-assisted extraction, Natural extracts, Microencapsulation, Spray-dry, Antioxidants, Antimicrobials, Meat industry, Hamburger

1 Introduction

In the meat industry, antioxidants are compounds that help to neutralize the free radicals which can oxidize lipids, proteins, and pigments, causing rancidity of fats and degradation of proteins and heme pigments, respectively [1]. Thus, oxidation reactions promoted by free radicals involve unwanted changes during the shelf

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life of meat and meat products generally related to sensory quality (including alterations in color, texture, and off-flavor and off-odor appearance), while antioxidant compounds prevent these deterioration processes [2–4]. Moreover, antioxidant compounds can prevent the formation of toxic substances (e.g., cholesterol oxides, malonaldehyde, 4-hydroxynonenal, carbonyl compounds, and hydroperoxides) also generated by free radicals during oxidation reactions that are harmful to human health [5–7] even proving to be potential carcinogenic and DNA disruptors [8, 9]. For their part, the antimicrobial substances prevent or reduce the growth of both pathogenic and nonpathogenic microorganisms [10], thus delaying the deterioration of food and avoiding food safety problems (e.g., intoxications, toxico-infections, etc.).

Due to the functions that antioxidant and antimicrobial compounds can have in food (both in the shelf life and quality of the product, as well as the implications in the eradication of the formation of toxic compounds and stopping the growth of pathogenic microorganisms), their use in the meat industry is inescapable to elude economic losses and safety issues because of the food spoilage and the presence of pernicious substances and microorganisms, respectively.

Initially, the food industry began to use synthetic antioxidants, the most common being butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ). These synthetic additives have high antioxidant capacities even when used at low concentrations. However, these compounds have been related to various adverse effects, including carcinogenicity, cytotoxicity, endocrine disruption, and oxidative stress induction [11–13]. Similarly, some synthetic antimicrobial compounds commonly used in the meat industry, such as nitrites, nitrates, and sulfites, have been associated with adverse health effects [14–16].

In order to avoid the harmful effects of synthetic additives, currently in the meat industry, more and more attention is being paid to the employment of antioxidant and antimicrobial compounds of natural origin with the aim of enhancing the conservation of meat products without harming the health of the consumers [4, 17, 18]. Nonetheless, the existence of a multitude of natural matrices (vegetables, fruits, plants, seeds, seaweeds, etc.) that can be utilized to obtain antioxidant and/or antimicrobial compounds and the lack of knowledge on how to use these natural ingredients in food products properly can hinder the successful utilization of these as additives in the meat industry. For this reason, the methodology proposed in this book chapter tries to provide a standardized way to produce natural antioxidants and/or antimicrobials from a specific natural matrix, as well as to standardize their subsequent use as an additive in the meat industry.

For employ as an additive in industry, the natural antioxidant and antimicrobial compounds must be extracted from the matrix in question. Currently, different extraction methods have been proposed over all those considered green technologies [19]. In this way, the extractions carried out are usually solid–liquid extractions assisted by different green technologies such as pulsed electric field (PEF), ultrasound (US), microwaved (MW), and high hydrostatic pressure (HHP) [20–23]. Thus, the target substances are released from the cells of the starting raw material into the solvent (generally water) used for their extraction. Despite the variety of technologies currently available for the extraction of antioxidant and antimicrobial compounds, this chapter describes a feasible and easy solid–liquid extraction technique using water as a solvent and the application of PEF (i.e., a solid–liquid extraction assisted by PEF). The PEF technology originates the appearance of pores in the membrane of the cells that constitute the sample to be treated and allows the release of the target compounds without the application of heat [20, 24, 25]. Once the aqueous extracts containing the antioxidant and/or antimicrobial compounds of natural origin have been obtained, this chapter proposes performing a microencapsulation of these substances through spray-drying, with the aim of protecting the antioxidant and/or antimicrobial compounds and making them stable during their storage until their use as additive in a meat product.

With all stated above, the purpose of this book chapter is to serve as a guidebook for the development of an antioxidant and/or antimicrobial additive of natural origin which can be used in the meat industry with the intention of increasing the shelf life of diverse meat products without the need to use synthetic additives that can be harmful to human health. Likewise, this book chapter includes the steps to follow in the preparation of a meat product to which said natural additive has been added with the purpose of guaranteeing its proper handling and integration into the food industry.

2 Materials

2.1 Preparation of the Natural Antioxidant and/or Antimicrobial Additive

For the preparation of natural antioxidant and/or antimicrobial additives, different materials, ingredients (i.e., plant by-products), and equipment (i.e., PEF equipment, microencapsulation equipment, etc.) are required that may be less common in the food industry. However, little by little, with the enhancement of innovative techniques in research and the generation of new knowledge, the use in the food industry of the methods proposed in this chapter (i.e., solid–liquid extraction assisted by PEF and microencapsulation by spray-drying) could gradually grow, and it could be

increasingly common to find these materials, ingredients, and/or equipment, making it possible to commercialize meat products without artificial additives.

The materials and ingredients necessary for the manufacturing process of the natural antioxidant and/or antimicrobial additive are indicated below.

2.1.1 Ingredients for the Preparation of the Natural Antioxidant and/or Antimicrobial Additive

For the preparation of the natural antimicrobial and/or antioxidant additive, few ingredients are necessary (listed below). Nevertheless, the starting raw material must be carefully selected. It is especially important that it be rich in antioxidant and/or antimicrobial compounds and that these are easy to extract and highly stable to guarantee their usefulness in the food industry.

1. Vegetable matrix (*see* **Note 1**).
2. Distilled water.
3. Maltodextrin (*see* **Note 2**).

2.1.2 Material and Equipment for the Preparation of the Natural Antioxidant and/or Antimicrobial Additive

In addition to the usual material used in the food industry (cutting board, knife, spatula, etc.), as mentioned above, less frequently used equipment is included in this chapter (namely, PEF generator and spray-dry microencapsulator). This could make implementation in the meat industry difficult. However, the use of new emerging technologies is a very interesting tool that can make it possible to develop meat products without artificial additives and with a high added value. Thus, it is interesting to study its use and the feasibility of its implementation at an economic level.

The equipment and material necessary for developing a natural antioxidant and/or antimicrobial additive for meat industry utilization are indicated below.

1. Usual food industry material (cutting board, knife, spatula, etc.).
2. Professional mill KN Knifetec (Foss).
3. Homogenizer HM 294 (FOS).
4. Analytical balance, with a resolution of 0.0001 g, mod. ME 614S (Sartorius, Göttingen, Germany).
5. Beakers of 250 mL.
6. Aluminum foil.
7. Polypropylene tubes of 250 mL with caps (Beckman Coulter, California, United States).
8. Magnetic stirrer bars.
9. Magnetic hot stirrer plate.

10. Pulsed electric field generator (semiconductor-based positive Marx modulator Epulsus-PM1-10) equipped with a batch treatment chamber (EnergyPulse Systems, Lisbon, Portugal).
11. Centrifuge, mod. Allegra TM X-22R (Beckman Coulter, California, United States).
12. Qualitative filter paper.
13. IKA T25 Digital Ultra-Turrax (IKA®-Werke GmbH & Co. KG, Staufen, Germany).
14. Microencapsulator Mini Spray Dryer B-290 (Büchi, Noble Park, VIC, Australia).
15. Compressed air bottle.
16. Vacuum bags.
17. Vacuum packing machine.

2.2 Preparation of the Meat Product with the Addition of the Natural Antioxidant and/or Antimicrobial Additive

As an example of a meat product in this chapter, a hamburger has been selected due to its high consumption, and also because it is an interesting matrix to test for antioxidant and antimicrobial compounds, since being made with minced meat, this product can be very susceptible to oxidation processes and microbial contamination. However, despite this particular specification, this model can be used as a guide for preparing other different meat products (e.g., other fresh meat products, cured products, pâté, cooked products, etc.).

2.2.1 Ingredients for the Preparation of the Hamburger with the Addition of the Natural Antioxidant and/or Antimicrobial Additive

The ingredients used in the preparation of hamburgers can vary according to the characteristics desired in the final product. Thus, for example, the meat of different animal species (beef, pork, chicken, turkey, etc.), oils that replace animal fat (e.g., algal mixtures, avocado, pumpkin seed, sesame, and walnut oils) [26–28], and distinct spices (black and white pepper, oregano, paprika, rosemary, thyme, etc.) can be employed. In this book chapter, ingredients commonly used in the preparation of hamburgers are proposed, in addition to the natural antioxidant and/or antimicrobial additive.

1. Lean beef meat (*see* **Note 3**).
2. Pork backfat (*see* **Note 3**).
4. Water.
5. Natural antioxidant and/or antimicrobial additive.
6. Common salt (NaCl).
3. Black pepper (*see* **Note 4**).
4. Oregano (*see* **Note 4**).

2.2.2 Material and Equipment for the Preparation of the Hamburger with the Addition of the Natural Antioxidant and/or Antimicrobial Additive

The production of hamburgers at an industrial level requires a specific material that allows the process to be carried out in optimal conditions and to obtain suitable products (*see* **Note 5**). These trademarks can vary according to the amount of production so that the selection of the appropriate brands and sizes for each case is left in the hands of the readers. Thus, the brands of the equipment that are shown below are indicative and are based on the previous experience of the authors.

1. Tables, knives, trays, containers, and utensils for regular use in the food industry.
2. Precision balance with a resolution of 0.01 g, mod. TE612 (Sartorius, Göttingen, Germany).
3. Refrigerated mincer machine (La Minerva, Bologna, Italy).
4. 6 and 8 mm plates for the refrigerated mincing machine.
5. Vacuum maceration tumbler (Fuerpla, Valencia, Spain).
6. Burger maker machine (Gaser, A-2000, Girona, Spain).
7. 300 mm polystyrene traits with a permeability of 2 mL/(m^2·bar·day) (VIDUCA, Alicante, Spain).
8. 70 mm polystyrene film with a permeability of 2 mL/(m^2·bar·day) (VIDUCA, Alicante, Spain).
9. Heat sealer LARI3/Pn T-VG-R-SKIN (Ca.Ve.Co., Palazzolo, Italy).
10. Gas cylinder of CO_2.
11. Gas cylinder of O_2.
12. Gas mixer (KM 100-2 ME PA; Witt-Gasetechnik GmbH & Co. KG, Witten, Germany).

3 Methods

The method provided below for the use of natural antioxidant and/or antimicrobial additives in meat products is a prototype of a general protocol where, initially, a process for obtaining natural antioxidant and/or antimicrobial compounds has been developed for its later use as an additive in the preparation of a hamburger (food used as a model of general meat product). It should be noted that there are many techniques that can be followed to obtain natural additives with antioxidant and/or antimicrobial power. In the same way, the preparation of the meat product in question varies according to the requirements in each case and according to the product itself. Therefore, the methods shown in this book chapter are proposed as a general example.

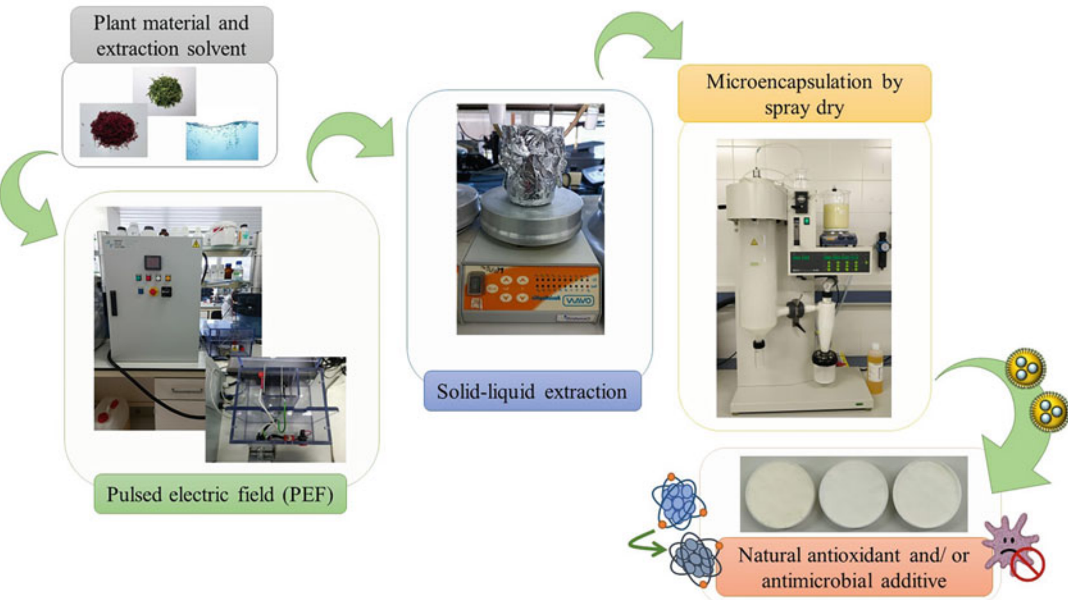

Fig. 1 Main steps for the preparation of natural antioxidant and/or antimicrobial additives for meat industry utilization

3.1 Preparation of the Natural Antioxidant and/or Antimicrobial Additive

Originally, the potential natural antioxidant and/or antimicrobial additive must be prepared (extracted and encapsulated) (Fig. 1), which will then be added to the meat product. To do this, the first step is to grind the plant material (vegetables, fruits, plant wastes, etc.), followed by an extraction and finally a microencapsulation.

The detailed methodology of these steps is shown below.

3.1.1 Raw Material Crushing and Homogenization

The first step is to cut the sample with the help of a cutting board and a knife. It will then be ground and homogenized using a professional KN 295 Knifetec mill (Foss) or an HM 294 homogenizer (Foss), depending on the type/size of the sample (*see* **Notes 6** and 7).

3.1.2 Extraction of Antioxidant and/or Antimicrobial Compounds

The extraction of the target compounds is carried out through a solid–liquid extraction assisted by PEF technology (*see* **Note 8**) and using water as solvent (*see* **Note 9**).

1. 50 g of crushed sample is mixed with 100 mL of distilled water in a 250 mL beaker with the aid of a magnetic stirrer bar in a magnetic stirrer for 15 min at 50 rpm (*see* **Notes 10** and **11**).
2. After stirring, the homogenates are placed in the batch treatment chamber (between two electrodes separated by 5 cm, reaching 1.8 cm of height) connected to the PEF generator (*see* **Note 12**).
3. For each batch, 7000 V of potential difference and 10 Hz frequency are applied and a number of 100 pulses of 20 μs pulse width (*see* **Note 13**) (Table 1).

Table 1
Conditions of pulsed electric field (PEF) technology employed for the extraction of natural antioxidant and/or antimicrobial compounds from plant material

Potential	7000 V
Frequency	10 Hz
Pulses number	100
Pulse width	20 μs

4. After applying the PEF to the samples, the homogenates are collected in a 250 mL beaker and kept under constant stirring at 150 rpm for 120 min (*see* **Note 10**).
5. After solid–liquid extraction, the sample is centrifuged in 250 mL polypropylene tubes with caps at 4200 rpm for 20 min.
6. The resultant supernatant is filtered through qualitative filter paper (*see* **Note 14**) and stored protected from light and refrigerated until subsequent microencapsulation.

3.1.3 Encapsulation of the Obtained Antioxidant and/or Antimicrobial Extract

The extract previously obtained, rich in natural antioxidant and/or antimicrobial substances, is incorporated into a maltodextrin matrix (*see* **Note 2**) that acts as a coating wall, wall material, or membrane. In this way, the protection of the active compounds is achieved, obtaining the natural additive that can later be used in the meat industry. Even this proposed natural additive could be used in other sectors of the food industry after testing.

The steps followed for the microencapsulation of the antioxidant and/or antimicrobial extract are detailed below.

1. 90 mL of the aqueous extract is mixed with 10 g of maltodextrin (*see* **Note 2**).
2. The mixture is heated on a magnetic hot plate stirrer at 40 °C with constant stirring (50 rpm) for 1 h.
3. Next, the sample is homogenized in a high-speed disperser at 11,000 rpm for 10 min.
4. The homogenate is microencapsulated in a spray-dryer employing the following conditions: inlet temperature of 130 °C; maximum outlet temperature of 60 °C; atomization air flow rate of 601 L/h; liquid feed pump rate of 4 mL/min; main drying air flow rate of 38 m^3/h; feed solution temperature of 70 °C; and feed solution of 70 mL (Table 2) (*see* **Notes 15** and **16**).
5. The microencapsulated antioxidant and/or antimicrobial extract are collected from the dry particles' collector of the spray-dry equipment giving rise to the natural additive in powder form.

Table 2
Encapsulation conditions for natural antioxidant and/or antimicrobial compounds

Encapsulation agent	Maltodextrin
Inlet temperature	130 °C
Maximum outlet temperature	60 °C
Feed solution temperature	70 °C
Atomization air flow rate	601 L/h
Liquid feed pump rate	4 mL/min
Main drying air flow rate	38 m^3/h

6. The new natural antioxidant and/or antimicrobial additive should be stored under vacuum and protected from light, preferably at refrigeration temperature (4 ± 2 °C).

3.2 Preparation of the Hamburger with Natural Antioxidant and/or Antimicrobial Additive

There is no single formulation for the preparation of hamburgers with natural additives, since this is characterized by being made from a mass of chopped or minced fresh meat, fat, and salt to which other ingredients have been added (including antioxidant and/or antimicrobial additives), so that there are many possibilities for its elaboration. In this way, the protocol shown below is indicative and can be adapted to the needs of each process.

The flowchart for the production of hamburgers with natural antioxidant and/or antimicrobial additives is shown in Fig. 2. Each ingredient is weighted in a tray or container in its correct proportion (Table 3) (*see* **Note 17**).

1. Initially, lean beef meat (79% of the final mass) must be minced in a refrigerated mincer machine with a 6 mm plate and gathered on a tray.
2. Next, pork backfat (8% of the final mass) must also be minced in a refrigerated mincer machine with an 8 mm plate and gathered on a tray (*see* **Note 18**).
3. Now, the minced lean beef and pork backfat are introduced into a vacuum maceration tumbler.
4. To the previous mixture, 0.5% of the natural microencapsulated additive is added (*see* **Notes 17** and **19**), homogenizing everything together for 1 min at 3 ± 2 °C.
5. Next, 10% of water, 1.5% of salt, 0.5% of black pepper, and 0.5% of oregano (of the initial mixture of beef lean meat and pork backfat) are added to the previous dough (*see* **Note 17**), and mixing is continued for 3 more min at the same temperature (3 ± 2 °C).
6. Following, the mass is left to rest for 4 h at 3 ± 2 °C.

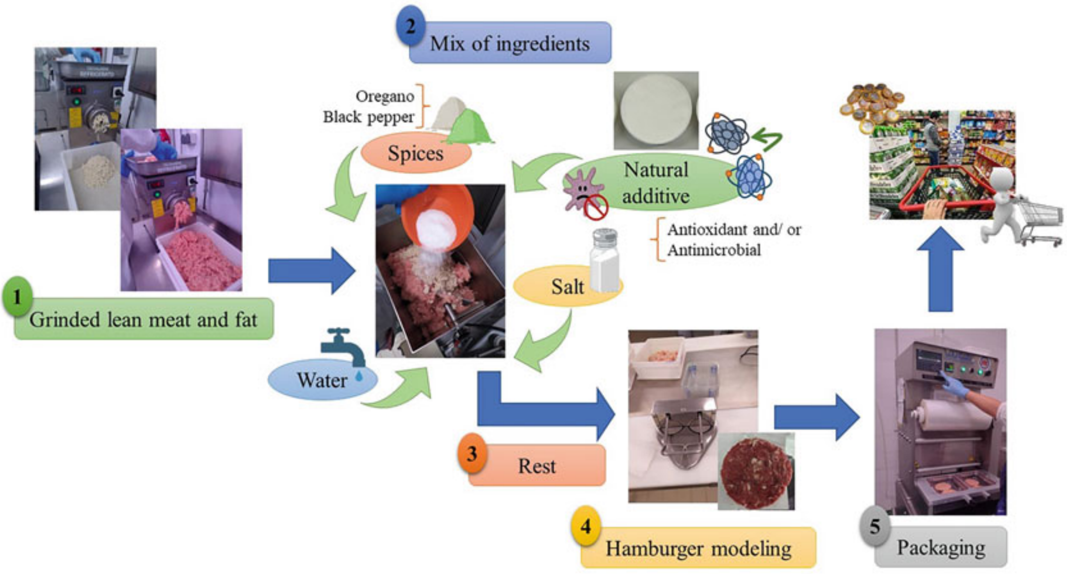

Fig. 2 General scheme for the preparation of hamburgers with the addition of a natural antioxidant and/or antimicrobial additive

Table 3
Proportions of the ingredients in the elaboration of hamburgers with natural antioxidant and/or antimicrobial additive

Ingredient	Proportion (%)
Meat	79
Fat	8
Water	10
Natural antioxidant and/or antimicrobial additive	0.5
Salt	1.5
Black pepper	0.5
Oregano	0.5

7. After resting, the meat dough is divided into 120 g portions, and hamburgers are made using a burger-maker machine.
8. The hamburgers are placed in 300 mm thick polystyrene trays with a permeability of 2 mL/(m^2·bar·day).
9. Finally, the trays are sealed with a 74-mm-thick polyethylene film with a permeability of 2 mL/(m^2·bar·day) using a protective atmosphere of 20% O_2 and 80% CO_2 (*see* **Notes 20** and **21**).
10. The packaged hamburger must be stored at refrigeration temperature (4 ± 2 °C) during its shelf life to guarantee its microbiological quality and, therefore, its safety.

4 Notes

1. The raw materials that can give rise to extracts with antioxidant and/or antimicrobial activity suitable for the food industry are multitudinous. However, among them, materials of plant origin stand out due to their high content of phytochemicals (polyphenolic acids, anthocyanins, carotenoids, etc.) [29–32]. In this way, the possible ingredients that can be used in this step are as many as the possible existing vegetable matrices that are available for food use.
2. Different coating agents (e.g., gum arabic, inulin, modified starch, whey protein isolate, etc.) for microencapsulation can be used in addition to maltodextrin. However, it should be noted that these agents must be soluble in water, have a low viscosity, and have a good fluidity [33].
3. The ingredients of animal origin can come from different species (beef, pork, chicken, turkey, etc.) according to the needs and tastes required.
4. This spice can be eliminated or replaced by another spice or mixture of species.
5. The entire process of manufacturing the hamburger must be carried out at refrigeration temperature. This involves that the work rooms used must be prepared to produce cold. Otherwise, the shelf life and safety of the product may be compromised.
6. The tools used for this case may vary depending on the volume to be processed.
7. This step is very important since it manages to increase the surface for the subsequent mass transfer by reducing the particle size and, consequently, improves the following extraction of the target compounds. In addition, sometimes, the sample can be previously dried at low temperatures to concentrate the objective compounds.
8. PEF technology involves the formation of pores in the cell membrane of raw materials (a phenomenon known as electroporation). In this way, the mass transfer of the target compounds to the extraction solvent is favored [25]. However, other technologies that favor the extraction of target compounds from sample cells (e.g., US, MW, HHP, etc.) could also be used in this step instead of PEF technology.
9. Solvents other than water can be employed (e.g., ethanol at different concentrations) depending on the extraction needs. Nevertheless, it must be considered that these have to be authorized for the food industry. Also, it must be taken into

account that if this solvent is not aqueous, it must be evaporated before the spray-drying process so that this process can be carried out properly.

10. The sample must be protected from light. For this, opaque containers or aluminum foil that covers the surface of the entire container can be used.
11. The solid-to-solvent ratio must be optimized for each case.
12. Before starting the PEF treatment, the conductivity of the homogenates must be determined in order to check the applicable voltage.
13. The applied conditions (voltage, frequency, number of pulses and pulse width) must be optimized for each extract in question using, for example, response surface methodology [34].
14. Different filters can be used at this stage according to the sample needs.
15. It must be taken into account that the feeding solution (solution containing the antioxidant and/or antimicrobial extract and the coating agent) must remain under constant agitation while it is pumped to the spray-dry equipment to prevent maltodextrin from precipitating.
16. These conditions can be adjusted to the characteristics/needs of each sample/process in question. In addition, the susceptibility to high temperatures of the compounds to be microencapsulated must be taken into account, and it must also be verified that the spray-drying technique does not excessively damage the antioxidant and/or antimicrobial activity of the future natural additive.
17. The proportions used in the development of the hamburger can vary according to the needs. Furthermore, depending on the potential of the natural antioxidant and/or antimicrobial additive, its concentration may be increased or decreased. For this, pertinent studies must be carried out prior to its utilization in the meat product.
18. The fat can be added frozen to avoid smearing.
19. The microencapsulated natural additive can be previously diluted in water to achieve a better homogenization.
20. Instead of using O_2-rich protective atmosphere, vacuum skin packaging could be interesting at this point (to maintain the activity of the natural antioxidant and/or antimicrobial compound). However, it should be considered that vacuum packaging provides colors that consumers may reject due to the possible formation of metmyoglobin [35].
21. The characteristics of the packaging can play a crucial role when it comes to selling the product, so these must be carefully studied if successful marketing is to be achieved.

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Chapter 10

In Vitro and In-Model Evaluation of the Antimicrobial Activity of Lactic Acid Bacteria Protective Cultures to Replace Nitrite in Dry Fermented Sausages

Eva Hierro, Xavier F. Hospital, Sergio B. Mano, and Manuela Fernández

Abstract

When investigating substitutes for nitrate and nitrite in meat products, it is essential to assess whether these alternatives have the same antimicrobial effect as the curing salts to be fully or partially replaced. Challenge tests on real products require a considerable amount of time and expense, and it is necessary to have precise information on the activity and viability of the compounds/ingredients to be investigated to carry out those tests. Here we describe the protocol for conducting in vitro (using agar diffusion test) and meat model studies with bacteriocin-producing lactic acid bacteria cultures to replace nitrite in fermented sausages, before proceeding to in-product challenge tests. We describe the protocol to investigate antilisterial activity, but it can be applied to other non-spore-forming meat pathogens. With some modifications, this protocol could also be adapted to test antimicrobial ingredients other than bacteriocin-producing cultures.

Key words Nitrite alternatives, Fermented sausages, Lactic acid bacteria, Antimicrobials, *Listeria*, Agar diffusion test, Meat model, Biopreservation

1 Introduction

Nitrate and nitrite are commonly used additives in cured meat products, where they exert important activities in the development of the typical color, the modulation of oxidative reactions, and the control of pathogenic and spoilage microorganisms [1]. Nitrate must undergo reduction to nitrite to be effective, and therefore it is mainly used as a precursor of nitrite, which is more rapidly and directly involved in curing reactions. In terms of antimicrobial activity, nitrite inhibits the outgrowth and toxin production by *Clostridium botulinum*, but it also inhibits other pathogens of relevance in the meat industry, such as *Listeria monocytogenes* or *Salmonella* spp., apart from spoilage bacteria such as *Enterobacteriaceae* [2].

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However, their role in nitrosation reactions, in which N-nitroso compounds such as nitrosamines are generated, has made the use of nitrate and nitrite and the consumption of cured meats controversial because of their association with certain types of cancer [3–5]. As a consequence, regulations on nitrate and nitrite are becoming increasingly restrictive, despite the fact that positive re-evaluations have been conducted by food safety authorities [6, 7].

In this context, different alternatives are being investigated with the aim to fully or partially replace nitrate and nitrite in cured meats. Apart from vegetable extracts (celery, spinach) which are rich in nitrate (and nitrite, if nitrate is pre-converted) [8, 9], other alternatives include spices and other vegetable extracts rich in phenolic compounds (cherries, blueberries, grapes, citrus peel) or carotenoids (tomato peel) [8, 10], the direct addition of organic acids [11], and the use of biopreservation strategies based on the direct addition of bacteriocins or protective cultures of bacteriocin-producing microorganisms [12–15].

Due to its multifunctional role, it is not easy to replace nitrate and nitrite with a single compound, and the control of pathogens is critical from a food safety point of view. Therefore, when investigating any alternative to nitrite, the antimicrobial activity against meat pathogens must be thoroughly studied to achieve the same effect of nitrite. This is of special relevance in cured raw meats, such as fermented products. *L. monocytogenes* is one of the major concerns in meat products due to their ubiquity and persistence in processing plants [16] and the high mortality rate caused by listeriosis [17].

To investigate the antimicrobial activity of nitrite alternatives, it is not sufficient to examine the microbial population of the reformulated products, but challenge tests are needed to ensure that food safety criteria are accomplished [18–20]. However, before conducting time-consuming and cost-intensive in-product challenge test studies, it is recommended to perform preliminary analyses, both in vitro and in meat models to assess the viability of the compounds/ingredients under study.

Regarding biopreservation, since the direct addition of bacteriocins requires regulatory approval, investigations are being conducted towards the use of microorganisms that can produce them in situ, being lactic acid bacteria (LAB) the most studied. This latter strategy is of particular interest in fermented products, in which it is highly compatible since these bacteria are responsible for fermentation.

Many factors may affect in-product bacteriocin production, including pH, temperature, oxygen tension, other ingredients (NaCl, spices), cell biomass, growth phase, and the competition with other microorganisms [15, 21]. These factors can interact to enhance or reduce production, such as nitrite and oxygen tension

[22]. Furthermore, the optimum conditions for bacteriocin production may not coincide with those for growth [22, 23]. On the other hand, bacteriocin production is usually low in monocultures, and the presence of other bacteria may act as a stress signal that triggers their synthesis [24]. Therefore, bacteriocin-producing LAB may show a good antimicrobial in vitro activity that may not be reflected during meat processing.

This chapter describes the protocol for conducting in vitro and in-model studies with bacteriocin-producing LAB cultures to replace nitrite in fermented sausages, prior to scaling up to in-product challenge tests. In this protocol we describe the steps for working with bacteria with proven bacteriocin-producing ability against *L. monocytogenes*, but it can be adapted to other non-spore-forming microorganisms. First, in vitro activity of bacteriocinogenic LAB strains is assayed by agar diffusion test (ADT) to screen for the ability to control *L. monocytogenes* under selected conditions. Afterwards, the LAB strains showing the highest inhibition are challenged against *L. monocytogenes* in a fermented sausage model. With some modifications, this protocol could also be adapted for testing antimicrobial ingredients other than bacteriocin-producing cultures. To implement these procedures, it is essential to know in depth the product in which nitrite is going to be replaced: composition, physicochemical properties, typical microbiota, potential pathogens, and processing conditions.

2 Materials

The laboratory must have biosafety measures adequate for working with Risk Group 2 microorganisms [25], including a laminar flow cabinet and all the facilities and equipment for working under sterile conditions. Personnel must be trained to work under these conditions.

General glassware and disposable material for microbiology, as well as general laboratory equipment (autoclave, balances, heaters, magnetic stirrers, Bunsen burners, water bath, incubators, centrifuge, pH meter, etc.), are required.

Equipment is also needed for mincing and kneading meat and other ingredients of the product model.

2.1 Microorganisms

1. Bacteriocin-producing bacteria. Different microorganisms produce bacteriocins, but LAB are usually the choice for fermented sausages (*see* **Note 1**).
2. Meat starter cultures. The experiment should include the typical starters used in the product that is going to be reformulated, e.g., gram-positive catalase-positive cocci (GCC+) such as *Staphylococcus* spp. in fermented sausages.

3. Target pathogens. Bacteriocins are mainly active against gram-positive bacteria [26]. In this protocol we include assays with gram-positive non-spore-forming pathogens, as it is *L. monocytogenes.* Use as a target a reference strain or isolate appropriate for the product category to be reformulated.

2.2 Culture Media

Different general culture media can be used, such as Tryptic Soy Broth (TSB), Brain Heart Infusion (BHI), and Tryptic Soy Agar (TSA) for the growth of pure cultures. Specific media are also needed, such as De Man, Rogosa, and Sharpe (MRS) broth and agar for LAB, Mannitol Salt Agar (MSA) for GCC+, and Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol (PALCAM) agar for *L. monocytogenes* (*see* **Note 2**). PALCAM medium requires a PALCAM *Listeria* selective supplement, which is added after sterilizing the agar (*see* **Note 3**). This supplement must be rehydrated with sterile distilled water before use, according to the product specifications.

For media preparation, follow the instructions of the supplier.

2.3 Strain Revival

1. Prepare fresh bacterial cultures from stocks stored at -80 °C (*see* **Note 4**).
2. Use a sterile inoculating loop to gently scrape the surface of the stock.
3. Streak the loop onto a general growth medium (e.g., TSA), and incubate 18–24 h at the proper temperature (32 °C for LAB and GCC+, 37 °C for *Listeria* spp.).
4. Observe that colonies have grown on the agar surface and that there is no contamination (as evidenced by the uniformity of the colonies). Pick one colony with a sterile loop, and transfer to a tube with 10 mL of an appropriate broth (e.g., MRS broth, TSB, or BHI). Incubate at the same temperature and time as in **item 3**.
5. Take 50 μL of the grown culture, and transfer to 10 mL of fresh broth. Incubate at the same temperature and time as in **item 3**.

2.4 Serial Dilutions

Serial decimal dilutions will be required for microbial enumeration. They can be prepared in general use test tubes (e.g., 16 × 160 mm) or in micro-centrifuge tubes (Eppendorf or similar). Peptone water (15 g/L) or saline solution (0.85%) can be used for this purpose.

2.5 Petri Dishes

1. 55 mm diameter dishes for the meat model assays.
2. 110 mm diameter dishes for microbial enumeration.
3. 140 mm diameter dishes for in vitro inhibition assays.

2.6 Nitrate and Nitrite

Curing salts are used in the fermented sausage meat model experiments, in which samples with different nitrite concentrations are prepared for comparison with the activity of the protective cultures. Sodium nitrate, potassium nitrate, sodium nitrite, and potassium nitrite may be used (at mg/kg levels). The incubation period in these experiments is usually short (around 7–10 days), so nitrite is more suitable to be included in the model instead of nitrate, because of its faster antimicrobial effect.

3 Methods

3.1 Preparation of LAB Cultures

The inhibitory activity of the bioprotective LAB should be tested on their own (monoculture) and together with typical meat starters, e.g., GCC+ such as *Staphylococcus* spp. (coculture), and under the conditions typical of the product in which nitrite is going to be replaced, in this case temperature and pH.

3.1.1 Preparation of Monocultures

1. Take 100 μL of fresh LAB culture, and transfer to a sterile centrifuge tube with at least 50 mL of fresh MRS broth added with 2% NaCl (*see* **Notes 5** and **6**).
2. Incubate at temperature and time mimicking the fermentation stage of the product, e.g., 18–22 °C, 2–3 days for Mediterranean-style sausages [27].
3. Centrifuge at 3000 × *g* for 20 min at 4 °C (*see* **Note** 7).
4. Recover the supernatant. Move to Subheading 3.1.2, **step** 7.

3.1.2 Preparation of Cocultures

1. Prepare separate cultures of LAB and meat starters as follows. Take 50 μL of each grown culture (as obtained in Subheading 2.3, **item 5**), and transfer to 10 mL of fresh corresponding broth in sterile centrifuge tubes. Incubate at the same temperature and time as in Subheading 2.3, **item 3**.
2. Centrifuge at 3000 × *g* for 20 min at 4 °C, and discard the supernatant.
3. Add 10 mL of fresh MRS broth with 2% NaCl to each tube, and stir vigorously until the pellet is completely resuspended (*see* **Note 8**). Repeat **steps 2** and **3** one more time.
4. Measure the absorbance of the suspension at 600 nm, and prepare dilutions to obtain the desired microbial concentration in the inoculum (*see* **Note 9**).
5. To prepare the coculture, mix each LAB with each GCC+ in a sterile centrifuge tube with at least 50 mL of MRS broth added with 2% NaCl (*see* **Note 6**). The concentration of microorganisms should replicate that of a real product, e.g., 10^7 cfu/g for LAB and 10^6 cfu/g for GCC+. Incubate the mixtures at

temperature and time mimicking the fermentation stage of the product.

6. Centrifuge at 3000 × *g* for 20 min at 4 °C.
7. Recover the supernatants, and aliquot into 10 mL fractions.
8. Measure the pH of the supernatants, and adjust to the values typical for the product manufacturing process, e.g., those of the initial batter and at the end of fermentation (*see* **Note 10**). Keep a volume of each supernatant at its original pH.
9. Prepare 10 mL fractions of non-inoculated MRS broth, and adjust pH at the selected values (as in the previous step) to discard inhibition of the target microorganism by pH.
10. Filter the supernatants and the non-inoculated MRS broth samples through 0.22 μm pore size filters using a sterile syringe (*see* **Note 11**).

3.2 Agar-Well Diffusion Test

The agar-well diffusion test (ADT) is a simple and fast method extensively used to assess the antimicrobial activity of different compounds [28]. This assay is based on the measurement of the size of a growth inhibition zone around the sample, which in our protocol we have placed into a well cut into the agar (Fig. 1).

Follow the steps below:

1. Revive the pathogen as described in Subheading 2.3 to start preparing the inoculum.
2. Centrifuge at 3000 × *g* for 20 min at 4 °C, and discard the supernatants.
3. Add 10 mL of fresh TSB to a test tube, and stir vigorously until the pellet is completely resuspended. Repeat **steps 2** and **3** one more time.
4. Measure the absorbance of the suspension at 600 nm, and prepare dilutions to obtain the desired microbial concentration in the inoculum.
5. Prepare a medium consisting of TSB with 0.8% of bacteriological agar (*see* **Note 12**). After sterilization, keep at 50 °C in a water bath.
6. Inoculate the medium with the target pathogen at a concentration of 10^5 cfu/mL. Mix gently.
7. Pour 40 mL of the medium into 140 mm diameter Petri dishes. Leave at room temperature to solidify.
8. Carve 6 mm diameter wells on the solid medium plates using a sterile cork borer.
9. Add 50 μL of the supernatants or MRS broth obtained in Subheading 3.1.2, **step 10** to the wells. Leave to diffuse for 2 h at 4 °C.

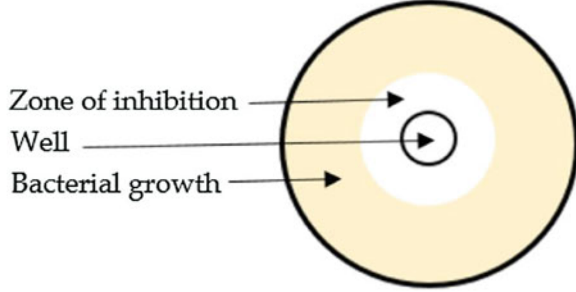

Fig. 1 Schematic representation of agar diffusion test (ADT). (Source [8])

Fig. 2 ADT assay against *L. monocytogenes*. Arrows indicate inhibition halos

10. In each plate, leave a well without any sample to monitor the growth of the pathogen (negative control) and another well with a sample containing an active bacteriocin against the target pathogen (positive control).
11. Incubate at 37 °C for 24 h.
12. Check for the presence of inhibition halos around the wells to which the supernatants were added (Fig. 2).
13. Measure the diameter of the halos (mm) with a caliper. Compare to halos around MRS broth adjusted to the same pH as the supernatants; if no inhibition is observed around the wells with MRS adjusted to the same pH, it means that inhibition in the wells with the supernatants can be attributed to bacteriocin production.

3.3 Study of Antimicrobial Activity in a Meat Model

Bioprotective cultures showing inhibition halos in the ADT should be challenged in a meat model prior to in-product challenge testing. The model must be prepared according to the composition of the real product to be reformulated. In the case of Mediterranean-style sausages, a typical formulation is 70% lean pork and 30% pork backfat. Calculate the amount of meat and fat according to the number of conditions to be assayed.

1. Grind meat and backfat together in a mincer. The particle size would be according to the specifications of each product. Meat and fat should be previously frozen, and before mincing they should be tempered (partial thawing) and ground at a temperature below 0 °C (*see* **Note 13**).
2. Transfer ground meat and backfat to a kneading machine, and add NaCl, sugars, and spices. A typical formula for Mediterranean fermented sausages includes 2–3% NaCl, 3–4% sugars (lactose, dextrose), and 0.25–0.5% spices, although other formulas can be prepared depending on the product to be modelled [27]. Knead the batter for 2 min (*see* **Note 14**).
3. Prepare the starters (GCC+) by reviving the strains as described in Subheading 2.3, and then proceed as in Subheading 3.2, **step 2**–**4**. Add the starters to the batter at a concentration of 10^6 cfu/g (*see* **Note 15**). Knead for 2 min. The following steps should be conducted under strict biosafety conditions.
4. Prepare the pathogen as described in Subheading 3.2, **step 1**–**4**. Inoculate the batter at a concentration of approximately 10^3 cfu/g [29] (*see* **Note 16**). Knead for 2 min.
5. Divide the batter into as many portions as bioprotective cultures are going to be investigated plus a control without any protective culture.
6. Revive each bioprotective culture as described in Subheading 2.3, and prepare the inoculum as described in Subheading 3.2, **step 2**–**4**. Add the cultures at a concentration of 10^7 cfu/g. Knead for 2 min.
7. Divide each portion into as many nitrite concentrations to be compared, e.g., 0 mg/kg and the desired reductions. It is recommended to prepare a control batch with the maximum amount of nitrite allowed by regulations and without any protective culture.
8. Dissolve nitrite in water, and add to the batter (*see* **Note 15**). Knead for 2 min (*see* **Note 17**).
9. Fill the 55 diameter mm Petri dishes with the different batters (approximately 40 g) up to the top. Cover the dishes with the lid, and press to release air in order to mimic low-oxygen tension conditions typical after stuffing (Fig. 3). Prepare three dishes per batter and sampling day.

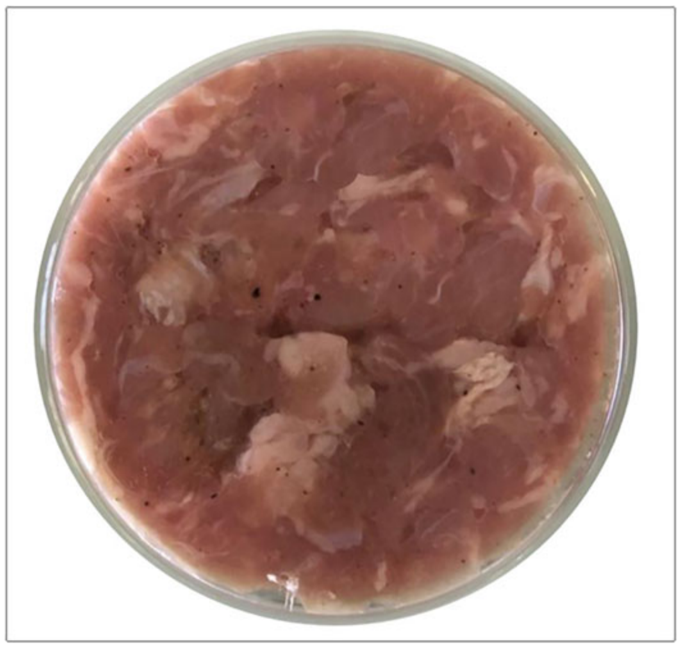

Fig. 3 Fermented sausage model "stuffed" in a 55 mm Petri dish

10. Incubate the dishes at 18–22 °C for 2–3 days (fermentation stage), and then lower temperature according to the typical conditions of the real product that is going to be reformulated, e.g., 12 °C for Mediterranean-style sausages [27].
11. Take samples (whole dishes) at day 0, after fermentation, and at the end of incubation for microbiological analysis (LAB, GCC +, and *L. monocytogenes*) as well as for pH measurement.
12. For microbiological analysis, place 10 g of the batter in a Stomacher bag (preferably with filter), and add 90 mL of peptone water. Homogenize in a Stomacher blender for 2 min. Prepare serial dilutions, if needed, by transferring 1 mL to a test tube with 9 mL of peptone water or saline solution (*see* **Note 18**).
13. Pour plate 1 mL of the corresponding dilutions in 110 mm dishes, and then add 20 mL of MRS agar, MSA, or PALCAM to the corresponding dishes for microbial enumeration. Gently stir the dishes. The dilution(s) to be plated will depend on the concentration expected. For instance, in the case of *L. monocytogenes*, as the inoculum was about 10^3 cfu/g, lower dilutions would be needed, while for LAB and GCC+, higher dilutions would be required. Plate three dishes for each dilution.
14. Incubate MRS and MSA dishes at 32 °C for 48 h, and PAL-CAM dishes at 37 °C for 48 h, and count the colonies (*see* **Note

19). Recognition of *Listeria* colonies is evident by the black discoloration of the medium due to esculin hydrolysis.

15. For pH analysis, insert the electrode in three different points of the batter, and obtain the mean pH (*see* **Note 20**).

4 Notes

1. Microorganisms producing antimicrobial compounds other than bacteriocins can also be assayed with this procedure.
2. Other culture media with similar characteristics can be used for each microorganism/group of microorganisms.
3. Remember that the supplement is added after sterilization and once the agar is cooled to about 50 °C.
4. Freeze dried cultures can also be used instead of frozen stocks.
5. The volume and/or the number of tubes will depend on the number of conditions to be assayed.
6. NaCl is added to MRS broth to simulate real product conditions.
7. Centrifugation speed and time can be adjusted depending on the centrifuge used. A higher speed will require a shorter centrifugation time.
8. Use MRS for both LAB and GCC+, since it will be the growth medium for the coculture.
9. For each microorganism prepare different cell concentrations, and measure the absorbance at 600 nm, to obtain the corresponding curves for correlating both parameters. As a rule of thumb, the microbial population achieved at the end of **step 3** would be about 10^8–10^9 cfu/mL.
10. Use 1M NaOH or 85% lactic acid to adjust pH.
11. Samples do not need to be processed immediately. If necessary, they can be stored at −20 °C until further analysis.
12. For better visualization of the inhibition halos, it is important not to increase the amount of agar in the medium. Keep at 50 °C until pouring onto the dishes to avoid premature solidification of the agar.
13. In order to control the microbiological quality of the raw materials, it is recommended to purchase whole pieces of meat and fat and grind them under maximum hygienic conditions.
14. Kneading can be done manually, but it is recommended to use pilot-scale meat processing equipment.

15. Use water (about 1% over the total weight of the batter) to suspend microorganisms and to dissolve nitrite and other additives. Divide the amount of water to adjust the volume to each ingredient. Distilled water is not necessary, but good-quality water is required.
16. If the inoculum concentration is too low, inhibition might be overestimated. If the concentration is too high, it may exceed the activity of the inoculum and lead to underestimating its effect. To establish the appropriate concentration, data on the prevalence of the pathogen in processing lines, contamination, and outbreaks can be found in the literature.
17. Adjust the volume of water to dissolve nitrite to the amount of batter to ensure a homogeneous distribution.
18. Dilutions can also be prepared by adding 100 μL to an Eppendorf tube with 900 μL peptone water or saline solution. In this case, it is preferable to plate the dishes on the agar surface with a Drigalski rod. Automatic spiral plating (or similar procedures) can also be used.
19. Remember that for an accurate enumeration, dishes should not contain less than 30 or more than 300 colonies. In the case of PALCAM dishes, the upper limit should be around 100 colonies, to better visualize the discoloration around the colonies.
20. pH must be measured after sampling for microbiological analysis, to avoid contamination.

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Chapter 11

Reduced-Sodium Meat Products

Maria Lúcia Guerra Monteiro, Eliane Teixeira Mársico, and Carlos Adam Conte-Junior

Abstract

Mohr's argentometric method is based on precipitation titration in a neutral or alkaline medium containing potassium chromate as the indicator. Briefly, chlorides bind to silver ions from titrant silver nitrate solution, generating white silver chloride precipitates. When all existing chloride in the sample reacts with silver, it binds to chromate, yielding brick-red coloration. Although recent techniques have been emerging for sodium chloride determination in meat products, Mohr's method is still extensively used due mainly to its ease and simple execution and cost-effectiveness, requiring little low-cost equipment. Nevertheless, there is no detailed guideline for Mohr's method to date. Thus, this chapter aims to share a practical protocol built from classical official Mohr's methods and performed routinely in the laboratory with tips based on day-by-day experience, allowing technique standardization and avoiding successive errors.

Key words Salt reduction, Sodium reduction, Mohr method, Argentometric method, Precipitation titration

1 Introduction

Salt or sodium chloride (NaCl) contains 40% sodium, which is considered a micronutrient of global public health concern because its excessive intake (above 5 g NaCl or 2 g sodium per day) has been related to causing cardiovascular, kidney, and neurological diseases, hypertension, osteoporosis, asthma, obesity, and gastric cancer [1–3]. Otherwise, processed meat products contain high salt levels (from 1.20% to 5.98%), being one of the main food responsible for dietary salt intake [3]. According to the World Health Organization [4], salt intake reduction is one of the most cost-effective strategies for better global population health since most people consume salt excessively, leading to approximately 2.5 million deaths yearly. For these reasons, developing reduced-sodium meat products is on the rise.

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In this context, analytical procedures to determine salt content are crucial to monitor and inspect it in processed meat products. In general, there are two reference methods to quantify salt or sodium in meat products: flame or inductively coupled plasma atomic-absorption spectrophotometry and titration techniques based on chloride binding to silver ions, such as the Mohr method. Automated portable devices with ion-selective electrodes for sodium or chloride have been developed mainly to save time and allow immediate action in loco (e.g., restaurant meals). However, this methodology has been proposed as a screening analysis, and thus meat products close to the salt threshold value would have to be forwarded to the laboratory to be evaluated by reference techniques [5].

Although much older than spectrophotometry techniques, Mohr's method has been used until today in several studies evaluating salt content in meat products, and it has been well accepted by reputable journals in the food field [6–15]. Mohr's method is based on the reaction of chloride with silver ions from titrant solution in the presence of potassium chromate indicator in a neutral or alkaline medium, resulting in a brick-red coloration from silver chromate formation. Therefore, it is an argentometric method with precipitation titration [16]. Besides being simple and easy, Mohr's method is a cost-effective technique for determining sodium chloride, which requires little low-cost equipment that can be used for other purposes, such as muffle furnace (e.g., ash determination, digestion step for further determination of trace elements, and even preliminary step for analyzing sodium content by spectrophotometry techniques), analytical balance (e.g., sample weighing), pH meter (e.g., pH determination and preparation of solutions), and magnetic stirrer (e.g., preparation of solutions with or without heating). Otherwise, Mohr's method needs a blank sample, it does not apply to iodine, and the reaction only takes place in pH between 7 and 10 [17].

There are several analytical protocols for Mohr's method, but none is described in detail with tips based on day-by-day experience, which is crucial to technique standardization and avoiding successive errors. Considering these facts, this chapter aimed to share an inside detailed practical protocol built from classical official Mohr's methods [16, 18].

1.1 Overview of Recent Studies Concerning Technological Strategies to Reduce Sodium in Meat Products

Research towards the development of reduced-sodium meat products started with the use of salt replacers, such as magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), and, mainly, potassium chloride (KCl). Within NaCl replacers, KCl is the most evaluated due to their high similarity in molecular terms, besides being considered a safe additive [19–21]. However, studies concluded that, in general, salt replacers, including KCl, changed the taste even when in association with flavor enhancers, making consumers

perceive it as bitter and metallic [22]. It is worth highlighting that flavor enhancers (e.g., monosodium glutamate, yeast extracts, vegetable protein hydrolysates, lysine, arginine, and taurine) contain up to 40% salt and thus must be added in a limited way [23]. Since developing reduced-sodium meat products is challenging for the scientific community and industries regarding quality maintenance, especially flavor, texture, safety, and preservation, studies have focused on emerging nonthermal technologies. They have shown successful outcomes in sensory and technological attributes compared to conventional NaCl replacers, including reduction of cooking loss and improvement of flavor, texture, and preservation, and thus have been suggested as promising strategies to produce desirable reduced-sodium meat products [24, 25].

Five years from now, several studies have evaluated the effect of isolated and combined emerging nonthermal technologies on the quality of reduced-sodium meat products, such as high-intensity ultrasound, high hydrostatic pressure, irradiation (gamma rays and UV-C), microwave, and pulsed electric field (PEF). Within technologies, high hydrostatic pressure (HHP) and high-intensity ultrasound (HIU) are the most studied in this context.

Orel et al. [26] investigated the effect of HHP on reduced-sodium ready-to-eat chicken breasts after tumbling (300 MPa/ 5 min) and in the final product (600 MPa/3 min). These authors concluded that unpressurized RTE chicken products could be reduced by 25% NaCl, while HHP at both conditions allowed a reduction of 50% NaCl with no changes or improvements in physicochemical, microbiological, and sensory qualities. Also, they reported that HHP at 600 MPa for 3 min extended the shelf life of chicken products by more than 60 days at 4 °C. Zhou et al. [27] replaced 25% NaCl with KCl, potassium lactate (K-lactate), or potassium citrate (K-citrate) in chicken sausage and observed no changes in weight loss, texture, color, and sensory parameters. Otherwise, they reported that applying HHP at 200 MPa for 10 min before cooking increased juiciness and firmness and decreased weight loss, but it also decreased flavor and saltiness perception when combined with K-lactate or K-citrate and did not affect or improve these attributes when combined with KCl. Barretto et al. [28] applied HIU at 600 W/cm^2 for 10 min in cooked ham reduced by 50% salt and observed that this technology increased the yield and improved color, taste, texture, and global acceptance with no changes in lipid oxidation. These same authors further evaluated the isolated and combined effect of the replacement of NaCl by KCl at 50% and HIU at 600 W/cm^2 for 10 min also in cooked ham and reported that KCl or HIU improved technological and sensory characteristics, but the combination of the treatments did not show a positive effect on these parameters in cooked ham [19]. Zhou et al. [29] investigated the addition of salt mixtures (0.125% KCl + 0.125% $CaCl_2$, 0.25% KCl + 0.25% $CaCl_2$,

and 0.50% KCl + 0.50% $CaCl_2$) in reduced-salt bacon by 37–53% treated with 600 W for 30 min. They concluded that 0.25% KCl + 0.25% $CaCl_2$ enhanced its flavor and overall sensory quality by releasing several volatile phenolic and aldehyde compounds. Leães et al. [30] evaluated different salt reduction levels (0–50%), times of HIU at 175 W (0–20 min), and the replacement of water by basic electrolyzed water (BEW) in meat emulsions. They reported that HIU for 20 min improved some quality properties (e.g., cooking yield, texture, and emulsion stability) in meat emulsions reduced by 10% and 20% salt, and BEW slightly improved fat and water retention. However, they obtained better emulsion stability and cooking yield in reduced-salt meat emulsion at 30%, combining BEW and HIU for 20 min. Souza et al. [31] observed that gamma radiation at 3 kGy was more effective than 5 kGy for inactivating pathogens while maintaining the nutritional and color attributes of uncooked lamb sausage reduced by 50% NaCl from KCl replacing. Rodrigues et al. [32] evaluated the effect of three gamma radiation doses (1.5, 3.0, and 4.5 kGy) on the quality of hot dog wieners reduced by 37.50% NaCl and concluded that 1.5 kGy was the best treatment—it delayed the bacteriological growth; did not affect the lipid oxidation, appearance, and aroma; and improved the texture, taste, and overall acceptability. Bhat et al. [33] observed that a previous pulsed electric field treatment (0.52 kV/cm, 10 kV, 20 Hz, 20 μs) in reduced-salt beef jerky by 40% increased sodium release and saltiness perception with tenderness improvement and no influence on cooking yield, oxidative degradation, microbial stability, and sensory characteristics. Rosa et al. [34] observed that replacing 50% of regular NaCl by micronized ones in combination with a low KCl level (0.5%) and HIU (480 W, 25 kHz, 20 °C) was a promising strategy for developing reduced-sodium Bologna-type sausages, considering technological, oxidative, and sensory parameters.

Szerman et al. [35] used the response surface methodology to evaluate the effect of different levels of NaCl (0–2%), sodium tripolyphosphate (STPP; 0–0.5%), pressure level (100–300 MPa), and holding time (1–5 min), aiming to find an ideal treatment to produce reduced-sodium beef patties. Although no color changes were induced by HHP, it increased cooking loss and texture parameters, especially when higher NaCl and STPP were added. Thus, they could not find an HHP treatment to reduce the use of additives without influencing the quality of beef patties.

Monteiro et al. [36] found that UV-C radiation at 0.310 J/cm^2 or HHP at 300 MPa for 5 min maintained cooking loss, color and texture parameters, and salty taste perception of ready-to-eat fish products reduced by 25% NaCl, while it is not possible at 50% NaCl reduction levels. Moreover, they did not recommend the combination of UV-C and HHP, which adversely affected the quality of fish products, mainly at 50% NaCl reduction. Santos et al. [37]

evaluated the effect of different levels of NaCl by KCl (12.17%–60%), ultrasound times (5–60 min), and intensities (7.53–30.14 W/cm^2) through composite central rotational design on technological, physicochemical, and sensory characteristics of fish products. These authors revealed that HIU at 12.12 W/cm^2 for 48.85 min with 47.83% substitution level of NaCl by KCl was the ideal condition to produce reduced-sodium "spam-like" products elaborated with tilapia filleting by-products. Wang et al. [38] reported that a water bath for 40 min combined with microwave heating for 10 min enhanced sodium release and saltiness perception of surimi gels with 15% salt reduction compared with two-stage water bath heating for 40 min followed by 90 min.

The mechanisms of these technologies for increasing saltiness perception are based mainly on their ability to increase water-holding capacity, denature proteins, or increase membrane permeability, allowing higher salt mobility and diffusion evenly through the food [36, 38, 39]. HHP can weaken protein and Na ion interaction, increasing the availability of free sodium to contact the tongue surface [23]. HIU induces mass transfer, facilitating salt distribution in food matrices [40]. PEF leads to pore formation, enhancing membrane permeability and salt diffusion [33]. According to Wang et al. [38] and Kuo and Lee [41], the sodium distribution ratio is dependent on the water distribution ratio since most sodium is in the food aqueous portion. Nevertheless, the effect on the saltiness perception depends mainly on technological treatment conditions and intrinsic food properties (e.g., composition, biochemistry); thus, the literature results are conflicting.

Considering the state of the art of this subject, the gap in the literature is the need for the optimal conditions of nonthermal treatments for each reduced-sodium meat product based on physicochemical, technological, microbiological, and sensory parameters, which could enable well-accepted healthy meat products and boost eco-friendly processing in food industries. Simultaneously, consumers should be repetitively exposed to low-sodium diets to decrease their saltiness perception threshold and thus increase the acceptability of reduced-sodium foods [42]. Ganesan et al. [43] carried out a sensory evaluation of cheddar and mozzarella cheeses reduced by 25–60% salt and concluded that a 30% NaCl reduction is perceived by consumers. They also reported that gradual eating habits with low sodium content are crucial to increase the acceptability of reduced-sodium foods. Furthermore, combined technological strategies should be investigated to revert the adverse flavor effects of NaCl reduction, and matters referring to the safety and price of reduced-sodium foods should be overcome [42].

In this context, standardized analytical methods to quantify NaCl content in the food matrices are crucial to boosting studies concerning the development of reduced-sodium meat products.

2 Materials

2.1 Equipment and Glassware

- Analytical balance.
- Crucible.
- Bunsen burner.
- Muffle furnace.
- Desiccator.
- pH meter.
- Glass rod.
- Funnel.
- Qualitative filter paper.
- 250 mL Erlenmeyer flasks.
- 1 mL volumetric pipettes.
- 5 mL volumetric pipettes.
- 10 mL volumetric pipettes.
- Magnetic stirrer with speed and temperature settings.
- 25 mL burette graduated in 0.05 mL.

2.2 Reagents

- Nitric acid (HNO_3) solution (v/v): 1 mL of nitric acid (65%, P. A.) in 9 mL of distilled water.
- Sodium hydroxide (NaOH) solution at 0.1 N (w/v): 0.4 g NaOH microbeads (≥ 99%, P.A.) in 100 mL of distilled water.
- Potassium chromate (K_2CrO_4) solution at 5% (w/v): 50 g of K_2CrO_4 (≥99%, P.A.) in 1000 mL of distilled water.
- Silver nitrate ($AgNO_3$) solution at 0.1 N (w/v): 16.987 g of $AgNO_3$ (≥99%, P.A.) in 1000 mL of distilled water.

It is worth highlighting that for preparing NaOH, K_2CrO_4, and $AgNO_3$ solutions, the amount in grams of the solute must be added to a beaker, followed by adding approximately 80% of the total amount of distilled water. Further, a stir bar must be added and the content homogenized in a magnetic stirrer without heating until complete dissolution. After that, the mixture must be transferred to a volumetric flask (100 or 1000 mL); it must be filled until the meniscus with distilled water, followed by inverting and mixing.

3 Methods

Mohr's method is based on two reactions in a neutral or alkaline medium containing potassium chromate as the indicator. The first one is the binding of chlorides with silver ions from titrant silver nitrate solution, leading to its precipitation as white silver chloride

Fig. 1 Carbonization steps for ash obtaining

Fig. 2 White ash after full incineration

($NaCl + AgNO_3 \rightarrow AgCl\downarrow + NaNO_3$). The second one starts when chlorides run out, and thus potassium chromate binds to silver ions forming silver chromate, which has brick-red coloration ($2\ AgNO_3 + K_2CrO_4 \rightarrow Ag_2CrO_4\downarrow + 2\ KNO_3$).

3.1 Ash Obtaining

- Cut the muscle portion (20 g) of different locations of the meat product, grind it in a ceramic mortar with a pestle, weigh it between 2 and 5 g in a crucible, and take note of the weight (*see* **Note 1**).
- Take the crucible to the Bunsen burner, and proceed with sample carbonization until black color to eliminate organic compounds (*see* **Note 2**; Fig. 1).
- Incinerate the sample in a muffle furnace at 550 °C until obtaining white ashes (*see* Fig. 2), and cool it in a desiccator (*see* **Note 3**; Fig. 3).

3.2 Filtrate Preparation

- Add two to three drops of nitric acid solution (*see* Subheading 2.2) to facilitate ash dissolution and 10 mL of hot distilled water.

Fig. 3 Crucible containing ash in the desiccator

- Stir the mixture with a glass rod, and transfer it to a 250 mL Erlenmeyer flask coupled to a funnel covered with qualitative filter paper, washing the crucible and filter paper well with hot distilled water (*see* **Note 4**).
- Adjust the filtrate pH between 7 and 10 with NaOH solution at 0.1 N (*see* Subheading 2.2), and add 1 mL of K_2CrO_4 solution at 5% (*see* Subheading 2.2) (*see* **Note 5**).

Observation Prepare a blank sample containing only 20 mL of distilled water, pH adjusted between 7 and 10, and 1 mL of K_2CrO_4 solution at 5%.

3.3 Titration

- Fill the burette with the $AgNO_3$ solution at 0.1 N (*see* Subheading 2.2), and adjust it until the 25 mL meniscus mark, verifying the presence of bubbles (*see* **Note 6**).
- Dispense dropwise the titrant solution in the 250 mL Erlenmeyer flask containing the filtrate until the appearance of brick-red coloration, and take note of the volume (*see* **Note 7**).

3.4 Calculation

$$\frac{\%\text{of chlorides in NaCl} = (V_s - V_b) \times f \times N \times 0.0585 \times 100}{P}$$

where V_s is the volume spent of $AgNO_3$ solution at 0.1 N on the titration of the sample in mL; V_b is the volume spent of $AgNO_3$ solution at 0.1 N on the titration of the blank sample in mL; f is the factor of $AgNO_3$ solution at 0.1 N; N is the normality of $AgNO_3$ solution at 0.1 N; 0.0585 is the milliequivalent gram of sodium chloride; and P is the sample weight in grams.

4 Notes

1. Take particular care with some specific meat products, such as ground beef, in which the test portion must have the fat distributed uniformly. A high fat amount tends to melt during carbonization, and the sample can overflow from the crucible, underestimating the NaCl content. Moreover, the sample must be weighted in analytical balance, and all decimals must be recorded to avoid under- or overestimation through calculation.
2. The sample must be carbonized until black coloration and the absence of fume, which is a trick in this step. Black ash with fume may still contain organic compounds and indicates incomplete carbonization. It may lead to flame formation within the crucible increasing the sample temperature to more than 550 °C, which can cause chloride volatilization. Moreover, the complete carbonization avoids small bursts from the sample due to its direct contact with high temperatures and thus avoids sample loss by extravasation, which would lead to chloride level underestimation in the sample.
3. The incineration time depends on food composition, but, in general, the time to achieve white ashes is about 3–4 h within the muffle furnace. If the time exceeds 4 h, two to three drops of distilled water can be added to disperse organic matter, thereby facilitating its burn. In these cases, the crucible is removed from the muffle furnace and transferred to the desiccator to cool it for 30 min, and two to three drops of distilled water are added to facilitate organic matter dispersion. Cooling in a desiccator is crucial to avoid water evaporation before dispersion. After that, carbonization in the Bunsen burner and incineration in the muffle furnace at 550 °C must be carried out, observing ash coloration eventually. If the ashes didn't get white after approximately two h, it is assumed that the meat product generates grayish ashes (*see* Fig. 4) due to its composition. It is also worth highlighting that after obtaining ash (white or gray) from the muffle furnace, the crucible must be cooled in the desiccator for at least 30 min to avoid the volatilization of the nitric acid solution, allowing a better ash dissolution in the next step (*see* Subheading 3.2).
4. The washing must be performed twice with a known amount of water. Overall, 5 mL of distilled water is measured in a volumetric pipette and used in each washing. The water temperature must be between the warm and boiling point (before starting the microdroplet dynamics).
5. It is crucial to calibrate the pH meter before measuring the filtrate pH. If pH is below 7, the H_3O^+ leads to chromic acid formation, which generates a bright-orange precipitate and

Fig. 4 Grayish ashes

impairs the binding between chromate and silver, making it necessary to add more silver titrant solution and thus overestimating the NaCl content. If pH is above 10, the silver hydroxide formation decreases the potential of the titrated solution and thus the availability of free Ag^{+}, generating a black precipitate and delaying its binding with chloride and chromate [44], which also may result in an overestimation of NaCl content. Moreover, the K_2CrO_4 solution must be measured in a 1 mL volumetric pipette.

6. Rinse the burette before titration, and discard the titrant solution properly. Do not use the beaker from rinsing to fill the burette for analysis. The titration must only be started if there are no bubbles inside the burette.
7. The first reaction in the neutral or slightly basic medium is the binding between chloride and silver from the titrant solution, where white precipitates are visualized. When all existing chloride in the sample reacts with silver, it interacts with chromate, generating brick-red coloration, indicating the titration's end point. Silver chloride is less soluble than silver chromate; thus, white silver chloride precipitates first (*see* Fig. 5).

General Notes

- *Sample thickness and particle sizes: when there is more ground sample, the contact surface increases, facilitating the carbonization.*
- *Crucible volume versus sample volume to be weighed: it must be proportional to avoid sample loss by extravasation during carbonization.*

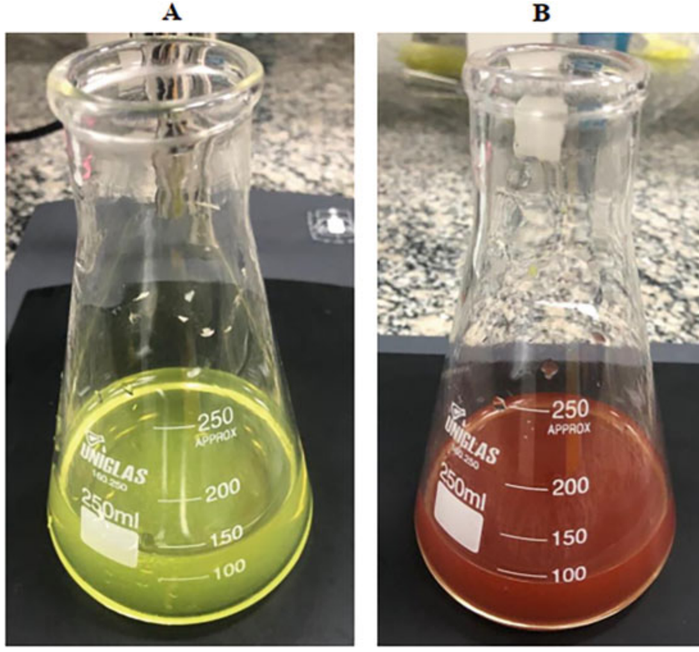

Fig. 5 (**a**) White precipitates referring to silver chloride formation and (**b**) brick-red coloration indicating silver chromate formation and the titration's end point

- *Crucible handling: it must be gripped and held with curling tongs to avoid touching the sample. On the contrary, part of the sample may remain in the grooves from the tong, underestimating the NaCl content. Crucible must also be handled with gloves because it is scalding.*

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Chapter 12

Direct Method for Simultaneous Analysis of Cholesterol and Cholesterol Oxides by HPLC in Meat and Meat Products

Vanessa Sales de Oliveira, Geni Rodrigues Sampaio, Elizabeth Aparecida Ferraz da Silva Torres, and Tatiana Saldanha

Abstract

Cholesterol and cholesterol oxides have numerous implications for human health, highlighting the importance of determining these compounds in highly demanded and consumed foods such as meat and meat products. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are the most used techniques for detecting and quantifying cholesterol and its oxides. However, the former requires a time-consuming derivatization step and uses high temperatures. Thus, HPLC is an alternative method to GC. In the present chapter, we describe the procedures to carry out the direct saponification of samples, which is the preferred method for hydrolyzing samples and separating these compounds from other interfering lipids, as it is cost- and time-effective. HPLC analysis using photodiode array (PDA) and refractive index (RI) detectors is presented for identification and quantification. Moreover, it can be more precise and accurate with the support of mass spectrometry (MS) to confirm the structures of the compounds.

Key words Cholesterol, Cholesterol oxides, Saponification, High-performance liquid chromatography, Mass spectrometry

1 Introduction

Cholesterol is the main steroidal lipid present in animal fats responsible for multiple biological functions in mammals. It may be endogenously synthetized in the body or obtained by the diet through the consumption of foods of animal origin [1–3]. This fact has drawn scientific interest since the intake of cholesterol, and its oxidized products, is a constant target of investigations, particularly due to their possible health implications [2–5].

Cholesterol presents a long chain of polycyclic alcohol with a tetracyclic ring, a hydroxyl group in carbon 3, an unsaturation between carbons 5 and 6, and an aliphatic side chain [1]. Therefore, its chemical structure makes cholesterol highly susceptible to

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oxidation when exposed to pro-oxidant factors (e.g., oxygen, light, heat, radiation, free radicals, metal ions), leading to the formation of mono- or polyoxygenated compounds called cholesterol oxidation products (COPs) or cholesterol oxides [1, 3, 6].

Cholesterol and cholesterol oxides have been extensively reported in meat and meat products [7–10]. Cholesterol concentration in meat is influenced by various factors, such as animal species, muscle fiber type, cut, breed, animal feeding and age, muscle fat, and others [11–13]. Regarding cholesterol oxidation and, consequently, the level of cholesterol oxides, meat presents pro-oxidant constituents like polyunsaturated fatty acids, heme pigments, and metallic ions, as well as several enzymes that catalyze lipid oxidation [14–16]. Moreover, fresh meat generally contains lower levels of COPs as several conditions to which foods are exposed during handling, processing, and storage may induce cholesterol oxidation [7, 8, 16].

The most common COPs present in meat and meat products are 20α-hydroxycholesterol (20α-OH), 25R-hydroxycholesterol (25R-OH), 5,6α-epoxycholesterol (5,6α-EP), 5,6-β-epoxycholesterol (5,6β-EP), 7-ketocholesterol (7-keto), 7-β-hydroxycholesterol (7β-OH), 7α-hydroxycholesterol (7α-OH), 22R- hydroxycholesterol (22R-OH), 22S-hydroxycholesterol (22S-OH), and 25-hydroxycholesterol (25-OH) (*see* Table 1, materials section). Cholesterol levels ranging from 50.00 to 71.2 mg/100 g were determined in beef [17, 18]. Contents of 96.2, 91.7, and 201.70 mg/100 g were found in commercial samples of fresh frankfurter sausage, loin ham, and bacon, respectively [19]. Fresh minced beef presented a total COP content of 1.930 mg/kg, which increased after boiling and frying [7]. A study showed that canning induced COP formation in a low-fat meat product, where the total COPs increased up to the sixth month of storage [8].

Although extensive research presents controversial findings that do not evidence an association between dietary cholesterol and an increase in blood cholesterol levels, the consumption of cholesterol-containing foods is still a topic of intense debate regarding risk factors for cardiovascular disorders [5, 20, 21]. In addition, high cholesterol levels have also been linked to human diseases, including neurodegenerative diseases and cancers [22, 23]. Moreover, dietary COPs may be even more harmful than cholesterol due to their cytotoxic, atherogenic, neurodegenerative, inflammatory, and carcinogenic effects [2, 3, 24, 25]. Thus, determining these sterols in highly demanded and consumed foods such as meat and meat products is crucial for food analysis and public health.

Chromatography is the most suitable technique for cholesterol and cholesterol oxides determination due to their ability to separate and quantify these compounds from other similar ones. Analytical instrumental approaches have been improved from primary

Table 1
Chemical and physical properties and identification of cholesterol and cholesterol oxides present in meat and meat products

Compound name	Abbreviation	Chemical formula	CAS number	MW (g mol^{-1})	Melting point (°C)	Boiling point (°C)	Chemical structure	Relative intensity % (m/z)[a]				
								367	385	369	401	403
5-Cholesten-3β-OL	Cholesterol	$C_{27}H_{46}O$	57–88-5	386.65	148–150	360		5	5	100	–	–
7α-hydroxycholesterol	7α-OH	$C_{27}H_{46}O_2$	566–26-7	402.66	168–170	515.3		100	30	10	–	–
7β-hydroxycholesterol	7β-OH	$C_{27}H_{46}O_2$	566–27-8	402.65	165–167	515.3		100	30	10	–	–
5,6α-Epoxycholesterol	5,6α-EP	$C_{27}H_{46}O_2$	1250-95-9	402.65	147	464.7		50	100	10	–	20
5,6β-Epoxycholesterol	5,6β-EP	$C_{27}H_{46}O_2$	4025-59-6	402.65	135–136	497.7		25	100	10	–	10
7-Ketocholesterol	7 K-OH	$C_{27}H_{46}O_2$	566–28-9	400.65	158–160	463.26		–	–	–	100	10

(continued)

Table 1 (continued)

Compound name	Abbreviation	Chemical formula	CAS number	MW (g mol^{-1})	Melting point (°C)	Boiling point (°C)	Chemical structure	Relative intensity % (m/z)[a]				
								367	385	369	401	403
25-hydroxycholesterol	25-OH	$C_{27}H_{46}O_2$	2140-46-7	402.65	177–179	513.1		100	20	–	–	–
20α-hydroxycholesterol	20α-OH	$C_{27}H_{46}O_2$	516–72-3	402.7	136–137	512.3		100	95	10	–	–
(25R)-26-hydroxycholesterol	25R-OH	$C_{27}H_{46}O_2$	20,380–11-4	402.7	172–174	517.1		100	100	10	–	–
22R-hydroxycholesterol	22R-OH	$C_{27}H_{46}O_2$	17,954–98-2	402.7	171–174	513.1		100	30	–	–	–
22S-hydroxycholesterol	22S-OH	$C_{27}H_{46}O_2$	22,348–64-7	402.7	150–154	513.1		100	50	–	–	–

[a]Relative intensity of the main ions of cholesterol and some of its oxides in HPLC-APCI(+)MS

thin-layer chromatography (TLC) to gas chromatography (GC) and high-performance liquid chromatography (HPLC), which are the most frequently used methods [8, 10, 25, 26].

In general, the first step involved in these methods is the preliminary lipid extraction, which can be performed using different solvent mixtures to provide total extraction of lipids for a reliable analysis. The choice of the sample extraction method presents implications for selectivity and sensitivity and, therefore, for the possibilities of identifying the substances in samples [27]. However, numerous studies have successfully suggested methods that directly treat the sample [10, 28, 29]. The extraction directly from the sample is recommended since it reduces the number of analytical steps, avoiding the formation of artifacts during the process. Artifacts are described as COPs that are not present in the sample before analysis, being generated during it, or COPs that are initially present in the sample whose quantities are increased or decreased due to generation during analysis. Therefore, artifacts may result in over- and underestimations of quantities and kinds of COPs present in a sample [30].

Prior to the qualitative and quantitative steps, sample preparation is necessary to eliminate interferences and increase sensitivity. Thus, cholesterol and COPs must be separated from other interfering lipids, especially fatty acids. It may be achieved by saponification based on an alkaline hydrolyse of esterified compounds [31, 32]. Moreover, since cholesterol oxides occur at lower levels compared to cholesterol, purification or cleanup step is commonly considered [32–34]. However, apparatus used in these steps, such as cartridges of solid phase extraction (SPE), may compromise the analysis accuracy due to the loss of lipophilic compounds resulting from solubility limitations when the water present in the medium is not completely removed [27]. Therefore, a method for simultaneously determining cholesterol and COPs, which can be conducted in the same chromatograph run, was developed and validated, demonstrating that there is no need for using SPE or cleanup [29]. In addition to the gains related to the lower formation of artifacts, it results in the lower consumption of solvents, effectively contributing to the concept of stimulating environmentally friendly methodologies.

Sample preparation is crucial regardless of the chromatographic method applied [26, 33, 34]. However, CG requires an additional derivatization step to enhance the volatility and thermal stability of the analytes, which extends the analysis time and affects the quantitation accuracy by forming artifacts, while HPLC does not require a derivatization step. Besides, HPLC has the main advantage of being performed at relatively low temperatures and avoiding cholesterol oxidation, while GC may thermally destroy compounds to form artifacts [11, 26, 31]. Thus, HPLC is an alternative method to GC (*see* Table 2, materials section). Moreover, the use of mass

Table 2
Studies regarding analyses of cholesterol and cholesterol oxides in meat and meat products by HPLC

Meat sample	Analysis	Extraction	Column/mobile phase	Chromatographic system/detector	Reference
Fermented sausages of pork meat	Cholesterol	Direct saponification: Methanolic KOH (0.5 M); diethyl ether/hexane (1:1, v/v) and ether/hexane (1:1, v/v) extraction	Phenomenex Luna C18 (150 mm × 3.0 mm × 5 μm) with C18 analytical guard column, 4.0 × 2.0 mm/acetonitrile/isopropanol (80:20, v/v)	RP-HPLC-PDA system	[38]
Cooked ham, Spanish serrano ham, minced beef	Cholesterol and cholesterol oxides	Direct extraction: Chloroform + hexane Silica cartridge: Hexane/diethyl ether (95:5, v/v) + hexane-diethyl ether (90:10, v/v) + hexane-diethyl ether (80:20, v/v). COPs were eluted in acetone	Tracer excel 120 ODSA C18 (150 × 4.6 mm, 3 μm) for oxides and tracer excel C8 (100 × 4.6 mm, 3 μm) column for cholesterol/acetonitrile/distilled water (90:10, v/v)	RP-HPLC-UV system	[34]
Meat hamburgers	Cholesterol and cholesterol oxides	Lipid extraction: Chloroform/methanol (2:1, v/v). COPs were eluted in hexane/isopropanol (97:3, v/v)	CN column (25 cm × 0.4 cm × 5 μm)/hexane/isopropanol (97:3, v/v)	NP-HPLC-UV system	[39]
Cypriot smoked meat	Cholesterol oxides	Method A: Direct saponification with aqueous KOH (50%) + ethanol; hexane. Silica cartridge: Hexane/diethyl ether (8:2, v/v) + hexane/diethyl ether (1:1, v/v). COPs were eluted with methanol Method B: lipid extraction with chloroform/methanol (2:1, v/v); hexane. Silica cartridge as applied in method A Method C: lipid extraction with hexane/isopropanol (3:2, v/v) Silica cartridge as applied in method A	Brava C18-ODS, column (250 × 4.6 mm × 5 μm)/acetonitrile/methanol/distilled water/isopropanol (67:27:5:1, v/v)	RP-HPLC-DAD-UV system	[40]

Processed meat (salami and bologna)	Cholesterol	Direct saponification: Aqueous KOH (50%) + ethanol; hexane extraction	Waters column, XTerra MS C18 (25 cm × 4.6 mm × 5 μm)/acetonitrile/ isopropanol (70:30, v/v)	RP-HPLC-UV system	[41]
Pork meat sausages	Cholesterol	Direct saponification: Aqueous KOH (50%) + ethanol; hexane extraction	Supelcosil (75 × 3.0 mm × 3 μm) column/hexane:1,4-dioxane (97.5: 2.5, v/v)	NP-HPLC-DAD system	[42]
Pork loin	Cholesterol and oxides	Direct saponification: Aqueous KOH (50%) + ethanol; hexane extraction	Nova-Pak CN HP (waters, Milford, MA) column (300 × 3.9 mm i.d. × 4 μm)/ hexane/isopropanol (97:3, v/v)	NP-HPLC-UV-RI system	[43]
Cypriot processed meat	Cholesterol oxides	Lipid extraction: Chloroform/methanol (2:1 v/v), followed by extraction with hexane	BEH shield RP18 column (50 × 2.1 mm id, 1.7 μm) 0.1% v/v formic acid in distilled water (A) and methanol (B)	RP-UPLC-MS/ MS system; ESI mode	[44]
Dry fermented sausages	Cholesterol oxides	Direct saponification: Ethanolic KOH (2 M) + dichloromethane; diethyl-ether and aqueous KOH (0.5 M) + NaCl + acetonitrile + hexane Solid-phase extraction: Strata Si-1 column (Phenomenex; 8B-S012-EAK-hexane/diethyl-ether (9:1, v/v); acetonitrile/isopropanol (55:45, v/v)	Phenomenex C18 column (100 mm × 2 mm × 2.6 μm)/water (A) and acetonitrile (B)	RP-HPLC-MS/ MS system; ESI mode	[45]
Beef	Cholesterol	Direct saponification: 11% w/v KOH + 55% ethanol v/v + 45% distilled water v/v; distilled water/ethanol/ hexane; hexane extraction	Waters sílica column SunFireTM prep silica (4.6 mm × 250 mm × 5 μm)/ hexane/isopropanol (98:2, v/v)	NP-HPLC-PDA system	[46]
Canned low-fat meat products	Cholesterol and oxides	Lipid extraction: Chloroform/methanol (2:1 v/v), followed by saponification with methanolic KOH (1 M) and diethyl ether; distilled water and hexane	Agilent Zorbax eclipse plus C18 column (4.6 × 100 mm, 3.5 μm)/acetonitrile/ methanol (3:1, v/v)	RP-HPLC-DAD system	[47]

(continued)

Table 2
(continued)

Meat sample	Analysis	Extraction	Column/mobile phase	Chromatographic system/detector	Reference
Canned low-fat meat products	Cholesterol and oxides	Lipid extraction: Chloroform/methanol (2:1 v/v), followed by saponification with methanolic KOH (1 M) and diethyl ether; distilled water and hexane	Agilent Zorbax eclipse plus C18 column (4.6 × 100 mm, 3.5 μm)/acetonitrile/methanol (3:1, v/v)	RP-HPLC-DAD system	[8]
Meat emulsions	Cholesterol and oxides	Lipid extraction: Chloroform/methanol (2:1 v/v), followed by saponification with methanolic KOH (1 M) and diethyl ether; distilled water and hexane	Agilent Zorbax eclipse plus C18 column (4.6 × 100 mm, 3.5 μm)/acetonitrile/methanol (3:1, v/v)	RP-HPLC-DAD system	[48]

HPLC high-performance liquid chromatography, *UPLC* ultra-high-performance liquid chromatography, *RP-HPLC* reversed-phase HPLC, *NP-HPLC* normal-phase HPLC, *DAD* diode array detector, *PDA* photodiode array; *UV* ultraviolet, *RI* refractive index, *MS* mass spectrometry, *ESI* electrospray ionization

spectrometry (MS) has supported the identification of these compounds, where MS and tandem MS/MS have been applied in recent studies for more selective detection.

Direct saponification and the simultaneous analysis of cholesterol and cholesterol oxides by HPLC were proposed by Saldanha and coauthors [29] and have been successfully used in foods. However, minor modifications must be considered for application in meat and meat products presented in this chapter. For example, the addition of anhydrous sodium sulfate is suitable to avoid emulsion formation. Thus, this chapter describes the procedures for the direct saponification of meat samples at room temperature, followed by the identification and quantification of cholesterol and cholesterol oxides by HPLC using photodiode array (PDA) and refractive index (RI) detectors. In addition, the structures of the compounds are confirmed using MS with atmospheric pressure chemical ionization (APCI) in the positive ion mode and selective ion monitoring (SIM) mode. Commercial standards are used for identification, which is performed based on their retention time and *m/z*, while the quantification is carried out by external standardization.

2 Materials

The solutions must be prepared using analytical grade reagents and ultrapure distilled water (e.g., Milli-Q generating system). Use solvents of HPLC grade for the chromatography analyses. Carry out all procedures at room temperature unless otherwise specified.

2.1 Saponification

- Food sample (meat and meat products).
- Potassium hydroxide (KOH).
- Distilled water.
- Ethanol.
- Hexane.
- Anhydrous sodium sulfate.
- Vortex.
- Magnetic stirring and magnetic stirring bar.
- Rotatory evaporator.
- Nitrogen gas (N_2).
- 50% aqueous solution of KOH: to prepare 100 mL of this solution, dissolve 25 g of KOH in 50 mL of distilled water.

2.2 HPLC/MS Analysis

- Hexane of HPLC grade.
- Isopropanol of HPLC grade.
- 0.45 μm syringe filter.
- Mobile phase: hexane/isopropanol (97:3, v/v).
- Compound standards (*see* Table 1).
- Column: CN Hyperchrome (250 mm × 4.3 mm × 5.0 μm) (Phenomenex, Colorado, USA).
- HPLC system equipped with PDA and RI detectors.
- MS detector: APCI.

3 Methods

3.1 Direct Saponification of Sample

Direct saponification is preferable since it reduces the number of steps and, consequently, the probability of errors, analysis time, and process complexity. In addition, it reduces the consumption of solvents as lipid extraction is not carried out, which is of great value since one of the disadvantages related to HPLC analysis is the excessive use of solvents. The direct saponification of samples has shown yields either similar or better recovery compared with the traditional two-step extraction/saponification procedure [28, 29].

Procedure

1. Add 4 mL of the 50% aqueous solution of KOH and 6 mL of ethanol into a tube containing 2 g of meat sample, and agitate the mixture using a vortex apparatus for approximately 30 seconds. The sample must be finely ground since lowering particle size provides better surface contact with extraction solvents.
2. Insert a magnetic stirring bar into the tube and close it. Submit the mixture to continuous agitation using a magnetic stirring at room temperature (25 °C) for 22 h in the absence of light. The sample must be protected from light to avoid photooxidation. Saponification may also be performed by hot saponification procedures; however, using high temperatures to facilitate the extraction may degrade compounds and form artifacts, by the degradation of 7-ketocholesterol and isomeric epoxides, for example [35–37].
3. For the extraction of the nonsaponifiable matter, add 10 mL of distilled water to dilute the alcohol. This procedure allows the easy formation of the saponified solution. Add 10 mL of hexane, and agitate the mixture using a vortex apparatus for approximately 30 seconds, allowing the tube to rest until the

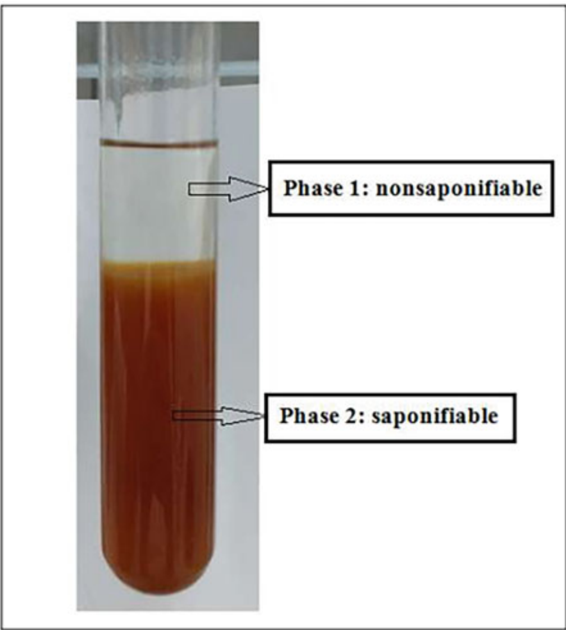

Fig. 1 Phases' separation. Phase 1: nonsaponifiable matter containing cholesterol and its oxides. Phase 2: saponifiable matter

mixture separates into two phases (*see* Fig. 1). Collect the hexane fraction. The extraction with hexane must be repeated three times, combining the hexane solution collected.

Note 1: Add 1 g of anhydrous sodium sulfate during the first extraction to facilitate the phases' separation. It may keep the water on the saponifiable matter, avoiding emulsion formation. This procedure may be considered an optimization, for meat and meat products, of the method described in a previous study [29].

Note 2: The hexane fraction must be collected and transferred to a flask suitable for rotatory evaporator drying.

4. Evaporate the solvent using a rotatory evaporator at 40 °C.

Resuspend the residue with 3 mL of hexane, and transfer the volume to a glass tube, which must be evaporated using N_2.

3.2 HPLC/MS Analysis

1. Resuspend the sample, contained in the glass tube according to step 5 of item 3.1, with 1 mL of the mobile phase (hexane/ isopropanol, 97:3, v/v), and filter it through a 0.45 μm syringe filter, transferring it to a glass vial.
2. Inject 20 μL sample and standards into the HPLC, and analyze using the following analytical conditions:

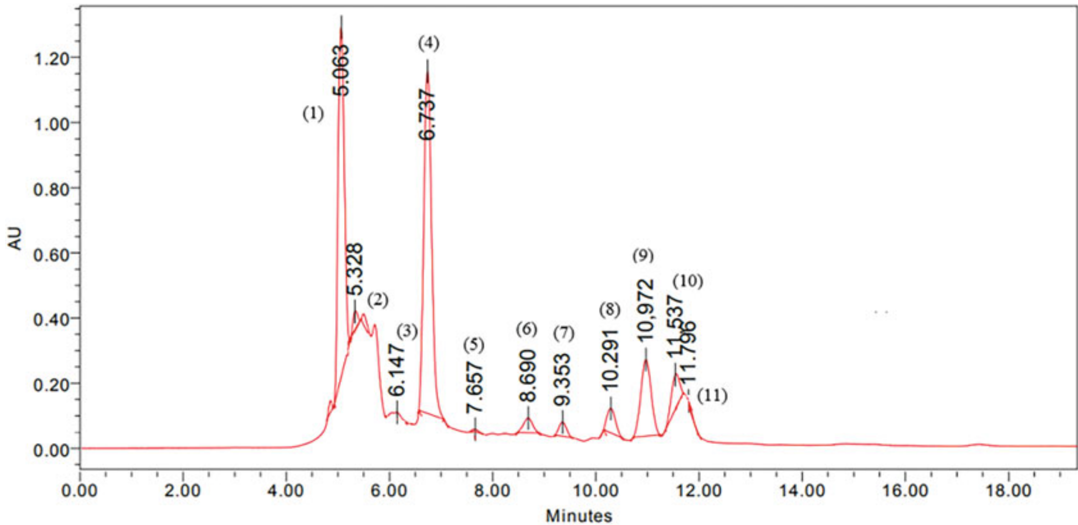

Fig. 2 Chromatogram of cholesterol and cholesterol oxide standards obtained by HPLC. (1) cholesterol; (2) 20α-OH; (3) 22R-OH; (4) 22S-OH; (5) 25-OH; (6) 5,6α-EP; (7) 5,6β-EP; (8) 25R-OH; (9) 7-keto; (10) 7α-OH; (11) 7β-OH

Column: CN Hyperchrome (250 mm × 4.3 mm × 5.0 μm) (Phenomenex, Colorado, USA).

Mobile phase: hexane/isopropanol (97:3, v/v) at 1 mL/min.

Detector: PDA at 210 nm.

Oven-heated column: 32 °C.

MS detection: in the positive ion mode with APCI ionization using the SIM mode. The selected ions: m/z 367, 369, 385, 401, and 403. The relative intensity of the main ions of cholesterol and some of its oxides in HPLC-APCI(+)MS is shown in Table 1.

3. Identification: identify the compounds in samples by comparing the retention time of standards with the peaks in sample and the *m/z*. Figures 2 and 3 present chromatograms of cholesterol and cholesterol oxide standards and a sample of salami, respectively.

4. Quantification: prepare a calibration curve for each compound (cholesterol and COPs) by plotting the average detector response (peak area) of the standard versus standard concentration (*see* Fig. 4). Use the peak area of the sample and the calibration curve to determine the amount of each compound.

Note 3: Cholesterol and the epimeric 5,6-epoxides must be quantified using the RI detector because these oxides do not absorb in the UV (ultraviolet) wavelengths.

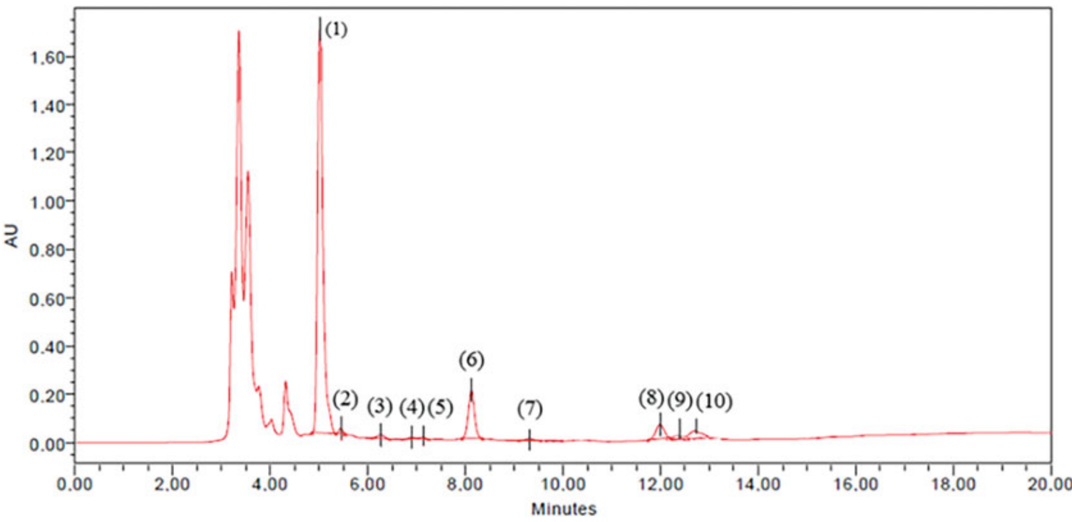

Fig. 3 Chromatogram of salami sample obtained by HPLC. (1) Cholesterol; (2) 20α-OH; (3) 22R-OH; (4) 22S-OH; (5) 25-OH; (6) 5,6α-EP; (7) 5,6β-EP; (8) 7-keto; (9) 7α-OH; (10) 7β-OH

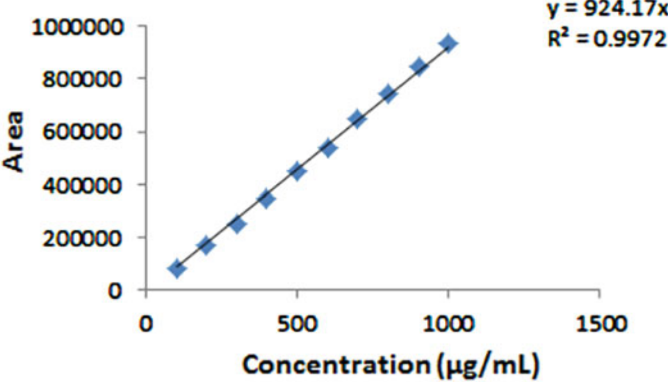

Fig. 4 Example of a calibration curve constructed for cholesterol

Note 4: The standards are diluted in ethyl acetate, the final concentration of cholesterol being 1 mg/mL and that of the oxides 1 μg/mL. The calibration curves must be constructed with at least six points ($R > 0.9$), and the concentrations may vary according to the sample evaluated.

Note 5: Internal standardization may also be used for quantification. In internal standardization, an internal standard of known amount and concentration is added to samples, so that a graph is constructed by relating the ratio (area of each standard/area of the internal standard) by the concentration of each standard. As the same amount of the internal standard is added to the sample, the concentration of the analyte can be obtained through the ratio of the areas obtained in the chromatogram of the sample. The internal standard must have a structure close to that of cholesterol/

cholesterol oxide and be absent in the sample. Due to the complexity of choosing the appropriate internal standard for each type of sample, external standardization is more applied in HPLC analysis of meat and meat products. Table 2 summarizes the studies carried out during the last years that used HPLC to analyze cholesterol and its oxides in meat and meat products.

4 Final Considerations and Future Perspectives

Meat and meat products are among the main sources of cholesterol and cholesterol oxides in the human diet. Thus, due to the legitimate concerns regarding the deleterious effects of these compounds, investigations to determine their occurrence in foods must be carried out, as well as studies regarding strategies to minimize cholesterol oxidation. The literature has shown extraction and saponification as crucial steps. Moreover, monitoring artifact generation requires considerable attention to guarantee consistent results. HPLC is considered an alternative method to GC, while MS and tandem MS/MS allow more selective detection. Based on the points above, the methods and procedures described in this chapter for the simultaneous analysis of cholesterol and COPs in meat and meat products represent a faster, cheaper, and environmentally friendly methodology without ignoring precision, accuracy, and sensitivity. However, as numerous procedures for extraction and saponification have been published, the choice between them remains a problem. Minor changes, during the procedure, can influence the final quantification of COPs. Therefore, the methodology must be evaluated prior to application for each different kind of food matrix to provide the most reliable results.

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Chapter 13

The Long-Lasting Potential of the DNPH Spectrophotometric Method for Protein-Derived Carbonyl Analysis in Meat and Meat Products

Rebeca Cruz, M. Madalena C. Sobral, and Susana Casal

Abstract

This book chapter explores protein carbonyl analysis in meat and meat products, focusing on the enduring power of the classic 2,4-dinitrophenylhydrazine (DNPH) spectrophotometric method. Understanding protein oxidation extent is crucial for product quality and safety. The chapter provides a thorough overview of the DNPH method, including its fundamental principles, advantages, and inherent limitations, along with details on experimental procedures and sample preparation. The DNPH method stands as a potent technique for measuring carbonyl compounds, playing a crucial role in upholding regulatory standards and satisfying consumer demands for minimally oxidized meat and meat products. Its versatility is showcased by its ability to evaluate different meat processing and preservation methods, supporting shelf life and sensory studies. By providing a comprehensive guide, this chapter empowers readers to unlock the mysteries of protein oxidation, ultimately enhancing product quality, reducing food loss, and increasing consumer satisfaction.

Key words DNPH, Protein carbonyls, Oxidative damage, ProtOx analysis

1 Introduction

Meat and meat products play a vital role in providing essential nutrients, including proteins, vitamins, and minerals, as part of the human diet. However, the quality and safety of these products can be compromised by a variety of chemical, physical, and microbiological factors that take place during production and storage leading to changes in their nutritional composition, processing characteristics, and sensory properties. Among the most significant chemical changes that occur in meat products, protein oxidation (ProtOx) stands out as a crucial process. ProtOx involves the modification of protein structures such as alterations in amino acid side chain, protein backbone cleavage, and protein cross-linkage, all caused by reactive oxygen species (ROS) and other free

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radicals [1, 2]. These modifications have notable effects on the functional properties of proteins, impacting functions like gelation, emulsifying, and water-holding capacities [1, 2]. Several mechanisms of ProtOx result in remarkable and measurable transformations in meat and meat products such as carbonylation, loss of sulfhydryl groups, and formation of protein cross-linking [2]. These transformations are significant and measurable, profoundly shaping the attributes of meat and meat products.

Carbonyls (aldehydes and ketones) are primarily formed through direct oxidation of vulnerable aliphatic amino acid side chains (e.g., lysine, arginine, proline, and threonine) resulting in the addition of a carbonyl moiety (-CO) to the molecular structure of proteins [3]. Furthermore, protein carbonylation can also occur through other routes like nonenzymatic glycation (also known as Maillard reaction) in the presence of reducing sugars (e.g., glucose, fructose, ribose, galactose, maltose, and lactose), by the oxidative peptide backbone cleavage by α-amidation or oxidation of glutamyl and prolyl residues, or by covalent binding to nonprotein carbonyl compounds from lipid peroxidation (e.g., malondialdehyde, 4-hydroxy-2-nonenal) [4–9].

The extent of carbonyl formation in meat is influenced by several factors, including the nutritional composition (such as myofibrillar protein content, oxidizing lipids, and metal catalysts), temperature, pH, oxygen availability, and the presence of antioxidants [1]. ProtOx leads to texture changes due to increased protein cross-linking and aggregation, resulting in tougher and less tender meat [10, 11]. It also contributes to the formation of volatile compounds, leading to off-flavors and off-odors that negatively impact the aroma and flavor of meat products [12]. Additionally, carbonylation reduces water-holding capacity, causing a higher drip loss and a drier texture [10]. It further impacts color stability, causing discoloration and diminishing its visual appeal [11]. These technological effects significantly impact the overall sensory quality of meat products. Proper handling, storage, and processing techniques are essential to minimize protein carbonylation and mitigate these negative consequences. Detecting and quantifying carbonyl compounds in meat products plays a crucial role in evaluating protein oxidation and developing strategies to prevent or minimize their formation and the resulting detrimental effects.

Several methods have been developed to detect carbonyls in meat products, including spectrophotometric [13], spectroscopic [14], chromatographic [15], mass spectrometry [16], and immunological techniques [17]. Among these methods, the DNPH method has gained widespread use due to its simplicity and sensitivity for carbonyl detection. This method has been used to analyze carbonyls in various food matrices, including meat products [18–21], with demonstrated reliability and reproducibility.

Fig. 1 The reaction between carbonyl groups and 2,4-dinitrophenylhydrazine. *DNP* dinitrophenylhydrazone; *DNPH* dinitrophenylhydrazine [1]

1.1 The Classic DNPH Method

The DNPH method is a well-established and extensively used colorimetric method operating through the nucleophilic addition of the DNPH molecule to the protein carbonyl group under acidic conditions, resulting in the formation of highly stable, yellow-orange-colored compounds known as dinitrophenylhydrazones (DNP) (*see* Fig. 1).

These hydrazone derivatives exhibit a prominent absorbance peak at approximately 370 nm, allowing for their quantification using spectrophotometric or chromatographic methods with UV detection [13].

The basic principles of the DNPH method involve the following steps:

1. *Protein sample preparation*: Isolation or extraction of the protein of interest while making sure it is in a suitable buffer or solvent for the reaction.
2. *Reaction mixture*: Combining the protein sample or extract with the DNPH solution. The reaction is typically performed in acidic conditions (e.g., pH 2–3) to promote the formation of protein carbonyl-DNP derivatives.
3. *Incubation*: Allow the reaction mixture to incubate at room temperature or a specific temperature depending on the protocol. Incubation times can range from a few minutes to several hours, depending on the experimental requirements.
4. *Quenching and precipitation*: Addition of a quenching solution (e.g., 10% trichloroacetic acid) to stabilize the protein-DNP derivatives. The quenching solution also helps remove excess DNPH.
5. *Wash*: Washing step with a suitable solvent (e.g., ethanol) to remove any residual DNPH or contaminants.
6. *Derivative purification*: Dissolution of the protein-DNP derivatives in an appropriate solvent (e.g., buffer containing guanidine hydrochloride).

Table 1
Modifications to the DNPH method for protein carbonyl measurements

Modification	Description	Benefits/implications	References
Sample preparation	Treatment with hydrochloric acid–acetone solution to remove potentially interfering chromophore substances (e.g., hemoglobin, myoglobin, and retinoids)	Enhances accuracy by eliminating sources of false-positive results	[23]
	Precipitation of the nucleic acids with 1% streptomycin sulfate	Enhances accuracy by eliminating sources of false-positive results	[15]
Derivatization conditions	Combination of TCA and 5% SDS, followed by heat and ultrasound treatment	Increases the solubility of the TCA-precipitated protein	[21]
	Optimization of DNPH dosage, reaction time, and temperature	Improves specificity and sensitivity leading to more accurate and reliable measurements	[26]
Measurement techniques	Incorporation of HPLC with UV or MS detection	Allows for enhanced detection and characterization of protein carbonyls, providing valuable information on specific carbonylated proteins	[25]

HPLC high-performance liquid chromatography, *MS* mass spectrometry, *SDS* sodium dodecyl sulfate, *TCA* trichloroacetic acid, *UV* ultraviolet

7. *Analysis*: Quantification of the purified protein-DNP derivatives and protein content using UV spectrophotometry.

The DNPH method was first introduced by Oliver and Stadtman (1987) [22] to investigate age-related changes in oxidized proteins in different tissues of young and old rats, and they used the DNPH method to measure protein carbonyls. Since its development, the DNPH method has received minor adjustments and was extended to other research fields, including food science [15, 21, 23–25]. The method has also been adapted to high-throughput screening methods to facilitate rapid and accurate analysis of protein oxidation in large sample sets. Some key modifications that have been made are summarized in Table 1.

The spectrophotometric DNPH method for protein carbonyl detection has both advantages and limitations. Firstly, it demonstrates high sensitivity, enabling the detection of low concentrations (within the low micromolar to nanomolar range) of protein carbonyls in samples [27]. This sensitivity is crucial for studying oxidative damage and assessing oxidative stress conditions accurately. Secondly, it demonstrates good specificity, enabling reliable measurements of oxidative damage and precise assessment of carbonylated proteins in complex samples [27]. Thirdly, the DNPH method

offers a straightforward and user-friendly approach, allowing for quick analysis using commonly available laboratory equipment [27]. These characteristics make it accessible to researchers with limited resources or technical expertise. Furthermore, the DNPH method is versatile, applicable to various samples including meat products, and capable of detecting different carbonyl compounds like aldehydes and ketones. It provides valuable insights into oxidative processes and food quality.

However, there are some limitations to the DNPH method. One limitation is its dependency on carbonyl compounds that can effectively react with DNPH. It may not be able to detect carbonyl compounds that have low reactivity or do not undergo reaction with DNPH. This restricts its scope in detecting the complete spectrum of carbonylated species. Furthermore, the DNPH method is susceptible to generating false positives. Interfering compounds present in the sample, such as lipids, nucleic acids, and reducing agents, may react with DNPH, leading to the detection of carbonyl-like signals that do not originate from true protein carbonyls. This can compromise the specificity of the method and requires careful interpretation of results. Also, during sample preparation and derivatization steps, there is a potential risk of protein degradation and loss. Harsh conditions or extended exposure to reagents can result in protein hydrolysis or modifications, including oxidation, leading to inaccurate quantification of protein carbonyls. Finally, the method's dynamic range may be limited, especially for sample extracts with high protein carbonyl levels (higher than 5 mg/mL), requiring dilution or concentration for accurate measurements.

It is important to consider these limitations while applying the DNPH method and to explore complementary approaches or validation techniques to enhance the accuracy and reliability of protein carbonyl analysis. Nevertheless, it has gained recognition as a well-established technique for protein carbonyl detection due to extensive utilization and validation in numerous studies. The wide application of the DNPH method allows for meaningful comparisons and meta-analyses, facilitating a comprehensive understanding of protein carbonylation across different contexts. The abundance of published studies utilizing the DNPH method underscores its credibility and reliability as a valuable tool in protein carbonyl analysis.

1.2 Application of the DNPH Method in Meat and Meat Products

The DNPH method is commonly employed in the examination of carbonyls within meat products. Its application enables the assessment of lipid oxidation and protein oxidation levels, both of which can significantly impact the quality and safety of such products. Moreover, this method serves to evaluate the efficacy of various processing and preservation techniques in minimizing carbonyl

Table 2
Selected articles on protein oxidation in meat using the DNPH method

Animal source	Meat product	Objective	Main findings	References
Pork	Frankfurters	ProtOx in frankfurters with varying levels of rosemary oil and its impact on color and texture during refrigerated storage	ProtOx increased in control frankfurters but was lower in those with 300 and 600 ppm rosemary oil, protecting heme molecules and sensorial properties	[28]
	Patties	Impact of phenolic-rich extracts from Mediterranean wild fruits on texture, color changes, and ProtOx in refrigerated storage of cooked burger patties	Refrigerated storage-induced ProtOx Dog rose (*Rosa canina* L.) extract, along with other fruit extracts, effectively reduced carbonylation and prevented color and texture deterioration	[29]
Chicken	Burgers	Oxidation in meat cooked via oven or microwave methods, with/without additional ingredients, and during in vitro digestion	Carbonyls' content decreased during cooking Oregano could be used to reduce LipOx and ProtOx, preserve meat quality, and minimize LOP exposure after ingestion	[20]
Cow	Beef homogenates	Influence of finishing mode (pasture- or mixed diet-finishing) on LipOx and ProtOx in beef homogenates and antioxidant activities	Although the pasture diet significantly reduced LipOx, no significant effect on ProtOx was observed	[23]
Lamb	–	Effects of pasture- and concentrate-based diets and 7-day refrigerated storage on meat oxidation	ProtOx increased during 7-day storage, with higher carbonyl levels in animals fed concentrate than pasture	[30]
Lamb	Loins	Impact of sous vide cooking conditions on LipOx and ProtOx in lamb	Total protein carbonyls increased over time at all cooking temperatures	[31]

LipOx lipid digestion, *LOP* lipid oxidation products, *ProtOx* protein oxidation

compounds in meat products. Given its wide utilization within the food industry, the DNPH method plays a vital role in quality control and regulatory compliance endeavors. Table 2 summarizes a selection of studies that have employed the classic DNPH method to assess protein carbonylation in meat products.

These findings highlight the multifactorial nature of ProtOx in meat products and the potential use of natural extracts and dietary interventions to mitigate oxidative processes. The following section describes the materials and methods used to carry out the protein oxidation analysis using the DNPH method, providing insights into the experimental procedures and data collection for a comprehensive evaluation of protein oxidation in meat products.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to achieve a resistivity of 18 MΩ.cm at 25 °C) and analytical grade reagents (*see* **Note 1**). Prepare and store all reagents and solutions at room temperature (unless indicated otherwise). Thoroughly follow all safety precautions of chemical manipulation as waste disposal guidelines when disposing waste materials.

1. Buffer solution A: 20 mM phosphate buffer with 0.6 M NaCl, pH 6.5. Add about 50 mL of water followed by 2 mL of 1 M phosphate buffer solution, pH 7.4 (e.g., Sigma-Aldrich P3619) to a 100 mL beaker. Weigh 3.51 g of NaCl, adjust the pH to 6.5 with HCl 1 M (*see* **Note 2**), transfer the solution to a 100 mL graduated cylinder, and complete the final volume with water. Transfer the buffer solution to an appropriate storage container.
2. Trichloroacetic acid 10% (w/v): Add about 50 mL water to a 100 mL graduated flask. Weigh 10.0 g of trichloroacetic acid. Mix and make up to 100 mL with water. Store at 4 °C until use.
3. Hydrochloric acid 2 M (*see* **Note 3**). Add about 10 mL water to a graduated cylinder. Transfer 2.67 mL of 12 M HCl with caution. Mix and make up to 20 mL with water.
4. 2,4-Dinitrophenylhydrazine (DNPH) 0.2% (w/v) in HCl 2 M (*see* **Note 4**). Weigh 30 mg of DNPH, and transfer it to a 25 mL graduated cylinder that already contains about 10 mL of HCl 2 M. Mix until complete dissolution, and make up the final volume of 15 mL with HCl 2 M. It can be prepared daily or stored at 5 °C for up to a week.
5. Washing solution: Add 25 mL of absolute ethanol to a 50 mL conical flask with standard ground joint, and mix with 25 mL of ethyl acetate.
6. Buffer solution B: 20 mM phosphate buffer with 6 M guanidine hydrochloride, pH 6.5. Add about 25 mL of water followed by 1 mL of 1 M phosphate buffer solution, pH 7.4 (e.g., Sigma-Aldrich P3619) to a 50 mL beaker. Weigh 28.65 g of guanidine hydrochloride, adjust the pH to 6.5 with HCl 1 M

(*see* **Note 2**), transfer the solution to a 50 mL graduated cylinder, and make up to 50 mL with water. Transfer the buffer solution to an appropriate storage container.

7. BCA protein assay kit (*see* **Note 5**).

3 Methods

Carry out all procedures at room temperature unless otherwise specified. Analysis of samples should be performed in triplicate to ensure reliable and reproducible results.

1. Accurately weigh 1.0 g of fresh minced meat sample into a clear 15 mL centrifuge tube.
2. Add 10 mL of buffer solution A, and provide a nitrogen atmosphere (*see* **Note 6**).
3. Vortex for 10 s at 150–200 rpm, and mix in a rotary agitator overnight (12–15 h).
4. Vortex again for 10 s, and centrifuge at 5000 × *g* for 5 min.
5. Transfer 300 μL of the clear supernatant to three separate 2 mL centrifuge tubes, being one for protein quantification (P) and the other two for carbonyl quantification (CX for sample X and CB for blank) (*see* **Note** 7). Identify each one intelligibly.
6. Add 1 mL of cold 10% TCA to all tubes to promote protein precipitation, and vortex for 10 s. Refrigerate (4 °C) for 15 min.
7. Vortex the solution once again for 10 s, and subsequently centrifuge it at 7500 × g for 3 min.
8. Discard the supernatants, and add 1 mL of 2 M HCl to the pellets on tube P and CB and 1 mL of 0.2% DNPH solution to the pellets on tube CX.
9. Allow to react for 1 h with mild agitation (30–50 rpm) in a vortex mixer.
10. Add 1 mL of cold 10% TCA to both tubes to promote protein precipitation, vortex for 10 s, and centrifuge again at 7500 × *g* for 3 min.
11. Discard the supernatant, and wash the pellets by adding 1 mL of the washing solution to remove any free DNPH reagent. Centrifuge at 7500 × *g* for 3 min. Repeat the washing procedure one more time (*see* **Note 8**).
12. Reject the supernatants (*see* **Note 8**), and solubilize the pellets with 1.5 mL of the buffer solution B.
13. Centrifuge at 7500 × *g* for 3 min to separate any insoluble fragments, and transfer 200 μL of the yellow supernatant from

tubes CX and CB to a 96-well plate, and record the absorbance at 370 nm (*see* **Note 9**). The supernatant from tube P will follow the recommendations defined by the BCA protein kit chosen by the user (*see* **Note 10**).

14. Determine the content of carbonyls in the sample extracts (nmol/mL) using the following equation (*see* **Note 11**):

$$Carbonyls\left(\frac{nmol}{mL}\right)=\frac{A370nm}{(22{,}000\times 0.58)}\times 10^{6}$$

15. Results are expressed as nanomol carbonyl per mg of protein after dividing the concentration of carbonyls in the extracts (nmol/mL) by the protein content (mg/mL).

4 Notes

1. The suggested quantities of all reagents are enough for ten individual test samples.
2. HCl 2 M can be used first to narrow the gap from the starting pH to the desired pH. As pH 7 is reached, it is best to use a series of HCl 1 M or 0.5 M to avoid a sudden drop in pH below the required pH.
3. Check the purity of the concentrated HCl. The density of HCl 37% (w/w) solution is 1.19 g/mL. Considering the molar mass of hydrogen chloride as being 36.46 g/mol, the molarity of HCl 37% (w/w) solution is 12.1 M. Transfer about 35 mL water to a 50 mL volumetric flask, and slowly add 8.3 mL of the concentrated HCl. Mix, allow to cool down, and make up to 50 mL with water.
4. To ensure the complete dissolution of DNPH and enhance the stability of derivatives, we suggest adding 2 mL of 97% (w/w) H_2SO_4. It is advisable to incorporate this solution into the final volume and prepare it daily. This approach facilitates optimal conditions for DNPH dissolution and promotes the formation of stable derivatives.
5. There are other alternatives for protein measurement, but we believe this approach is less time-consuming and more accurate. Check if your BCA protein assay kit includes bovine serum albumin (BSA) standard for calibration purposes.
6. By working under a nitrogen atmosphere, the risk of oxidation is minimized, ensuring more accurate and reliable DNPH measurements. Allow the nitrogen gas to flow through the experimental setup for a sufficient duration to ensure complete displacement of air. The purging time may vary depending on the specific apparatus and setup, but typically a few minutes of purging is recommended.

7. The supernatant should have a protein concentration of approximately 1 mg/mL. If needed, dilute the supernatant with buffer A.
8. Special care must be taken when rejecting the supernatants to not lose any protein particles that could result in a high variability. You should use a 1 mL micropipette to slowly remove the supernatant at once.
9. In general, DNPH derivatives are relatively stable when stored under appropriate conditions. When kept in a cool, dry, and dark environment, most DNP derivatives can maintain their stability for several months to a year. However, it's important to note that their stability can decrease over time, especially if exposed to light, heat, or reactive substances such as strong acids or bases.
10. The BSA calibration curve is prepared with buffer solution B as in the sample extracts.
11. The molar absorptivity of the hydrazone is 22,000 $M^{-1}.cm^{-1}$ at 370 nm, and the path length of a 200 μL aliquot in a 96-well plate is 0.58 cm.

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Chapter 14

Functional Molecules Obtained by Membrane Technology

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Abstract

Meat by-products and coproducts, including slaughtered wastes, represent an actual challenge for the food processing industry due to their prejudicial impact on the environment. Membrane technologies can be helpful for the valorization of these streams according to the principles of a sustainable circular economy. This chapter described selected experimental models that have been used to valorize meat by-products, like blood, and wastewater through the application of membrane-based processes. The aim of this study is to provide protocols outlining important factors related to sample handling, membrane selection, operation process, and membrane performance.

Key words Microfiltration, Ultrafiltration, Wastewater, Meat by-products, Meat proteins

1 Introduction

Actually there is a significant increase in the world population and, consequently, in meat consumption. According to Bouwman et al. [1], there is a projected continuous increase in meat production, expected to double by 2050. Meat can be defined as the muscle of different species, including poultry, pigs, cattle, and others. Today, one of the major problems of the meat industry is related to the disposal of by-products and the usage of water during the processing [2–4]. The meat processing industry generates substantial quantities of wastewater from animal slaughter and cleaning of processing facilities. It is responsible for utilizing 24% of the total freshwater consumed by the food and beverage sector [5, 6].

These wastes are concentrated in small areas and are potential pollutants. They cannot be fully absorbed by the environment due to their high loads of nutrients and organic matter. One of the common practices on rural properties is the use of these wastes

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directly as soil fertilizer, which is not recommended due to the high loss of nitrogen in the form of ammonia, causing environmental pollution [7].

In general, industrial waste can be divided into two large groups, wastewater and coproducts, with emphasis on blood. The industrial wastewater is rich in organic compounds such as proteins, fats, and carbohydrates from meat, blood, skin, and others. An improper discharge of wastewater presents a significant risk of polluting freshwater sources, leading to environmental and health hazards like deoxygenation of rivers, groundwater contamination, eutrophication, and the spread of diseases. Also, due to the high protein content, this wastewater is prone to putrefaction and emits unpleasant odors [4, 8–11]. Blood is primarily composed of cellular components and plasma serum in which the cells are suspended. Plasma, which constitutes the liquid part of blood, is made up of approximately 91% water, 8% organic compounds, and 1% inorganic compounds. Proteins and lipoproteins, fatty acids, cholesterol, triglycerides, hormones, glucose, and vitamins are the primary organic compounds found in blood [12].

On this way, one of the challenges of the scientific community is based on finding alternatives that can be cost-effective on the treatment of these wastes, mitigating the environment damage. Several methods have been studied, and some are already well developed and widely used. These treatments can include decantation, flotation and flocculation, adsorption, precipitation, enzymatic treatment, and others [3].

The treatments actually used have several disadvantages, as the recycling of organic materials into valuable proteins and fats involves treating them with chemicals to separate them through flocculation or also breaking them down through digestion to generate biogas or carbon dioxide. The primary cause of this issue is that both coagulants and flocculants alter the structure and functional properties of proteins by binding to them and precipitating them. Furthermore, the flocculants and coagulants employed are toxic [2, 10, 13]. In addition, traditional techniques also add to air pollution and necessitate more energy and materials input, resulting in associated emissions [11]. Fatima et al. [4] emphasized in their work also the importance of the reuse of water, as the conventional techniques only treat and discharge water into the environment without recycling it.

One alternative to these processes is membrane separation, which has a significant appeal for waste valuation. The membrane technology has several advantages, among which are the nonuse of additives, the absence of phase change, and an easy scaling-up. Moreover, a low capital expenditure is required for launching. These advantages can address the majority of the limitations exhibited by both traditional and recently developed techniques [2, 3, 8].

So the membrane process can be used for the isolation and concentration of protein-based molecules and fragments from blood wastewater. Considering these advantages, the final product may have some important characteristics, such as maximizing the production of the desired molecules, reducing the degradation and loss of bioactivity of the molecules, and guaranteeing a high quality of the final product. The recovery and valuation of these compounds are important since they have biological activities such as hypotensive, hypocholesterolemic, antihypertensive, and others. One example was related by Avula et al. [9] to the usage of a combined method of membrane, specifically the ultrafiltration and dehydration. These authors related the obtaining of a by-product consisting of 30–35% protein and 24–45% fat, from wastewater collected from a poultry abattoir. They also evaluated the economic viability of this process, based on a protein recovery of 60%, and a plant that processes 100,000 chickens per day can generate a daily income of around US $24,000.

In this way, it is quite evident the necessity of valorizing meat by-products, like blood, and wastewater through the application of membrane-based processes. These protocols outline important factors related to sample handling, membrane selection, operation process, and membrane performance.

2 Recovery of Protein-Based Composites from Meat Sub-products by Assisted Membrane Processes

The membrane acts as a barrier that separates the components of a solution. It achieves this by concentrating the solution on one side, known as the concentrate, while allowing the solution to pass through on the other side, known as the permeate. When it comes to the recovery of protein-based molecules from meat coproducts, UF membranes are deemed highly suitable based on the molecular weight of the proteins. To provide a theoretical guideline in this regard, Castro-Muñoz et al. [3] propose the following approach:

1. UF membranes with a wide pore size, ranging from 50 to 100 kDa, should effectively recover larger molecules (e.g., ω-lactalbumin).
2. UF membranes, possessing a 10–30 kDa cutoff, are more efficient for the extraction of macro- and microsolutes (e.g., protein hydrolysates).
3. Narrow UF membranes, ranging from 1 to 5 kDa, should selectively separate low-molecular-weight compounds (e.g., peptide fractions and fragments).

Slaughterhouse waste, including pork and beef coproducts, comprises significant amounts of blood, viscera, and intestinal tissues. These materials are primarily protein-based but often go to waste. Bovine blood, in particular, is a common by-product in the meat processing industry [7]. From the early 1970s, researchers began exploring the use of reverse osmosis (RO) and ultrafiltration (UF) for blood treatment. Notably, they discovered that the filtration of blood plasma and red blood cells is heavily influenced by concentration polarization. This means that specific operational conditions, such as low pressure and high velocity, are the most effective approach [3].

2.1 Recovery of Proteins from Bovine Blood Serum

Blood is a product generally obtained in an animal slaughterhouse that provides a great basis for the growth of pathogenic bacteria. Hence, the separation of the blood into plasma and hemoglobin usually is carried out [14]. Posteriorly, the plasma is dried or frozen to obtain a product in which both the stability and the properties are improved. In the production processes of dried or frozen blood plasma, water plays a relevant role. Therefore, an initial plasma concentration is used to reduce the costs and time of drying or freezing. In this way, Makara et al. [12] suggested a membrane protocol. An integrated system was proposed, microfiltration followed by ultrafiltration.

2.1.1 Materials

1. Animal blood plasma.
2. Microfiltration membranes 0.07 μm cutoff.
3. Ultrafiltration membranes 300 kDa cutoff.
4. Pump.
5. Jacketed processing tank.
6. Heat exchanger.

2.1.2 Methodology

1. Test the material to determine the protein content on the feed solution.
2. Fill the processing tank with the animal blood plasma.
3. Heat up the plasma until 35–40 °C.
4. Set up the membrane system so that the microfiltration concentrate is used as a feed for the ultrafiltration process (Fig. 1).
5. Start the membrane system with total removal of the permeates and recirculation of the final concentrate to the processing tank.
6. Set the transmembrane pressure to 2 bars.
7. Control the permeate flow to evaluate the membrane performance, and estimate the end of the process.

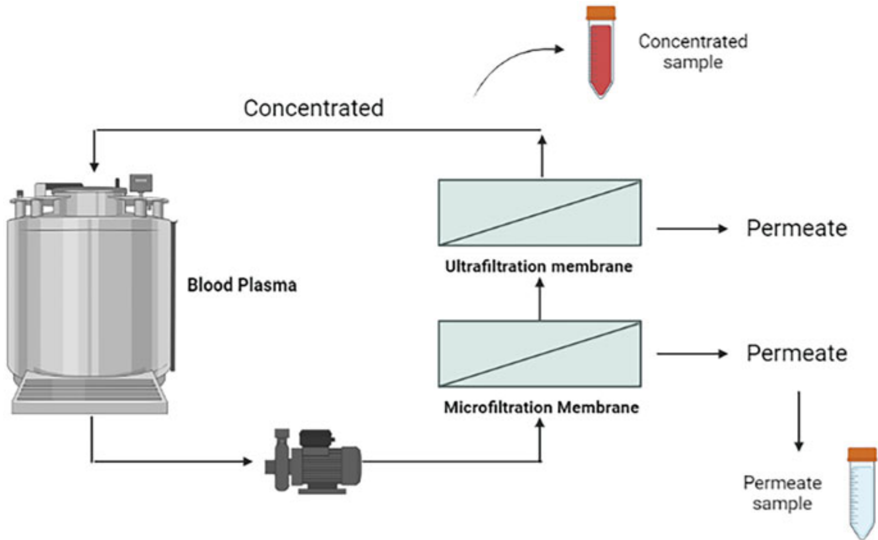

Fig. 1 Flow diagram of an experimental unit for the recovery of proteins from animal blood plasma. (Created inBioRender.com)

8. Collect samples to evaluate the protein content on the final concentrated solution and permeate.
9. Volume reduction factor (*VRF*) (3.5-fold concentration) is calculated as the ratio between the initial volume (L) of the blood plasma used in the feed and the final volume (L) of the concentrate after the ultrafiltration. The permeate flux (J) (L h^{-1} m^{-2}) during the filtration can be calculated: $J = V_p$ $t{\cdot}A$, where V_p (L) is the amount of permeate collected during the period of time t (h) and A (m^2) is the permeation surface area of the membrane. The quality of the filtration process was measured based on the protein content present in the concentrate.
10. After the filtration process, the equipment is cleaned with alkaline solution (0.1%) according to manufacturer's instructions.

2.2 Recovery of Proteins from Porcine Liver

The porcine liver is an example of an edible meat processing waste. This organ is cheap and is easily disposable from butchers. However, its singular color and odor mean it is mostly used for animal feed or meat derivate. At the same time, the nutritional profile of the liver is unique, so it is considered one of the most valuable protein and nutrient sources [15]. The high levels of carbohydrates and polyunsaturated fatty acids (PUFA) and the low levels of monounsaturated fatty acids (MUFA) are some of the characteristics of this coproduct. Also, the porcine liver is rich in numerous vitamins, such as retinol (A), riboflavin (B2), niacin (B3), pyridoxine (B6), folacin (B9), cobalamin (B12), and ascorbic acid (C), and has a high mineral content, especially iron and manganese.

For the purpose of finding attractive applications for a large amount of wasted porcine liver, Borrajo et al. [16] suggested a protocol using enzymatic hydrolysis in order to release the biopeptides through protein cleavage. Thereafter, a recovery step is carried out using low-molecular-mass cutoff membranes to separate small peptides from high-molecular residues and remaining enzymes.

Ultrafiltration is the main perm-selective barrier usually used to separate proteins from meat processing by-products, with pore sizes ranging from 4 to 30 kDa being the most effective for the separation of macro- and microsolutes, such as protein hydrolysates [17]. Previous studies established that low-molecular-weight peptides were stronger as antioxidants than high-molecular-weight peptides [18, 19].

2.2.1 Materials

1. Fresh porcine livers.
2. Cutter.
3. Papain.
4. Orbital shaker.
5. Centrifuge.
6. Vacuum pump.
7. Cellulose ultrafiltration membrane (4–30 kDa pore size) can be adjusted to concentrate specific compounds.

2.2.2 Methodology

1. Remove fat and connective tissue from the liver.
2. Cut into small cubes, and freeze until −20 °C.
3. Mix with ice at a ratio of 1:1 (w/w) in a cutter.
4. Incubate the sample with papain (1:100 w/w) at 37 °C and pH 6.0 for 30 min.
8. Incubate the sample with bromelain (1:100 w/w) at 40 °C and pH 6.0 for 30 min.
9. Incubate the sample with alcalase (1:100 w/w) at 50 °C and pH 8.0 for 30 min.
10. Leave the samples and the proteases reacting for 10 h in an orbital shaker with agitation of 125 rpm.
11. Stop the enzymatic reaction heating the samples until 95 °C for 3 min.
12. Cool the samples in bath ice.
13. Centrifuge the samples at 9000 RPM for 10 min.
14. Separate the supernatant.
15. Filtrate the supernatant with vacuum ultrafiltration membrane of 4–30 kDa.
16. Collect the final peptides.

A schematic diagram of the process is demonstrated on Fig. 2.

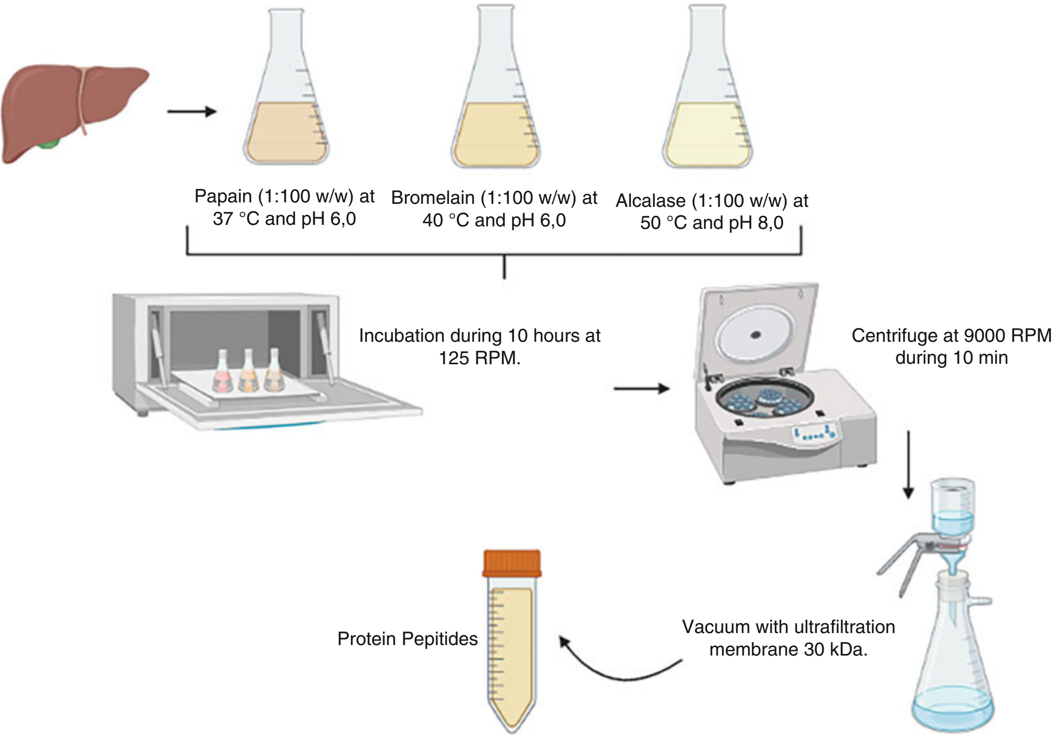

Fig. 2 Flow diagram of an experimental process for the recovery of proteins from porcine liver. (Created inBioRender.com)

2.3 Recovery of Proteins from Pork and Beef Lungs

For economic and environmental reasons, it is, therefore, necessary to find new manners to get the increased value of slaughterhouse coproducts. For instance, meat coproducts present a high protein content, between 15% and 20% (w/w), with many essential nutrients such as amino acids, minerals, vitamins, and fatty acids [20]. As a consequence, a new way to raise the value of slaughterhouse by-products would be to extract their proteins for use as functional ingredients in meat products, for instance, as emulsifying or gelling agents, using similar technologies. In this way, membrane process can be used to purify these proteins without considerably altering their properties. According to Selmane et al. [21], microfiltration can be used to filter colloidal and suspended particles, as well as bacteria, in the range 0.1–10 μm, while ultrafiltration can be used to concentrate the solutes when their colloidal or molecular structure ranged between 1 and 100 nm. Based on this, a protocol for proteins recovery from pork and beef lungs is proposed.

2.3.1 Materials

1. Fresh pork and beef lungs.
2. Grinder.
3. Mixer.
4. Centrifugal pump.

5. Microfiltration membrane with average pore size of 1.4 μm.
6. Ultrafiltration membrane with pore size between 100 and 200 nm.
7. Centrifuge.
8. Cloridric acid.
9. Rotoevaporator.

2.3.2 Methodology

1. Crush the fresh pork and beef lungs into small pieces with a grinder at 3000 RPM for 3 min.
2. Mix 200 g of the sample and 1 L of water (20% w/v), in a mixer at 1100 RPM for 5 min.
3. Incubate the samples at pH 9.0, temperature of 20 °C, and 60 min of operation.
4. Place the solution on the feed processing tank.
5. Set up the membrane system so that the microfiltration permeate is used as a feed for the ultrafiltration process (Fig. 3).
6. Start the membrane system with removal of the ultrafiltration permeate and recirculation of the concentrates to the processing tank.

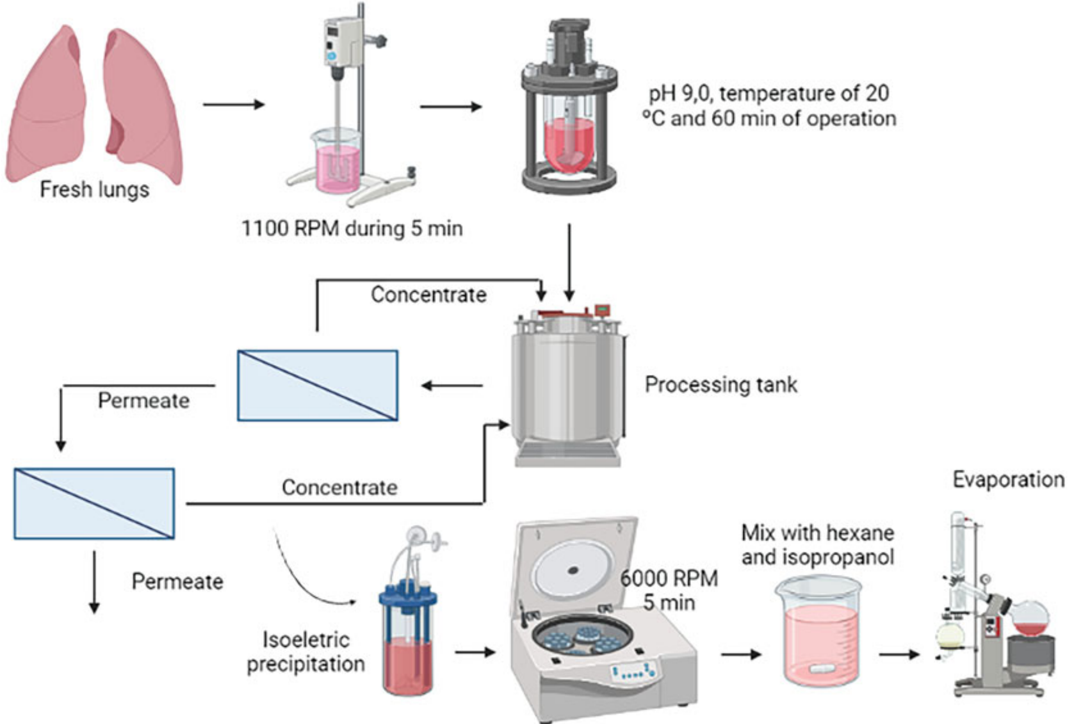

Fig. 3 Flow diagram of an experimental process for the recovery of proteins from porcine and beef lungs. (Created inBioRender.com)

7. Set the transmembrane pressure to 2 bars.
8. Control the permeate flux to evaluate the membrane performance, and estimate the end of the process.
9. The concentration is performed in an open system, using the volume reduction factor (VRF) as the end of the process. The permeate flux (J) can be collected every 5 min, and the efficiency of the process can be evaluated with the retention coefficient $R = (1 - C_P)/C_C$, where C_P is the concentration of the protein in the permeate and C_C is the protein concentration in the concentrate.
10. Collect the final sample, and add 37% HCl until isoelectric precipitation.
11. Centrifuge the mixture at approximately 6000 RPM for 5 min.
12. Mix the precipitate with hexane and isopropanol (3:2, v/v) under mechanical stirring for 1 h at 20 °C using a 1/5 proportion proteins/solvent.
13. Evaporate the residual solvent.

2.4 Recovery of Proteins from Wastewater of Meat Production

Membrane separation is widely employed in various industries such as dairy, beverage, fish, and poultry, including its application in wastewater treatment [22, 23]. In the context of meat processing wastewater, an average composition per ton of slaughtered live weight includes 12 kg of solids, 14.6 kg of biological oxygen demand (BOD), and 1.7 kg of nitrogen. Taking into account the nitrogen content in the wastewater, approximately 10% of the protein derived from the slaughtered live weight is lost. While it is possible to recover half of this protein, this would result in an additional 200 million kg of protein becoming available annually in the United States, with an estimated value of $200 million per year [24].

2.4.1 Materials

1. Meat processing wastewater.
2. Preliminary filters.
3. Depth filter.
4. Pump.
5. Ultrafiltration membrane with 10 kDa cutoff.
6. Reverse osmosis membrane.

2.4.2 Methodology

1. Preliminary treatment for the elimination of traces and fat particles: the initial stage consists of a protein chain with filters that allow the passage of coarser materials, depending on the origin of the effluent (minerals, pieces of bones, etc.).
2. Application of a depth filter, with the purpose of eliminating traces of fat that were not removed in the initial treatment and providing the effluent ready to be treated in the membranes.

3. Use of ultrafiltration membrane (total retention of protein molecules in the form of amino acids, based on 10 kDa ultrafiltration membranes) or application of preliminary depth filters for effluent treatment, with a 3 mm mesh opening, prior to the use of wide-open microfiltration membrane with a pore size of 0.5–2 microns.
4. Separation of the permeate phase (indication of salts, destructured proteins, polysaccharides, and molecules of reduced size that passed through the previous steps) and the retentate phase (protein).
5. Posttreatment with osmosis membrane for final polishing and to generate reuse water (permeate) and separated protein (retained).
6. Biological treatment and associated biotechnologies for advances in the degradation of the resulting matter and generation of cleaner water. A schematic diagram of the process is demonstrated on Fig. 4.

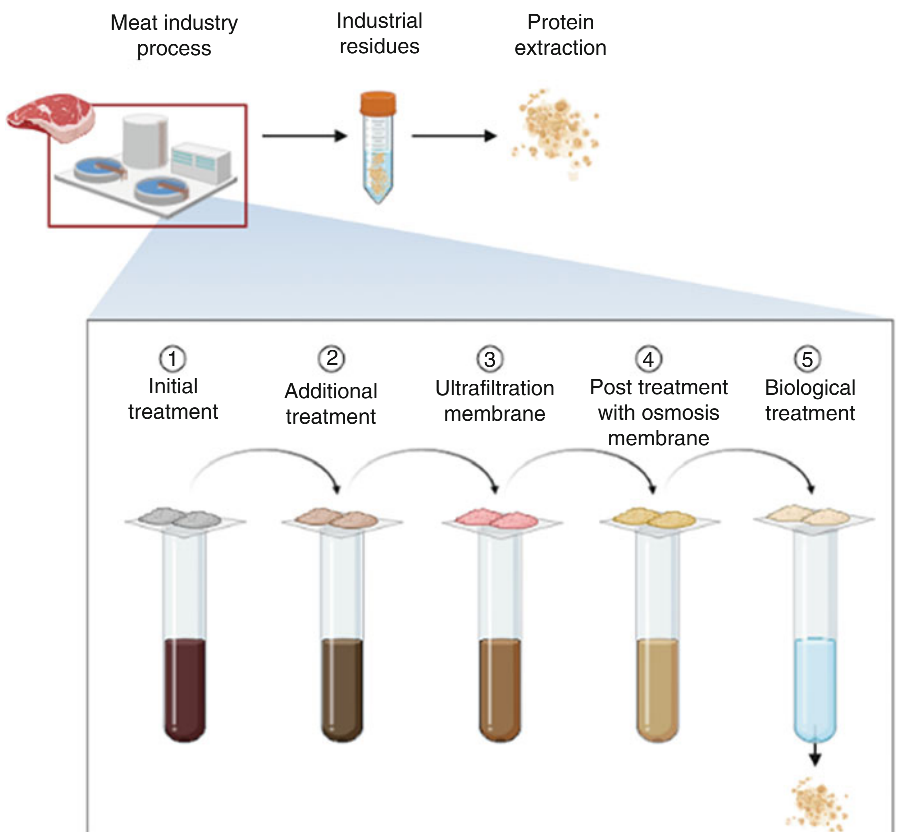

Fig. 4 Diagram of the different stages of the process of obtaining protein from the filtration and membrane separation process (MSP) for the meat industry

3 Notes

1. Before the test, take care to keep the raw material, which in this case is the protein solution, at a temperature below 10 °C so that it does not promote microbiological proliferation and that if the temperature is above this, it should be for a time less than 10 min.
2. The efficiency of the process is directly related to the temperature at which the process takes place.
3. The efficiency of the process is related to the porosity of the membrane used; the greater the porosity, the greater the volume of filtrate and the lower the amount of protein in the concentrate.
4. The efficiency of the process is related to the pressure at which the process occurs; the higher the pressure, the faster the fluid will be filtered.
5. The use of membranes for the recovery of meat peptides presents a promising potential for the food and pharmaceutical industries. The application of membrane filtration techniques, such as microfiltration, ultrafiltration, and nanofiltration, has shown to be efficient in the separation and concentration of peptides of interest.
6. The membranes used in these processes play a fundamental role in the selective retention of peptides, allowing the passage of smaller molecules and rejecting undesirable substances, such as fats, high-molecular-weight proteins, and impurities. In addition, the proper choice of membrane characteristics, such as pore size, can be adjusted to meet the specific needs of each application, enabling the recovery of peptides with different molecular weights.
7. It is expected that these technologies can contribute to the production of high-quality peptides, with potential application in functional foods, nutritional supplements, and drugs, bringing benefits both to human health and industry.

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Chapter 15

Bioactive Peptides Obtained from Meat Products

Mirella Romanelli Vicente Bertolo, Josemar Gonçalves de Oliveira Filho, Sinara Teixeira do Brasil Morais, Keila Nascimento Cavalcante, Daniel Rodrigues Cardoso, and Stanislau Bogusz Junior

Abstract

Meat and meat products stand out as significant sources of bioactive peptides, due to their high content of high-quality proteins and the presence of all the essential amino acids that the human body needs. Here we describe the main methods involved in obtaining and characterizing bioactive peptides from meat and meat products, from sample preparation to enzymatic hydrolysis protocols and characterization techniques, such as MALDI-TOF-MS and LC-ESI-QqQ.

Key words Bioactive peptides, Meat, Meat products, Enzymatic hydrolysis, Matrix-assisted laser desorption/ionization-time of flight, Liquid chromatography, Triple quadrupole

1 Introduction

Bioactive peptides are small protein fragments composed of 3–20 amino acid residues that have shown beneficial physiological effects when consumed at appropriate levels [1]. While rare, some exceptional bioactive peptides may contain more than 20 amino acid residues [2]. These peptides can be found in a variety of food sources, including grains, legumes, milk, eggs, fish, meat, and seaweed [3]. These sources offer a wide range of options for obtaining bioactive peptides, allowing a diversified approach to incorporating these compounds into a healthy diet.

The bioactivities of bioactive peptides are inherently linked to their amino acid composition and their position within the peptide sequence [4]. The scientific literature has reported that bioactive peptides derived from food proteins can exhibit a wide range of activities, including antioxidant activity [5], antimicrobial activity [6], antihypertensive effects [7], anticancer properties [8], antidia-

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betic effects [9], and others. These findings demonstrate the potential of these peptides as therapeutic and functional agents in promoting health and preventing diseases.

Meat and meat products stand out as significant sources of bioactive peptides, due to their high content of high-quality proteins and the presence of all the essential amino acids that the human body needs [10]. Moreover, meat proteins are easily digested and can be safely consumed, being preferred over other sources of proteins (like dairy products). The high availability of meat proteins leads to the obtention of low-molecular-weight peptides with different bioactive properties. In particular, most bioactive peptides from bovine and porcine meat have angiotensin-converting enzyme (ACE) inhibitory and antioxidant properties; antimicrobial, antidiabetic, anticancer, and antithrombotic properties are also reported for poultry, venison, and mutton meat [11].

Ensuring the safety of bioactive peptides is of utmost importance, requiring comprehensive toxicity studies and the establishment of appropriate dosage levels for consumption [12]. To prevent consumer deception and false claims regarding the efficacy of peptide-based products, several countries have implemented regulations and standards to guide foods claiming to contain functional or bioactive ingredients [11]. The primary objective of these regulations is to protect consumers against potential risks. However, these stringent requirements may limit the commercialization of functional peptide-based products. In addition to regulatory and safety considerations, the effectiveness of bioactive peptides in humans plays a crucial role in the commercial viability of such products [13]. Therefore, it is essential to ensure compliance with established standards and regulations to guarantee the safety and quality of marketed products.

Therefore, the aim of this chapter is to present and describe the main methods involved in obtaining and characterizing bioactive peptides from meat and meat products, from sample preparation to enzymatic hydrolysis protocols and characterization techniques.

2 Materials

2.1 Sample Preparation

Sample preparation is a crucial step prior to the production of bioactive peptides from meat: factors such as the presence of skin, nerves, or other parts that will not be used in the enzymatic hydrolysis step, as well as the non-homogeneity of the sample once mixed with the reaction medium for the enzymatic attack, will affect the yield of the process, as well as the profile of the produced bioactive peptides. Moreover, knowing the chemical composition of the meat (in terms of moisture, protein, fats, and ash) can be interesting for calculating the efficiency of peptide production.

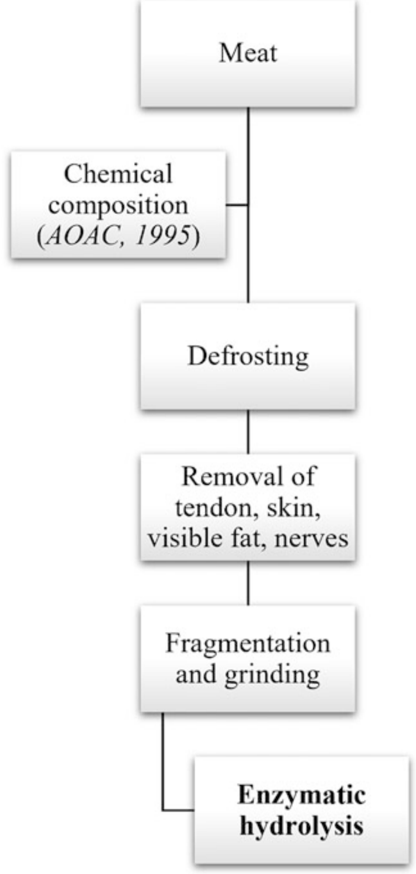

Fig. 1 Diagram of the main steps involved in the sample preparation of meat and meat products

Figure 1 presents a diagram of the main steps involved in the sample preparation of meat and meat products so that the enzymatic hydrolysis and the production of bioactive peptides can occur. The chemical composition of the samples can be determined according to the official procedures described by AOAC (1995) [14].

Meat by-products like trimmings and blood can also be used as matrixes for bioactive peptide obtention; in this case, they need to be selected from the rest of the meat and go through the other sample pretreatment steps (homogenization, fragmentation, grinding). Trimmings are the remaining portions of meat after the preparation of the primal cuts and include meat, gristles, fat, and bones; they can be homogenized and submitted to enzymatic hydrolysis or be used as a source of collagen, the main protein found in cartilages, bones, and skin. Collagen, in turn, can also undergo hydrolysis to produce bioactive peptides. The plasma fraction of meat blood, another by-product, can also be used as a matrix for bioactive peptide production [15].

2.2 Production of Bioactive Peptides from Meat

An enzymatic hydrolysis procedure (like the one described in Subheading 3.1.2) will require:

1. The commercial enzyme, with known provenance (supplier) and classification (animal, plant, or microbial origin), as well as with known activity value and optimum pH, preferably.
2. Ultrapure water as the medium of reaction.
3. A 2 mol L^{-1} NaOH solution for pH adjustment (*see* **Note 1**); in the case of the procedure described in Subheading 3.1.2, the optimum pH of subtilisin is 7.5, which justifies the use of NaOH for pH adjustment. Different enzymes will have different optimum hydrolysis pH, which may lead to the need for buffers or other acidic/basic solutions.
4. A shaker (or similar equipment) with speed and temperature control.
5. A centrifuge for the separation of the supernatant (containing the peptides of interest) after the necessary hydrolysis time.
6. Molecular weight cutoff (MWCO) membranes (*see* **Notes 2** and **3**) for the ultrafiltration of the hydrolysate.
7. A lyophilizer and a − 20 °C freezer for peptide recovery and storage.

2.3 Mass Spectrometry (MS) Analysis

All solutions must be prepared using ultrapure water and MS-grade reagents.

1. Prepare mobile phases A and B by diluting 0.1% trifluoroacetic acid (TFA) in water and acetonitrile (ACN), respectively.
2. Prepare a saturated matrix solution by resuspending 2 mg of α-cyano-4-hydroxycinnamic acid (CHCA) in 200 μL ACN/-water containing 0.1%TFA (50:50, v/v), and vortex for 10 min.
3. Prepare an angiotensin I 1 mM stock solution by dissolving 1.3 mg in 1 mL water. Dilute 5 μL of this solution in 995 μL water to prepare a 5 μM solution, and store at −80 °C until use.

3 Methods

3.1 Production of Bioactive Peptides from Meat

3.1.1 Autolysis

Autolysis is one of the main mechanisms for producing bioactive peptides from meat and meat by-products and consists of the action of endogenous enzymes (mainly calpains and cathepsins) during meat postmortem aging [16]. These enzymes will cleavage meat proteins, releasing free amino acids and peptides. The enzymatic activity (and, consequently, the efficiency of autolysis and the number of bioactive peptides produced) is affected by the physiological conditions of the meat during storage, like pH and temperature

[17]. The main advantage of this method is the zero cost of the enzymes (since they are endogenous), which makes it simple and [18].

3.1.2 Enzymatic Hydrolysis

The most common mechanism to produce bioactive peptides is enzymatic hydrolysis, which simulates the action of digestive enzymes from the gastrointestinal tract by using different commercial exogenous proteinases, like pepsin and trypsin; other exogenous enzymes can also be used, like the ones of plant origin (like papain and bromelain) and microbial sources (like collagenase, subtilisin, corolase, etc.) [19].

In general, the enzymatic hydrolysis is carried out in reactors with controlled temperature for hours; when the aimed degree of hydrolysis is reached, the action of the enzymes is stopped, and the product is fractionated and purified by filtration and/or chromatography. Factors like pH and temperature need to be strictly controlled during hydrolysis, as well as the process duration, which will influence the size, composition, and bioactivity of the bioactive peptides. The number of peptides produced from enzymatic hydrolysis is usually large since it is an intense process [15].

The use of immobilized enzymes and ultrafiltration membranes can overcome some challenges faced during the enzymatic hydrolysis process, like the low yields and the production of secondary metabolites due to the enzymatic activity [20]. The enzyme immobilization is carried out in two phases, one containing the enzyme and the other one containing the matrix; this method facilitates the separation of the enzyme and the matrix and allows the enzyme to be reused, which is economically advantageous due to the high cost of enzymes [20]. The ultrafiltration membranes, in turn, allow the passage of only small and hydrolyzed particles, while the larger ones (polypeptides, enzymes, non-hydrolyzed fraction) are recycled back to the hydrolysis tank; this has become the most efficient process for obtaining bioactive peptides [20, 21]. Recent research is also focusing on improving the hydrolysis efficiency and the release of bioactive peptides by using pretreatment methods such as ultrasound, microwave, high-voltage pulsed electric field, and high hydrostatic pressure, among others [18].

A general enzymatic hydrolysis protocol for meat and meat products is described next, adapted from Cunha et al. (2021) [22]. The parameters presented below (pH, sample/water ratio, enzyme concentration, time, and temperature of hydrolysis) were optimized for subtilisin (a protease of microbial origin) during the enzymatic hydrolysis of mussel meat and are just an example of work conditions. Each enzyme will have optimum work conditions which can vary according to the hydrolysis matrix, and that can be assessed with the use of chemometric tools, like Box–Behnken designs and central composite designs.

1. Mix the meat sample (prepared according to the details described in Subheading 2.1) and ultrapure water at the ratio of 1:2 (w/w).
2. Adjust the pH of the mixture to 7.5 with 2 mol L^{-1} NaOH.
3. Add the enzyme (subtilisin) at a concentration of 1.5% (w/w).
4. Incubate the mixture at 52 °C in an orbital shaker (200 rpm) for 3 h (*see* **Note 1**).
5. After 3 h, incubate the mixture at 90 °C for 10 min to inactivate the enzyme and stop the reaction (*see* **Note 4**).
6. Centrifuge the sample at 5000 × *g* for 30 min, and collect the supernatant.
7. Ultrafiltrate the supernatant using molecular weight cutoff (MWCO) membranes (*see* **Notes 2** and **3**).
8. Lyophilize the hydrolysate, and store it at −20 °C until further characterization analyses.

3.1.3 Other Mechanisms

Cooking and freezing processes can also affect the generation and availability of bioactive peptides in meat and meat products; ice formation and variations in pH and temperature during freezing may lead to chemical and physical stress, which can cause protein denaturation and, consequently, peptide formation. During cooking, on the other hand, heat can change the native conformation of the proteins and break their intramolecular forces [19, 23]. Curing, fermentation, and ripening processes in meat products, like dry-cured hams and fermented sausages, also lead to the formation of peptides and free amino acids [24].

3.2 Characterization of Bioactive Peptides from Meat

The main goal of peptidomics is to identify as many peptides as possible, and the most widely used approach for this purpose is based on MS techniques [25]. The workflow involves a “top-down” approach in which the most difficult aspect is to deal with highly complex samples because of an unspecific hydrolysis process [26]. In this regard, a frequent strategy to reduce sample complexity is to submit the sample to one or a combination of different separation techniques [27, 28]. The most used methodologies are based on tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), considering that more than half of already described bioactive peptides are comprised of peptides smaller than six residues length; however, chromatography techniques are more appropriate, especially for biopeptides [25, 29].

Given the huge sort of meat and meat products matrices to be analyzed, it is impossible to point to a single standard methodology for biopeptides analysis. Herein, based on literature, we describe MS-based methods which have unequivocally proven their ability in peptide profiling with minimal preparative steps aiming at the characterization of bioactive peptides regarding their identification and quantitation [27, 30].

3.2.1 Sampling

Sample preparation step is common for all of the three identification MS-based techniques described in this protocol (MALDI-TOF-MS; LC-ESI-MS; nLC-ESI-MS).

1. Resuspend lyophilized hydrolysates samples in water containing 0.1% TFA, and submit to homogenization by stirring at room temperature.
2. Centrifuge the homogenate at 10,000 rpm for 10 min at 8 °C.
3. Filter the supernatant solution containing peptide extract through a 0.2 μm cellulose acetate membrane filter.
4. Clean up and concentrate an aliquot of 200 μL of each sample extract using ZipTip C18 tips according to the manufacturer's guidelines.
5. Store at −80 °C until analysis.

3.2.2 MALDI-TOF-MS Peptide Profile and Sequencing

Compared to other MS-based techniques, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) is easier to handle, affording short data acquisition (seconds), requiring minimal sample and solvent amounts (microliter), thus comprising a cheaper analysis. Especially for meat and derivatives products, this technique suits as a very convenient methodology due to its higher salt contamination tolerance, proven to be relatively versatile for analyzing peptides in biological samples [30].

As a "soft" ionization technique, MALDI-TOF yields essentially nonfragmented ions allowing analyte identification even in complex mixtures, such as peptide extracts. Additionally, direct MALDI analysis of peptide extract enables an overview of predominant peptides allowing straightforward monitoring on changes in the peptide profile in different samples, requiring no deconvolution step due to the major monocharged ion production [31].

In this context, herein we describe a strategy considering the high throughput and reliable identification of MALDI-TOF/TOF as a fast-screening method for bioactive peptide characterization.

MALDI-TOF-MS Acquisition

1. Mix an aliquot of each sample to CHCA matrix solution 1:1 (v/v), directly apply 1 μL of this mixture onto MALDI target in triplicate, and allow to dry at room temperature before analysis (*see* **Note 5**).
2. Perform the acquisition of full-scan spectra (MS spectra) in positive ion reflector mode, recording m/z signals between 700 and 4000 Da. Accumulate ion signals from 5000 consecutive laser shots (2 kHz) to acquire MS spectra (*see* **Note 6**).

3. To perform method calibration on plate model and acquisition method, a peptide standard mixture should be used as an external calibration.
4. Once the MS spectrum is calibrated, select the top 20 (or more) abundant ions to undergo fragmentation analysis by LIFT-TOF/TOF-MS/MS.
5. Acquire MS/MS spectra of the selected ions with an average of 1000 laser shots.

Identification Analysis To elucidate the specific peptide sequences, the current mainstream sequencing method is to match the obtained MS/MS spectra with theorical existing databases via integrated commercially available algorithms [30, 32, 33].

6. Load MS/MS spectra raw data on Mascot (Matrix Science, Boston, MA, USA) search engine, perform searching against Swiss-Prot database including the subsets *Sus scrofa* for pork, *Bos taurus* for cattle, *Gallus gallus* for chicken, or Teleostei for fish [34, 35].
7. The following parameters must be considered: enzyme, none; number of missed cleavages allowed, none; peptide mass tolerance, 20 ppm; fragment mass tolerance, 0.5 Da; variable and fixed modifications, oxidation of methionine residues and carbamidomethyl of cysteine, respectively (*see* **Notes 7, 8,** and **9**).
8. As a result, only peptides with significant hits must be considered as defined by Mascot probability analysis ($p < 0.05$) of fish [34, 35].
9. Completely unknown peptides can be identified through de novo peptide sequencing approach. The assignment is based on manual inspection of MS/MS spectra to directly analyze the amino acid sequence. This analysis requires very-well trained personnel, but the description of this procedure is beyond the scope of this protocol [36].
10. The identified peptides can be investigated regarding their bioactivity based on previously reported in literature using specific databases such as BIOPEP, BioPep DB (BioPep Database), and AHTPDB (antihypertensive peptide database) [29, 37].

MALDI-TOF-MS Quantitation MALDI-TOF-MS limitations on achieving absolute quantitation must be carefully considered [38]. Nevertheless, to minimize signal reproducibility and subsequent poor accuracy and reliability of quantitative outcomes, strategies aiming relative quantitation using a standard peptide are highly recommended. Thus, by adding a reference peptide, such as angiotensin I, in constant quantities in the samples, the relative

intensity for each target peptide is calculated from its area compared with the added standard [39].

11. Mix an equal volume of 5 μM aqueous angiotensin I solution to peptide sample prior to cleanup step using ZipTip C18 tips (*see* Subheading 3.2.1, **step 4**).
12. Set the intensity of angiotensin I as 100%, and compare it to intensities of the endogenous peptides in different samples in order to identify a suitable internal standard with constant intensity (*see* **Note 10**).
13. Set the selected peptide intensity as 100%, and use it as an internal standard for all further quantitative analysis.

3.2.3 LC-MS/MS

Since the measurement of bioactive peptides in complex samples can present challenges, the coupling of liquid chromatography (LC) and mass spectrometry (MS) offers a valuable solution. Through LC, compounds can be isolated based on their physical–chemical properties, while MS can provide information on their fragmentation profiles. For the fragmentation, electrospray ionization (ESI) has become a prevalent technique for peptides and polypeptides, due to its ability to generate multiple charges. Utilizing tandem MS analysis further enhances the reliability of results and enables peptide quantification [30, 40].

As an untargeted study, the use of full-scan (FS) analysis is appropriate to determine the sample profile, to examine the entire peptide composition or find new peptides in a complicated sample. The use of tandem mode as multiple reaction monitoring (MRM) is referred to quantify specific peptide targets accurately and selectively. This mass spectrometry technique is based on selecting a peptide precursor ion and one or more characteristic fragment ions, to verify the species specificity of the corresponding peptides. Triple quadrupole (QqQ) mass analyzer is the most used detector in this technique [40, 41].

The utilization of nanoflow liquid chromatography coupled with tandem mass spectrometry (nLC-MS/MS) offers enhanced selectivity and sensitivity, thereby improving detection limits [40]. This technique provides notable advantages through its rapid separation capabilities and environmentally friendly solvent consumption, with a flow rate typically around 300 nL min^{-1}. Consequently, nLC-MS/MS has emerged as the primary method for determining bioactive peptides. Furthermore, when combined with high-resolution analyzers such as Orbitrap, it enables even more precise identification and characterization of the analytes [42].

LC-ESI-QqQ Acquisition

1. In a LC-MS/MS with triple quadrupole (QqQ) as analyzer,

couple a C18 column (150 mm × 2.1 mm, 2 μm). Models as Hypersil Gold (from Thermo Fisher Scientific) and Zorbax (from Agilent) are more robust in peptide analysis. Apply the temperature of 40 °C to improve the resolution of spectra (*see* **Note 11**).

2. Prepare mobile phases A and B, and inject 5 uL of the samples using the autosampler after the purge lines. Use flow of 300 uL min^{-1} and gradient from 5 to 95% B in 0–60 min; 95–5%B in 0.1 min; and an isocratic of 5% B for 4.9 min for reconditioning of column. The elution of bioactive peptides is in the range of 20 ~ 40%B.
3. Analyses should be done on positive ion mode. Use initial parameters on tune of spray voltage, 3500 V; capillary temperature, 250 °C; gas flow, 8 L min^{-1}; nebulizer in 30 psi; sheath gas heater, 250 °C; sheath gas flow, 11 L min^{-1}; and 3 micro scans and scan time of 0.675 s [43] (*see* **Note 12**).
4. Use the range of spectrum of 200–1500 m/z for a FS for untargeted measures and general profile.
5. Use the MRM mode for analyses of the target type. Apply 20 eV as energy collision (CE) and resolution for the first and third quadrupoles at 0.7 Da. In accordance with the literature, choose the precursor and product ions for quantifying the bioactive peptide of interest [43]. For better results, add a second transition, which will be used for the fragmentation confirmation.

nLC-ESI-LTQ-Orbitrap Acquisition

1. Couple a C18 column as PepMap10 (75 mm × 5 cm, 2 μm) into a nLC-LTQ (linear trap quadrupole). The system should be connected into a nano-ESI. Use a 75 μm diameter PicoFrit with a reverse phase for better resolution.
2. Inject 2 uL using the autosampler after the purge lines. Flow rate at 300 nL min^{-1} with a linear gradient of 5–95%B for 60 min, 95–5%B for 0.1 min, and an isocratic of 5% B for 4.9 min for reconditioning (*see* **Note 11**).
3. Apply a tune positive ion mode on the nano-ESI, with settings of spray voltage at 2800 V, capillary temperature at 180 °C, capillary voltage at 24 V, and tube lens offset at 700 V. Use sheath gas, ion sweet gas, and auxiliary gas flow rate at 0 [42].
4. Acquire the FS from m/z 200–1500 and automatic gain control target in 5×10^5 with resolution (FWHM) of 60,000 and width of 1 m/z [42].
5. For the tandem analyses, select the top 10 most intense (or with intensity higher than 1×10^4) peaks with +2 or + 3 of charge, and fragment them in LTQ with 35% of CID and 2500 V of spray voltage.

Identification and Quantitation Relatives Proteome Discoverer (Thermo Fisher Scientifics) is a powerful software tool that uses LC-MS/MS data for robust identification and relative abundance of peptides in complex samples. Programs such as MaxQuant are also usually available. Below is a basic workflow for Proteome Discoverer:

1. Import the data (. raw format) into Proteome Discoverer with preprocessing already. The initial identification can be performed in Sequest HT, with database Swiss-Prot, from UniProt. Use the entire UniProt species from animals' meats of interest.
2. Use tolerance of precursor ±10 ppm and for fragment to 0.6 ± Da, high; length, 2–20; mass precision, 2 ppm; coverage, 50%; and intensity ionic greater than 1×10^5 [42]. Apply the same enzymatic and PTMS conditions of topic 7 from MALDI-TOF identification.
3. Use the percolator tool to filter false MS2 notations. Apply a strict false discovery rate (FDR) of 0.01 and a relaxed rate of 0.05. Set validation based on q-value.
4. On the consensus work, in "Peptide and protein quantification," choose intensity threshold 1, S/N threshold 5. Ideally co-isolation can be set to 30%.
5. For better accuracy and quantification, data normalization is usually applied. Go to "Peptide and protein quantification," and use "Total peptide amount" or "specific protein amount," according to your results [44] (*see* **Note 13**).

Absolute Quantitation A calibration curve can be used to determine the range where there is linearity between the obtained signal and the sample concentration. And so, the concentration of the sample can be inferred by the application of the first-degree equation.

1. Inject in triplicate the synthesized or isolated peptide of interest in different (known) concentrations. Apply the same method that will be applied to the analyte of interest.
2. Analyze the normality of the data and the residuals. Apply a linear fit in points; use this obtained equation to quantify the samples, if $r^2 > 0.99$ [45].
3. Using the peptide in its isotopic form is another preferred method of quantification.
4. The addition of the labeled peptide is at the beginning of the extraction and quantified by the standard addition method [40].

5. In the absence of internal standards equal to the analysis, the use of molecules with a similar profile, such as angiotensin I, can be used for a relative quantification in the MRM mode, applying the same methodology quantification on MALDI-TOF/MS [30].

4 Notes

1. The pH of the mixture (meat and ultrapure water) during enzymatic hydrolysis may vary during the hydrolysis time; check and adjust the pH to 7.5 (initial pH of the hydrolysis) with 2 mol L^{-1} NaOH every 15 min to ensure that there are no pH variations.
2. Ultrafiltration can be a step for purifying the hydrolysate after conventional enzymatic hydrolysis; a previous filtration step with 0.2 μm membranes can facilitate ultrafiltration, eliminating secondary metabolites and non-hydrolyzed substances from the sample.
3. The size of the MWCO ultrafiltration membrane pores (3–14 nm) can be chosen according to the desired molecular weight of the bioactive peptides (3–100 kDa); different membranes can be combined, and the fractions can be separated according to their weight.
4. The step of stopping enzymatic activity by increasing the temperature of the reaction medium is crucial and must be performed as soon as the hydrolysis time is over; different temperatures and times can be used, and the higher the temperature (e.g., if the temperature is so high that the sample boils), the lesser the time it takes to inactivate the enzyme and stop hydrolysis.
5. Different runs can occasionally identify different peptide fragments, so to reduce missing peptides, it is advisable to perform a triplicate analysis and use data from all of the three experiments for identification purposes.
6. Coupling MALDI-TOF to separation techniques such as liquid chromatography (SEC or RP-HPLC) to reduce sample complexity and improve ionization sensibility to subsequent mass detection enhances the performance in identification.
7. The search parameter "enzyme" refers to the enzyme used for sample digestion; thus, if the sample is a product of an unspecific autolysis process, fill as "none"; otherwise, fill with the employed enzyme.
8. The number of missing cleavages allowed depends on the enzyme employed in sample digestion.

9. A previous parameter optimization regarding mass tolerance is recommended using a standard peptide extract such as BSA. Good results can be obtained if all parameters are well adjusted.
10. Variation on intensity signals must be statistically tested.
11. The flow and gradient conditions are only suggestions; before applying it, consult the manual for the maximum allowable column pressure used.
12. It is highly recommended to perform tune for parameter optimization.
13. If your data show that the bulk of peptides are relatively constant among samples while just a tiny part of peptides changes across samples, use the total peptide quantity.

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Index

A

Abnormal coloring 8
Absorbance 86, 100, 129, 130, 133, 171, 177
Acceptability of reduced-sodium foods 141
Acceptable daily intake (ADI) 82
Acetic acid 41, 46, 102
Acidic conditions 42, 171
Acidification 2, 42, 96
Active biocompounds 91
Additive 4, 7–8, 34, 39, 48, 58–60, 70, 73, 111–122, 125, 135, 138, 140, 182
Adverse health effects 112
Agar diffusion test (ADT) 127, 130–132
Agar streaking 88
Agar-well 130
Agitation 20, 74, 78, 122, 160, 176, 186
Air cavities 8
Air drying chamber 4, 6
Air flow rate 118, 119
Aldehyde 46, 140, 170, 173
Algae 64, 82
Alginates 14, 15, 17, 20, 21, 58, 59, 77, 82
Alkaline hydrolyse 155
Alterations 112, 169
Alterations in color 112
Alterations in texture 112
American law 71
Amino acids 25, 39, 44–46, 169, 170, 187, 190, 193, 194, 196, 198, 200
Analysis 47, 63, 85–87, 95–107, 133, 138, 146, 155, 159, 160, 162, 164, 172, 173, 175, 198
Analytes 97, 155, 163, 199, 201, 203
Analytical instrumental approaches 152
Analytical steps 155
Anhydrous sodium sulphate 159, 161
Animal fat 29, 33, 59, 60, 63–65, 70, 71, 76, 115
Animal fat replacers 76
Animal origin 121
Annatto dye 70, 76, 79
Antimicrobial 2, 4, 44, 73, 79, 112–122, 125–135, 193
Antimicrobial activities 121, 122, 125–135, 193
Antimicrobial ingredients 127
Antioxidant 4, 7, 41, 43, 44, 48, 73, 79, 81–83, 111–122, 170, 186, 193, 194
Antioxidant capacities 44, 112
Applicable voltage 122
Argentometric method 138
Aroma 4, 8, 32, 46, 47, 140, 170
Artifacts 155, 160, 162
Artificial cases 69
Artificial casings 4, 8, 34, 49
Ash 138, 143–145, 194
Atmospheric Pressure Chemical Ionization (APCI) 159, 160, 162
Atomization 118
Atomization air flow rate 118, 119
Autoclave 102–104, 127

B

Back fat (lard) 17, 30, 83
Bacon 140, 152
Bacterial stock 128
Bacteriocin-producing microorganisms 126
Bacteriocins 21, 41, 44, 126–128, 131, 133
Bacteriological analytical manual 87
Balance 44, 114, 116, 127, 138, 142, 145
Bamboo 82, 83, 91
Barley 82, 83
Barley malt pomace 83
Batch treatment chamber 115, 117
Batters 15, 35, 74–76, 79, 130, 132–135
B-complex 25, 95–107
Beef 6, 17, 20, 29, 41, 48, 69, 71, 101–104, 115, 119, 121, 140, 145, 152, 156, 157, 174, 184, 187–189
Beef jerky 140
Bees wax 71, 78

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Bifidobacteria....14, 45
Bioactive compounds....44–46
Bioactive peptides....44, 193–204
Bioassays....96
Biopreservation....126
Biosafety....127, 132
Black pepper....64, 69, 73, 115, 119, 120
Blanching....17, 19
Blueberries....83, 126
Bologna sausages....28, 45, 71
Bologna-type sausages....140
Brain....17, 19, 20, 43
Brain heart infusion (BHI)....87, 128
Brain heart infusion agar....87, 128
Brazilian law....71
Brick-red silver chromate precipitates....138, 146, 147
Bunsen burner....127, 142, 143, 145
Burger....59, 61, 63, 64, 116, 174
Burger maker machine....116, 120
Butylated hydroxyanisole (BHA)....112
Butylated hydroxytoluene (BHT)....112

C

Calcium chloride ($CaCl_2$)....138
Calibration curve....86, 162, 163, 178, 203
Caliper....131
Candelilla wax....71, 73
Canola oils....71
Capsules....15, 19–21
Capsule sizes....15, 21
Carbonization....143, 145, 146
Carbonylation....170, 173, 174
Carbonyl compounds....112, 170, 172, 173
Carcinogenic....112, 152
Carnauba wax....71
Casing....8, 48, 49, 76, 77
Cell concentrations....133
Cell membrane....121
Cell pores....113, 121, 186, 188, 190, 191
Cellulose....29, 58, 70, 73, 76, 82, 90, 186, 199
Cellulose derivatives....70
Centrifugation....19, 133
Centrifugation speed....133
Centrifuge....103, 115, 127–130, 133, 176, 186, 188, 189, 196, 198, 199
Cereal fiber....90, 98
CFU/g....21, 32, 129, 132, 133
Challenge tests....126, 127
Chemical changes....169
Chicken....48, 69, 71, 101–104, 115, 121, 139, 174, 183, 200
Cholesterol....14, 39, 41, 42, 45, 58, 112, 151–163, 182
Cholesterol oxidation....152, 162
Cholesterol oxidation product (COP)....152, 155, 156, 162
Cholesterol oxides....112, 151–162, 164
Chopped....7, 19, 34, 119
Chopping....3, 4, 6–7, 31, 50, 73, 74
Chroma....84
Chromatography....96–98, 100, 107, 152, 155, 158, 159, 197, 198, 201, 204
Cleaning....65, 99, 181
Clean-up....199
Clostridium perfringens....87
Coagulase-positive staphylococci....87
Coating wall....118
Cocultures....129, 133
Colonies....87, 88, 128, 133–135
Color....32, 34, 41, 48, 49, 62, 64, 65, 71, 73, 76, 78, 79, 83, 84, 90, 139, 140, 143, 170, 174, 185
Color changes....9, 140, 174
Colorimetry....85, 97, 171
Column....99, 100, 103–105, 156–158, 160, 162, 202, 205
Commercial mixes....4–7
Comminuted products....69
Comminution....74, 75, 78
Common salt (NaCl)....115
Compaction....4
Conductivity....122
Conservation chamber....6, 9
Consumer demand....152
Consumption of solvents....155, 160
Cooked ham....139, 156
Cooked meat products....15, 16
Cooking....16, 17, 20, 61, 73, 76, 79, 84, 139, 140, 174, 198
Cooking loss....76, 139, 140
Cooling....20, 70, 76, 145
Cork borer....130
Coronary heart disease....91
Cross-linking....170
Crucible....142–147
Culture media....96, 128, 133
Cured meats....60, 125, 126
Curing salts....129
Curing stage....9

Curling tongs ... 147
Cutter ... 20, 30, 32, 34, 69, 74, 83, 84, 186
Cutting ... 65, 74, 114, 117

D

Darkness ... 9, 84
Degradation ... 41, 105, 111, 140, 160, 173, 183, 190
De Man, Rogosa and Sharpe agar ... 128, 133
De Man, Rogosa and Sharpe broth ... 128–131, 133
Derivatization ... 97, 100, 104, 155, 172, 173
Desiccator ... 142–145
Detailed Mohr's protocol ... 138
Detection ... 96, 98, 100, 101, 107, 159, 162, 170–173, 201, 204
Deterioration ... 65, 106, 112, 174
Deterioration processes ... 2, 112
Dextrose ... 4, 7, 48, 132
Dietary cholesterol ... 152
Dietary fibers ... 43, 57–65, 82, 90, 91
Dietary salt intake ... 137
Dinitrophenylhydrazine (DNPH) ... 169–178
Dinitrophenylhydrazone (DNP) ... 171, 178
Direct dispersion ... 70, 74, 78
Direct saponification ... 156, 157, 159–161
Discoloration ... 7, 8, 134, 135, 170
Dissolution ... 74, 78, 142, 143, 145, 171, 174, 177
Distilled water ... 19, 86, 101–103, 105, 114, 117, 128, 135, 142–145, 156–160
DNA disruptors ... 112
Dough ... 2, 4, 6–9, 27, 30–32, 34, 35, 84, 119, 120
Drigalski rod ... 135
Drip loss ... 170
Dry fermented sausages ... 28, 41, 42, 47, 49, 125–135, 157
Drying ... 4, 6–9, 48, 49, 84, 113, 118, 119, 122, 161, 184
Drying processes ... 4
Dry particles ... 118
Dry-ripened sausage ... 59
Dyeing ... 70, 76

E

Eating habits ... 141
Economic losses ... 112
Electrodes ... 84, 117, 134, 138
Electroporation ... 121
Emerging non-thermal technologies ... 139
Emerging technologies ... 114
Emulsified meat product ... 14, 16, 20, 70, 72–74, 76, 78, 79, 81–91
Emulsified products ... 16, 73, 83–88
Emulsifier ... 35, 69, 74
Emulsifying capacity ... 170
Emulsion ... 14, 28, 59, 63, 69–76, 78, 84, 140, 159, 161
Encapsulation ... 14–17, 20, 21, 118, 119
Encapsulation agent ... 119
Enumeration ... 128, 133, 135
Environmentally friendly methodologies ... 155, 162
Equipment ... 4, 6, 17, 27, 65, 74–78, 113, 114, 116, 118, 122, 127, 138, 142, 173, 185, 196
Escherichia coli ... 16, 41, 87
External standardization ... 159, 164
Extraction ... 43, 84, 96–99, 101–105, 107, 113, 117, 118, 121, 155–158, 160–162, 183, 203
Extraction solvent ... 121, 160
Extrusion ... 14

F

Fat ... 3, 19, 27, 39, 58, 69, 81, 106, 111, 132, 140, 151, 182, 194
Fat hydrolysis ... 9
Fat-reduced foods ... 90
Fat replacers ... 29, 58, 59, 63, 90
Fat-soluble ... 97, 98
Fatty acid ... 14, 16, 26, 39, 43, 45, 47, 59, 63, 85, 152, 155, 182, 185, 187
Feed solution temperature ... 118, 119
Fermentation ... 2, 4, 8–10, 31, 32, 39–41, 44–46, 48, 49, 59, 61, 62, 126, 129, 130, 133, 198
Fermented sausage ... 3, 4, 6, 8, 9, 27, 28, 30–32, 35, 40, 42, 43, 45–50, 59, 61, 90, 91, 125–135, 156, 198
Fiber ... 26, 28, 40, 43, 57–65, 81–91, 152
Filter ... 19, 84, 103–105, 115, 118, 122, 130, 133, 142, 144, 160, 161, 187, 189, 199, 203
Filtration ... 107, 184, 185, 190, 191, 197, 204
Fish ... 140, 141, 189, 193, 200
Flavin adenine dinucleotide (FAD) ... 99
Flavin mononucleotide (FMN) ... 99
Flavor ... 3, 4, 27, 32, 39–43, 46–50, 69, 71, 73, 88, 138–141, 170
Flavor enhancers ... 3, 138, 139
Flour ... 15, 40, 43, 83
Fluidity ... 121
Fluorescence detector ... 100, 103–105
Fluorimetry ... 96, 97, 100

Food analysis 97, 106, 152
Food composition 145
Food loss 106
Food pathogen control 126
Food pathogens 126
Food quality 173
Food safety 2, 112, 126
Foods of animal origin 98, 99, 151
Food spoilage 112
Formation of pores 121
Formulation 3, 16, 26–30, 33, 42, 48, 59, 63, 64, 70, 73, 81, 83, 84, 90, 91, 119, 132
Frankfurter 69–72, 76, 77, 152, 174
Free probiotics 20, 21
Free radicals 44, 111, 112, 152, 169
Free riboflavin 99
Freezing 184, 198
Frequency 117, 118, 122
Fruits 15, 43, 82, 83, 90, 91, 112, 117, 174
Fuets 2–10, 40
Functional food 90, 191
Functional ingredients 15, 57, 81, 82, 90, 187
Functions 16, 40–45, 50, 73, 78, 95, 112, 151, 170

G

Gamma rays 139
Garlic 3, 26, 27, 48, 69, 73, 83
Gas chromatography (GC) 155
Gel 28, 31, 34, 35, 59–65, 70, 73, 74, 90, 141
Gelation 170
Gloves 147
Glycation 170
Gram positive bacteria 128
Gram-positive catalase-positive cocci (GCC+) 127
Grayish ashes 145, 146
Green technologies 113
Grinding 4, 31, 74, 78, 195
Ground allspice 17
Ground black pepper 17
Ground carnation 17
Ground cinnamon 17
Ground nutmeg 17
Gut health 16

H

Hamburgers 115, 116, 119–122, 156
Healthier ingredients 57
Healthier meat products 26
Health implications 151
Heat 14–17, 20, 21, 40, 59, 73, 76, 84, 105, 113, 116, 152, 172, 178, 184, 198
Heater 20, 127, 202
Heat treatment 15–17, 20, 40, 59, 73, 76
Hedonic test 88
Heme pigments 111, 152
High added value 114
High hydrostatic pressure (HPP) 113, 139, 197
High-intensity ultrasound (HIU) 139–141
High-performance liquid chromatography (HPLC) 96–105, 107, 151–164, 172
Hot dog wieners 140
Human health 42, 45, 57, 58, 112, 113, 191
Humidity 4, 9, 46, 48, 105
Hydrazone derivatives 171
Hydrolysis 9, 42, 44, 45, 98, 99, 102–104, 107, 134, 173, 186, 194–198, 204
Hydroperoxides 85, 112
4-Hydroxynonenal 112

I

Ice 27, 29, 32–34, 58, 73, 74, 83, 84, 103, 105, 186, 198
Identification 98, 106, 153, 159, 162, 198–201, 203, 204
Immune system 16, 26, 41
Immunological techniques 170
Incineration 145
Incubation 87, 88, 129, 133, 171
Incubators 127
Inflammation 16
Ingredient 3, 4, 8, 10, 27–32, 34, 48, 57–61, 63, 65, 71, 73, 74, 81–84, 90, 91, 112–115, 119–121, 126, 127, 135, 174, 194
Ingredient cost 84, 91
Inhibition 27, 127, 128, 130–133, 135
Inhibition halos 131–133
Injection volumes 103–105
Inlet temperature 118, 119
Inoculating loop 128
Inoculum 129, 130, 132, 133, 135
Inulin 26–31, 33–35, 40, 43–45, 47, 90, 91, 121
In vitro analysis 125–135
Irradiation 139

K

Ketones 46, 170, 173
Kneading machine 132

L

Lactic acid 1, 9, 13, 14, 21, 40–44, 47–49, 126, 133
Lactic acid bacteria (LAB) 2, 4, 13–15, 32, 40, 43–45, 47, 48, 96, 125–135
Lacticaseibacillus rhamnosus 4, 5, 42, 43, 45–47

Lactose 41, 43, 60, 88, 132, 170
Laminar flow cabinet 127
Lean beef meat 115, 119
Lean pork........27, 83, 132
Lecithin........70, 71, 78
Legislation 60, 71, 73
Light 9, 90, 104, 105, 107, 118, 119, 122, 152, 160, 178
Lightness........84
Lipid........27, 41–43, 46, 71, 72, 74, 79, 83, 84, 111, 139, 140, 151, 152, 155, 156, 160, 170, 172, 173
Lipid extraction 155–158, 160
Lipid oxidation 27, 42, 79, 83, 139, 140, 152, 172, 174
Lipid peroxidation........170
Lipid profile 71, 72
Liquid feed pump rate 118, 119
Listeria monocytogenes........42, 125–128, 131, 133
Listeria selective supplement 128
Liver 16–20, 29, 185–187
Lyophilized powder 7

M

M/z........159, 162, 199, 202
Maceration........116, 119
Magnesium chloride ($MgCl_2$) 138
Magnetic stirrer........103, 105, 114, 117, 127, 138, 142
Malonaldehyde 112
Malondialdehyde (MDA) 85, 86, 170
Maltodextrin........114, 118, 119, 121, 122
Mannitol Salt Agar (MSA) 128, 133
Mass spectrometry (MS)........97, 98, 155, 158, 159, 170, 172, 196, 198–201
Mass transfer........121, 141
Matrix 4, 9, 14, 40, 45, 50, 73, 91, 97, 98, 101, 113, 115, 118, 162, 195–197, 199
Maximum outlet temperature 118, 119
Meat 1, 13, 25, 39, 69, 81, 112, 125, 137, 152, 169, 181, 193
Meat curing 9, 44, 65, 71, 73, 125, 129, 198
Meat emulsions 69–71, 73–76, 78, 91, 140, 158
Meat industry 44, 76, 111–114, 117, 118, 125, 181, 190
Meat ingredients 3–5, 8, 26, 27, 30–32, 48, 59–61, 112, 126, 127, 187
Meat microbiota........14, 40, 127
Meat models........126, 128, 129, 132
Meat preservation 32, 61, 105, 139, 172
Meat processing equipment 133
Meat product........1, 13, 25, 39, 58, 70, 82, 96, 112, 126, 137, 152, 169, 187, 194
Mechanically separated poultry meat (MSPM) 71, 72, 74
Mediterranean style sausage 129, 132, 133
Merma........9
Metabolism........14, 43
Metal catalysts 170
Methods........4–9, 14, 30, 48, 60–63, 70, 84, 96, 130, 138, 155, 170, 182, 194
Microbial development........65
Microbial growth 39, 106
Microbiological analysis........133, 135
Microbiological assay 87, 96
Microbiological changes 139
Microbiological quality........20, 120, 133
Microbiological spoilage........9
Micro centrifuge tube 128
Microencapsulation........113, 117, 118, 121
Microfiltration 184, 187, 188, 190, 191
Micronized salt........140
Micronutrient 95, 137
Microorganisms........2, 4, 7, 8, 10, 13–16, 21, 26, 27, 31, 32, 39–43, 47, 48, 58, 76, 96, 112, 125–127, 129, 130, 133, 135
Microwave 139, 141, 174, 197
Minced........6, 50, 59–61, 115, 119, 152, 176
Minced meat........20, 50, 59, 115, 176
Mincer........4, 7, 116, 119, 132
Mincer machine........116, 119
Mincing machine........4, 6, 116
Mixing........4, 7, 8, 31, 50, 73, 119, 142
Mixing process 7
Mixture of spices 4, 7, 30, 115, 121
Mobile phase 101, 102, 104–107, 158, 160–162, 196, 202
Moisture 8, 9, 27, 32, 71, 82, 84, 106, 194
Moldiness........8
Molds........2, 4, 5, 8, 9, 62, 65
Monocultures 127, 129
Monoglycerides 70, 71, 78
Mortadella 26–28, 32–34, 69, 70, 81–87, 90, 91
Mortadella formulation 27, 82, 83, 90
MRS 128–131, 133
Muffle furnace 138, 142, 143, 145
Myofibrillar proteins 27, 73, 74, 78, 84, 170

N

Nanofiltration........191
Natural additives 113, 116, 118, 119, 122
Natural antimicrobial 114
Natural antioxidant 83, 112–120, 122
Natural matrices 112

Natural origin 112, 113
Negative control 131
Neutralize 111
New technologies 114
Niacin 96–101, 185
Nicotinamide 99
Nicotinic acid 95–107
Nitrate 83
Nitrate and nitrite concentration in mg/kg 129, 132
Nitrate and nitrite regulations 126, 132
Nitrates 4, 9, 39, 41, 48, 59, 60, 112, 125, 126, 129
Nitric acid 142, 143, 145
Nitrite 83, 90, 125–135
Nitrite alternatives 126
Nitrite replacement 125–135
Nitrites 4, 9, 27, 29, 33, 43, 48, 59, 60, 73, 74, 112, 125–127, 129, 132, 135
Nitropigments 9
N-nitroso compounds 126
Nonsaponifiable matter 160, 161
Non-spore-forming pathogens 128
Nourishing 81
Nucleophilic addition 171
Nutraceutical 83
Nutrition 44
Nutritional values 45

O

Oat 28, 29, 82, 90
Off-flavor 112
Off-odor 65, 112
Oleogel 69–79
Oleogelators 70, 71, 78
Olive oil 71
Omega 3 81
Onions sauteed with butter 17
Oregano 115, 119, 120, 174
Organoleptic properties 9
Oven 76, 84, 174
Overestimation of salt content 146
Oxidation 41, 46, 49, 79, 97, 99, 104, 111, 112, 115, 140, 151, 152, 155, 160, 162, 169, 170, 172–175, 177, 200
Oxidation reactions 111, 112
Oxidative damage 172
Oxidative processes 14, 86, 173, 175
Oxidative stress 112, 172
Oxygen tension 126

P

Packaging 20, 48, 49, 70, 76, 100, 107, 122
Partial thawing 132
Particle size 74, 121, 132, 146, 160
Pate 16–20
Pate molding 16
Pathogenic microorganisms 65, 112
Pediococcus 4, 5, 15, 41, 44, 47, 48
Peeling 76, 79
Pellet 129, 130, 176
Penicillium candidum 4, 5, 8
Pepper 3, 27, 48, 59, 60, 69, 73, 115, 119
Peptone water 87, 128, 133, 135
Pernicious substances 112
Petri dishes 87, 128, 130, 132, 133
pH measurement, , ... 130, 133, 135
Photodiode array detector 100, 159, 160, 162
Physalis 83
Physical changes 9, 169
Physicochemical changes 9, 139
Physicochemical parameters 83
Physicochemical quality 139
Phytochemicals 82, 121
Phytosterols 70, 71, 78
Pigment 27, 111
Plant by-products 43, 113
Polyethylene casings 83, 84
Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol agar (PALCAM) 128, 133, 135
Polyphenols 43, 83, 91
Polystyrene film 116
Polystyrene traits 116
Pores 113, 141, 183, 186, 188, 190, 191, 204
Pork 3, 4, 6, 7, 17, 19, 45, 48, 50, 60, 69, 71, 72, 83, 90, 101–104, 115, 119, 121, 132, 156, 157, 174, 184, 187–189, 200
Pork backfat 69, 72, 115, 119, 132
Pork belly 6, 7
Pork fat 4, 71
Pork lean 3, 4, 6, 7
Pork sausage 29, 90
Positive control 131
Positive ion mode 159, 162, 202
Post-fermentation period 9
Potassium chloride (KCl) 102, 138
Potassium chromate 138, 142, 143
Potassium nitrate 4, 60, 129
Potential 2, 3, 15, 20, 28, 41, 42, 47, 70, 83, 90, 101, 112, 117, 118, 122, 127, 146, 169–178, 181, 191, 194
Pour plating 88, 133
Powder 28, 29, 31, 34, 35, 59, 60, 63, 118
Prebiotics 14, 15, 26–28, 30, 31, 33, 34, 40, 42–43, 45, 47, 50
Precipitation titration 138

Preservative 4, 7, 83
Probiotic 2, 13, 27, 40
Probiotic strains 4, 5, 15, 16, 20
Process 1, 14, 30, 40, 57, 70, 84, 96, 112, 130, 155, 169, 183, 194
Processed meat 14, 137, 157
Processed meat products 14, 137, 138
Processing conditions 14, 44, 59, 127
Pro-oxidant 152
Propionic acid bacteria 14
Propyl gallate (PG) 112
Prosella 59–65
Protect 4, 8, 14, 16, 21, 78, 105, 194
Protective atmosphere 120, 122
Protective coatings 14
Protective cultures 125–135
Protein backbone cleavage 169
Protein carbonyl analysis 173
Protein coagulation 8
Protein cross-linkage 169
Protein hydrolysis 44, 173
Proteins 6, 25, 39, 69, 81, 98, 111, 139, 169, 182, 193
Protein source 39, 81, 185, 194
Proximal composition 84
Public health 137, 152
Pulse 71, 117, 122
Pulsed electric field (PEF) 113–115, 117, 118, 121, 122, 139–141, 197
Pulsed electric field generator 114, 115, 117
Pulse width 117, 118, 122
Purification 96–99, 155, 171

Q

Quality 2, 14, 26, 40, 58, 70, 81, 95, 112, 135, 169, 183, 194
Quality control 174
Quality of meat 170
Quantification 98, 99, 159, 162, 163, 171–173, 176, 201, 203, 204

R

Rancidity 9, 15, 111
Raw material 4, 6–7, 16, 19, 50, 59, 65, 71, 74, 113, 114, 117, 121, 133, 191
Raw meat sub-products 19, 20, 183–190
Reactive oxygen species (ROS) 169
Ready-to-eat chicken breasts 139
Ready-to-eat fish products 140
Reagents 97, 99, 106, 142, 159, 173, 174, 176, 177, 196
Redness 9, 84
Reduced-sodium content 140, 141
Reducing microorganisms 9
Reformulations 70
Refractive index (RI) detector 159, 160, 162
Refrigeration temperature 9, 10, 34, 59, 119–121
Regulatory standards 194
Relative humidity (RH) 9, 32, 49, 61, 62
Release 14, 98, 99, 113, 132, 140, 141, 186, 197
Resistance 13
Retention time 106, 159, 162
Riboflavin 95–107, 185
Ripening 7, 9, 31, 32, 44, 49, 61, 62, 65, 198
Ripening period 9, 62
Risk group 2 microorganisms 127
Running times 103–105

S

Safety 13, 14, 16, 21, 44, 48, 112, 120, 121, 126, 139, 141, 169, 172, 174, 194
Safety problems 112
Salami 29, 39, 40, 43, 50, 157, 162, 163
Salchichón 2, 5, 6, 9, 45, 59–62, 65
Saline solution 86, 128, 133, 135
Salmonella/25g 87
Salt 3, 4, 7, 8, 14, 20, 32, 39, 48, 58–61, 69, 73, 74, 83, 84, 119, 137–141, 190, 199
Saltiness perception 139–141
Salt penetration 8
Salt reduction 140, 141
Salt replacers 138
Sample 15, 40, 74, 84, 97, 113, 129, 138, 152, 171, 183, 194
Sample preparation 97, 99, 101, 105, 107, 155, 171–173, 194, 195, 199
Saponifiable matter 161
Saponification 155, 157–160, 162
Saturated fat 58, 69–79
Sausage 3, 14, 26, 39, 59, 78, 83, 127, 139, 152, 198
Sausage tying machine 4, 6
Sauteing 17, 19
Seasoning 3, 27, 29, 33, 34, 59, 60, 73, 83
Selective ion monitoring (SIM) mode 159, 162
Semi-automatic stuffer 5, 6
Sensory analysis 63, 64, 88
Sensory attributes 2, 35, 39, 43, 60
Sensory changes 9
Sensory characteristics 21, 73, 139–141
Sensory properties 32, 35, 41, 59, 61, 64, 169
Sensory quality 9, 112, 139, 140, 170
Sensory studies 89, 91

Serial dilutions 128, 133
Shelf life 1, 14, 16, 21, 30, 39, 49, 76, 111–113, 120, 121, 139
Silver chloride 142, 146, 147
Silver chromate 138, 143, 146, 147
Silver ions 138, 142, 143
Silver nitrate 142
Simultaneous determination 97, 98
Slicing 76, 78
Sodium 70, 73, 74, 105, 137–146
Sodium ascorbate 4, 59
Sodium chloride (NaCl) 17, 27, 29, 30, 33, 59, 60, 73, 104, 115, 126, 129, 132, 133, 137–141, 143–147, 157, 174
Sodium chloride determination 138
Sodium citrate 4
Sodium content 138, 141
Sodium erythorbate 29, 33, 60, 73
Sodium hydroxide (NaOH) 133, 142, 144, 196, 198, 204
Sodium nitrate 129
Sodium phosphate 4
Sodium tripolyphosphate (STPP) 27, 33, 73, 140
Solid-liquid extraction assisted by PEF technology 113, 117
Solid phase extraction (SPE) 155
Solid-to-solvent ratio 122
Soluble proteins 69
Solvent 70, 96, 113, 117, 122, 155, 159–161, 171, 189, 199, 201
Soybean oil 28, 71
Soy protein 28, 71, 73
Spectrophotometry 138, 172
Spice extract 4, 7
Spices 3, 4, 7, 20, 30, 46, 48, 69, 73, 115, 121, 126, 132
Spiral plating 135
Spores 5, 8, 16, 76
Spray drying 14, 113, 122
Spray dry microencapsulator 114
Stability 9, 13–15, 21, 27, 39, 58, 73, 78, 79, 84, 85, 91, 96, 97, 140, 155, 170, 177, 178, 184
Stabilization process 3
Stabilizer 4, 7, 83
Standardization 27, 31, 60, 138, 159
Standardize 112
Standardized Mohr's protocol 138
Standards 59, 70, 71, 75, 86, 90, 96, 98, 105–107, 159–164, 174, 177, 194, 198, 200, 201, 203, 205
Staphylococcus 40, 41, 87, 127, 129
Staphylococcus carnosus 4, 5, 30
Staphylococcus xylosus 4, 5, 41, 48
Starter cultures 2, 4, 5, 7, 9, 30, 40–42, 44–48, 50
Sterility 19, 34, 127–130
Sterilization filters 130
Stirring 19, 20, 78, 104, 117, 118, 159, 160, 189, 199
Storage 4, 8, 9, 13, 14, 16, 21, 42, 44, 49, 60, 97, 105–107, 152, 169, 170, 174, 176, 196
Storage stability 13, 16
Strain selection 13, 14
Structured fat replacers 59, 63
Structured scale 89
Structuring agents 70, 73
Stuffing 4, 5, 8, 9, 30, 31, 73, 75–77, 132
Sugar 3, 9, 17, 20, 46, 48, 58, 82, 83, 132, 170
Sulfites 112
Sunflower oils 64, 71
Supernatant 118, 129–131, 176–178, 186, 196, 198, 199
Surimi gels 141
Survival rates 15, 40
Synbiotic 44–46
Synthetic additives 112, 113
Synthetic antioxidants 83, 112
Syringe 130, 160, 161

T

Taste 8, 27, 32, 47, 64, 88, 121, 138–140
TBARS 85
Technological quality 139
Temperature 9, 14, 27, 46, 58, 74, 84, 103, 118, 126, 142, 155, 170, 188, 196
Tertiary butylhydroquinone (TBHQ) 112
Test tubes 101, 102, 128, 130, 133
Texture 9, 32, 35, 40, 48, 59–61, 63–65, 73, 83, 86–88, 90, 91, 139, 140, 170, 174
Texture modification 9
Thermal treatment 30, 34
Thiamine 95–107
Thiochrome 97, 99, 100, 104
Time 1, 7, 9, 48, 49, 57, 74, 78, 86, 106, 126, 128–130, 133, 138, 140, 145, 155, 160, 172, 174, 176–178, 184, 185, 191, 196, 197, 199, 202, 204
Titration 138, 144, 146, 147
Toxic substances 112

Transition temperature 74
Tryptic Soy Agar (TSA) 128
Tryptic Soy Broth (TSB) 128, 130

U

Uncooked lamb sausage 140
Underestimation of salt content 145
Uniform dehydration 8
Unsaturated fatty acids 26, 42, 45, 58, 63, 64
Unwanted changes 111
UV-C 139, 140

V

Vacuum grinder 4, 6
Vacuum maceration tumbler 116, 119
Vacuum stuffer 61, 76
Vegetable 58, 70, 71, 73, 78, 82, 91, 98, 112, 117, 126, 139
Vegetable matrices 114, 121
Vegetable oil 29, 58
Vegetable proteins 71, 73, 82, 139
Vegetable waxes 70, 78
Viability 14, 15, 47, 126, 183, 194
Viscosity 121
Vitamin 14, 25, 39, 41, 83, 91, 95–107, 169, 182, 185, 187
Voltage 197, 202

W

Washing 144, 145, 171, 174, 176
Water 4, 14, 27, 49, 59, 69, 84, 101, 113, 127, 140, 155, 174, 181, 196
Water activity 14, 32, 49, 86, 91
Water bath 87, 103, 105, 127, 130, 141
Water-holding capacity 141, 170
Water loss 8
Water solubility 155
Water-soluble 14, 97, 98
Wavelengths 103–105, 162
White ashes 143, 145
White silver chloride precipitates 146

Y

Yellowness 84