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The journal "Theory and practice of meat processing" is an international peer-reviewed scientific journal covering a wide range of meat science issues.

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- targeted modification (selection, hybridization, operative manipulation);
- processing of meat raw materials;
- improvement of technologies for meat product manufacture;
- study of effects of meat and meat product consumption on human health;
- rational use of secondary resources of the meat industry and the ecological problems of the industry

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ASSESSMENT OF HYGIENE OF SLAUGHTER AND DISTRIBUTION OF BUSHMEAT IN ZOGBODOMEY MUNICIPALITY

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Keywords: *Bushmeat, pathogen, quality, Bénin*

Abstract
Bushmeat production process influences its quality. The objective of the study is to take stock of the hygiene of slaughter and distribution of bushmeat in southern Benin. Therefore, data on the bushmeat production process were collected and analyzed for wild species slaughtered or captured in the village of Tègon. It has been found that except for snakes and ruminants, two types of bushmeat production schemes were used according to the practices identified by category of wild species: small mammals and birds. The first type (Practice 1) was done without application of fresh blood to carcasses and the second one (Practice 2) was characterized by application of fresh blood to carcasses just after evisceration. Tools used by operators in general were poorly maintained from the hygienic point of view. No operator had a specific location suitable for storing tools. Operators did not wear mufflers, clean gloves, clean clothes and appropriate footwear. The state of animal health also remained unknown to all these operators. There was no cleaning and disinfection program for processing areas and work tools. Forward movement was not practiced at any meat processing station. Among the respondents, 3.16% did not wash carcasses, 46.88% did it poorly (with dirty water or water already used) and 50% did it unsufficiently (with very little water). Blood applied to carcasses was not cleaned by 3.13% of respondents, poorly cleaned (with dirty water) by 40.63% and unsufficiently cleaned with a little water by 56.25%. The study shows that in Tègon, the bushmeat production process is not hygienic and measures must be taken to protect the health of consumers.

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Introduction

Animal and plant natural resources are used by humans for their survival and well-being. Regarding protein requirements, wildlife resources are exploited through the consumption of wild animal meat, also known as bushmeat [1]. This meat is preferred for its taste and makes a better nutritional contribution, in terms of proteins, essential amino acids and mineral salts (iron, potassium, magnesium, zinc, etc.) [2]. It is also a profitable source of income for the generally poor rural population [3,4]. Bush animal organs are also used for other purposes such as decoration and traditional medicine [5–9].

In Benin, like in most tropical countries, bushmeat is consumed from north to south not only because of eating habits in rural areas but also because of food insecurity [4,10,11]. This consumption of bushmeat poses two fundamental problems, namely the disappearance of wildlife and the transmission of diseases to consumers. Regarding the threats of disappearance of wild animals, efforts have been made for their conservation. These efforts include inventorying threatened species, creating conservation reserves, and adopting laws to protect heavily hunted species [12–14]. Measures to preserve endangered species are supplemented by the domestication and breeding of certain less aggressive species such as the grasscutter, the Gambian pouched rat (*Cricetomys gambianus*), the guinea pig, etc. [15–17]. Attempts have been taken to protect wild animals and ensure the continued availability of bushmeat. Unfortunately, the latter has never been the subject of a health study. However, cases of disease transmission between wild animals and humans are frequent and are sometimes responsible for endemic diseases as in the case of the Ebola virus disease and coronavirus disease (COVID-19) [18–21]. In Benin, cases of death due to the Lassa virus are often reported [22]. The microorganisms responsible for these diseases can be accidentally or intentionally introduced into meat from sick animals, processors, equipment or the environment during food preparation and other vectors [23,24]. Based on this observation, quality control of bushmeat is necessary to preserve the health of consumers. However, on the outskirts of the Lama forest, bushmeat is produced in the open at the edge of the runway, under sheds called posts without any control and is then exposed at the edge of the same runway to be sold to passengers. This practice does not guarantee the quality of meat and an improvement in the slaughtering, processing and sale pro-

cesses is essential. The objective of the study is to take stock of the conditions of slaughter and distribution of bushmeat in southern Benin.

Material and methods

Study framework

The study was carried out at the bushmeat market in the Tègon village, on the edge of the Lama classified forest, Zogbodomey municipality. This village defends the largest market for the marketing of these meats in the municipality. The municipality of Zogbodomey is located in the heart of the Lama forest and is the place for hunting and the following marketing of bush animals. Protected by the State by decree No. 05574/SE/F of December 24, 1946, the Lama classified forest is located in the south of Benin and extends between 6°55' and 7°00' north latitude and between 2°4' and 2°12' east longitude. Its total surface is estimated at 16,250 ha, distributed between the departments of Atlantique (9,750 ha in the municipality of Toffo) and Zou (6,500 ha in the municipality of Zogbodomey). The Lama classified forest occupies the phytogeographic zone with Guinea-Congolese affinity in southern Benin [25]. Intensive hunting activities take place in the Lama forest. In fact, the central core of the Lama classified forest is home to relatively various hunting fauna (mammals, birds, reptiles, amphibians, fish, mollusks, insects) and is partially dense. Data were collected at bushmeat sales points in the village of Tègon near the Lama forest from bushmeat processors.

Equipment

The material used consisted of survey sheets created for bushmeat producers to obtain as much information as possible on operations, conditions and hygiene of slaughter of wild animals, as well as practices of processing of wild animals on different bushmeat processing sites.

Methodology

The study was carried out among bush animal processors. In total, 10 processors working in five stations (two per processing station) were recorded and monitored from reception of game to cutting to make observations during processing. Processors bought slaughtered game from hunters. As the felling was not planned in advance, site visits were carried out every day of the week. The respondents were chosen at random with the support of guides or resource people. Their availability and agreement to be investigated and monitored was negotiated in advance.

Investigation

The survey was carried out using a semi-structured interview coupled with observations made in the field. In total, 10 respondents were interviewed, an average of two respondents per station.

A questionnaire was used for the interview and addressed points such as: the identity of processors, animal species processed, the cause of death of an animal to be processed, processing practices.

Concerning the observations, an evaluation grid was made on the basis of the points considered important to ensure the hygiene of the finished product according to the technical specification standard ISO/TS22002–1:2009 (F)[1]. These include environmental hygiene of premises, hygiene of slaughtering material, water, energy and other supplies, cleaning and disinfection programs, adequacy of equipment and its accessibility for cleaning, maintenance, ancillary services, in particular, services for the elimination of waste and wastewater. Each point was assigned to a category according to the observation: satisfactory (S: hygiene measures were well applied), unsatisfactory (PS: some measures were well applied, and others not), poor (P: all measures were poorly applied) and zero (0: if nothing was done).

A description of hygiene practices was also done according to the 5M (raw material, equipment, method, labor and environment).

Statistical analyzes

After counting and coding, the data were analyzed with SAS software [26]. The observed frequencies were calculated using the Proc FREQ procedure for each modality and the relative frequencies were compared two by two using the two-sided Z test. For each relative frequency, a 95% confidence interval (CI) was calculated according to the equation:

$$IC = 1{,}96\sqrt{\frac{P(1-P)}{N}} \quad (1)$$

Where:

P is the relative frequency;

N is the sample size.

Results and discussion

Description of preparation practices and assessment of bushmeat preparation hygiene

Hygiene of bushmeat preparation areas and general services

Most of the game processed was *Thryonomys swinderianus* (31.25%) followed by *Varanus niloticus* (12.25%), *Cricetomys gambianus* (9.38%), and then by *Xerus erythropus*, *Lepus crawshayi*, *Python sebae*, *Bitis arietans*, *Tragelaphus scriptus* with the same percentages (9.25%) and finally by *Pternitis* sp, *Streptopelia semitorquata*, *Philantomba walteri*, *Naja nigricolis* with a frequency of 3.13% each (Table 1). None of the respondents had a suitable building for processing of these animals. Animals were processed in sheds, 90.63% of which had a sheet metal roof and 9.38% a straw roof. The viscera were thrown away not far from the workstations, which caused unwanted odors at the processing sites (Table 1). Not all operators had trash bins, changing

[1] ISO/TS22002–1:2009. Prerequisite programmes on food safety. Part 1: Food manufacturing. Technical Committee: ISO/TC34/SC17. ICS: 67.020

rooms, toilets or a sufficient number of taps for drinking water. Only 3.13% of respondents had water taps compared to 96.87% who did not. The activities were all carried out without separating the clean circuits from the dirty circuits and this was observed in all positions. Almost all operators threw garbage at or near the workplace. The water used by many respondents was neither clean nor in sufficient quantity. Thus, 59.38% of respondents had insufficient quantity of well water (non-potable water) compared to 40.63% who had water of satisfactory quality (drinkable pump water) but still in insufficient quantity.

Table 1. Types of meat, buildings and general services

Variable		Percentage (N = 32)	CI
TYPES OF MEAT	LOCAL NAME (FON)		
Bushbuck or Antelope *(Tragelaphus scriptus)*	Agbanlin	6.25[b]	8.39
Grasscutter *(Thryonomys swinderianus)*	Hô	31.25[a]	16.06
Blue duiker or doe *(Philantomba walteri)*	Zoungbô/ Tegbô	3.13[b]	6.03
Python *(Python sebae)*	Hon	6.25[b]	8.39
Cobra *(Naja nigricolis)*	Klibo	3.13[b]	6.03
Squirrel *(Xerus erythropus)*	Awassagbé/Don	9.25[b]	10.04
Hare *(Lepus crawshayi)*	Azui	6.25[b]	8.39
Francolin *(Pternitis* sp)	Assôklé	3.13[b]	6.03
Gambian rat *(Cricetomys gambianus)*	Atchou	9.38[b]	10.10
Doves *(Streptopelia semitorquata)*	Houélé	3.13[b]	6.03
Monitor lizard *(Varanus niloticu)*	Vê	12.25[ab]	11.36
Viper *(Bitis arietans)*	Djapkata	6.25[b]	8.39
Evaluation of buildings			
Straw roof shed		9.38[b]	10.10
Tin roof shed		90.63[a]	10.10
ODORS AT TREATMENT SITES			
Yes		18.75[b]	13.52
No		50[a]	17.32
Yes, but weak		31.25[ab]	16.06
ODORS AROUND THE TREATMENT SITE			
Non-existent		6.25[b]	8.39
Existing		93.75[a]	8.39
PRESENCE OF DEAD ANIMALS SLAUGHTERED			
Non-existent		37.5[b]	16.77
Existing		62.5[a]	16.77

N: number; NS: not significant; CI: Confidence interval. Intra-class percentages followed by different letters are significantly different at the 5% level.

Description of bushmeat preparation practices

Observation of the process of slaughtering hunting animals and their processing for meat at the Tègon market revealed that the bushmeat preparation operations were generally the same for all operators and species with the exception of applying blood to the meat surface (painting), which was done differently by different operators. Some did not do any painting before smoking (Practice 1) and others did it with blood before smoking (Practice 2). The established slaughter patterns for species are shown in Figures 1 to 6. Species with the same meat production pattern were grouped together. Generally, application of oil to carcasses (oil painting of carcasses) took place just after animals were smoked. Some operators brushed meat with blood after eviscerating animals to give it a better presentation and better preservation over time. The smoked meat production diagram for *Guttera pucherani*, *Pternitis* sp and *Streptopelia semitorquata* is shown in Figure 3 for Practice 1 and Figure 4 for Practice 2. The production diagram for snake carcasses (*Python sebae*, *Bitis arietans* and *Naja nigricollis*) is presented in Figure 5. Finally, the production diagram for smoked carcasses of *Tragelaphus scriptus* and *Philantomba walteri* is presented in Figure 6 for Practice 1. There was no Practice 2 for snake and ruminant meats.

Description of the flowchart steps

Transportation and reception of dead animals

Game hunted in the Lama Forest was sold by hunters to merchants and other customers in places dedicated for this purpose. These dead animals were then transported to processing sites by traders in bags or on two-wheeled vehicles. The animals were also transported by hunters themselves for sale to processing sites on two-wheeled vehicles. These animals usually arrived dead for at least 24 hours because they were animals killed by firearms or fatally trapped. In some cases, animals arrived alive and once received at the processing site, an operator hit the transport bag containing an animal against the ground with a sharp blow to knock it over. This was especially the case with monitor lizards or snakes. A part of the hunted animals was resold without processing to travelers and restaurateurs who preferred it this way, the second part was processed.

Skinning/scaling

After the transportation and reception steps, comes skinning/scaling, which consists of removing hairs or feathers in birds or scales in reptiles. This operation was done by flaming for certain animals such as *Thryonomys swinderianus, Xerus erythropus, Varanus niloticus*, *Cricetomys gambianus*, *Lepus crawshayi* and *Genetta* spp, *Pternitis* sp and *Streptopelia semitorquata*. The flaming was done over a wood fire. An animal was brought into contact with flames several times and then stripped of its coat. In the case of animals with fur or with scales, such as reptiles, this was done gradually using a knife to ensure scraping until there was nothing left on the body of an animal.

Skinning of some animals, such as *Tragelaphus scriptus* and *Philantomba walteri* was done by simply tearing off the skin.

Washing

A skinned animal was then washed: the first time with simple water and the second time with simple water or water already used to rinse other carcasses (Figure 3). This second washing was carried out only by operators using Practice 1.

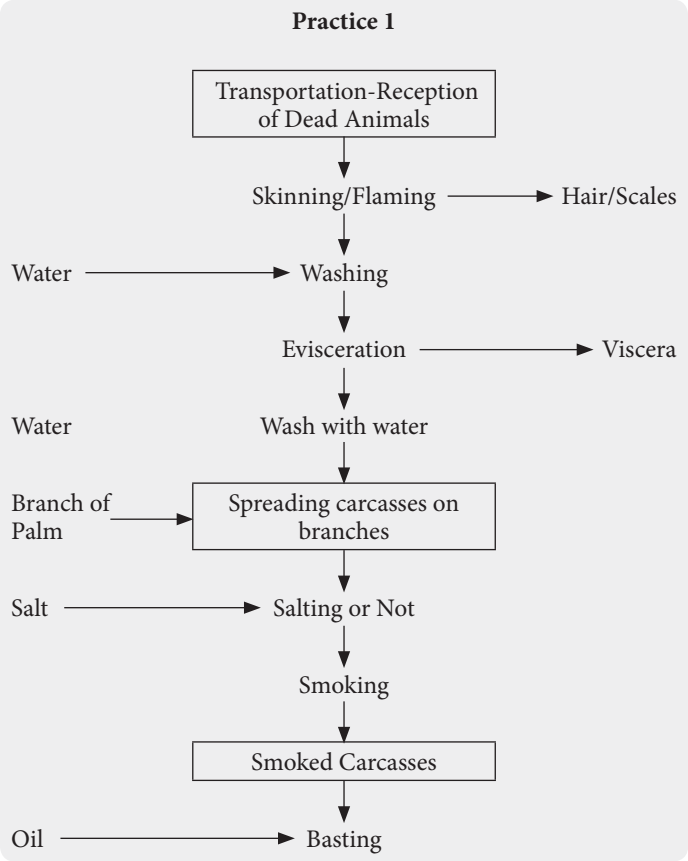

Figure 1. Production diagram for smoked carcasses of *Thryonomys swinderianus, Xerus erythropus*, *Varanus niloticus*, *Cricetomys gambianus*, *Lepus crawshayi* and *Genetta* spp. (Practice 1)

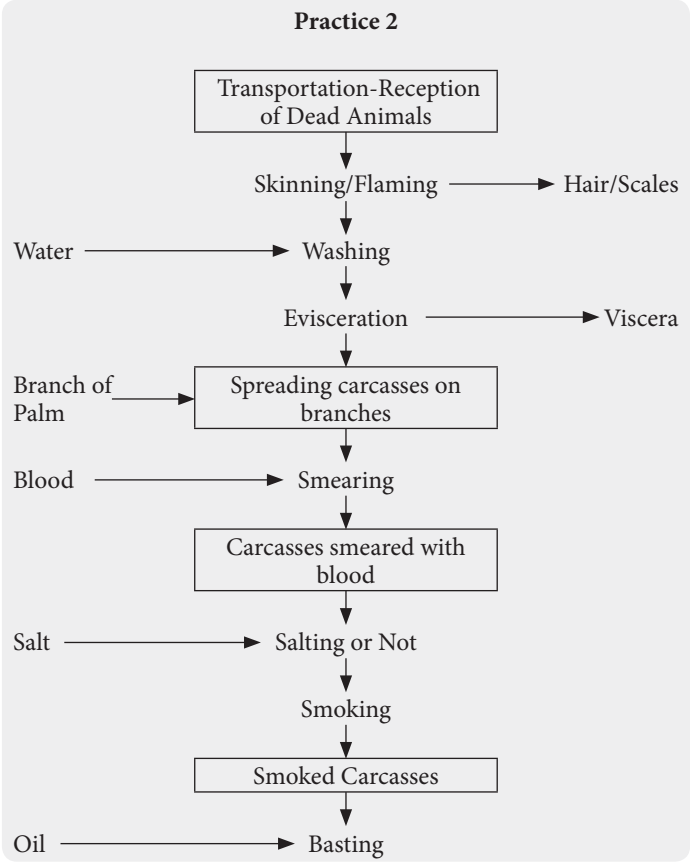

Figure 2. Production diagram for smoked meat carcasses of *Thryonomys swinderianus, Xerus erythropus*, *Varanus niloticus*, *Cricetomys gambianus*, *Lepus crawshayi* and *Genetta* spp. (Practice 2)

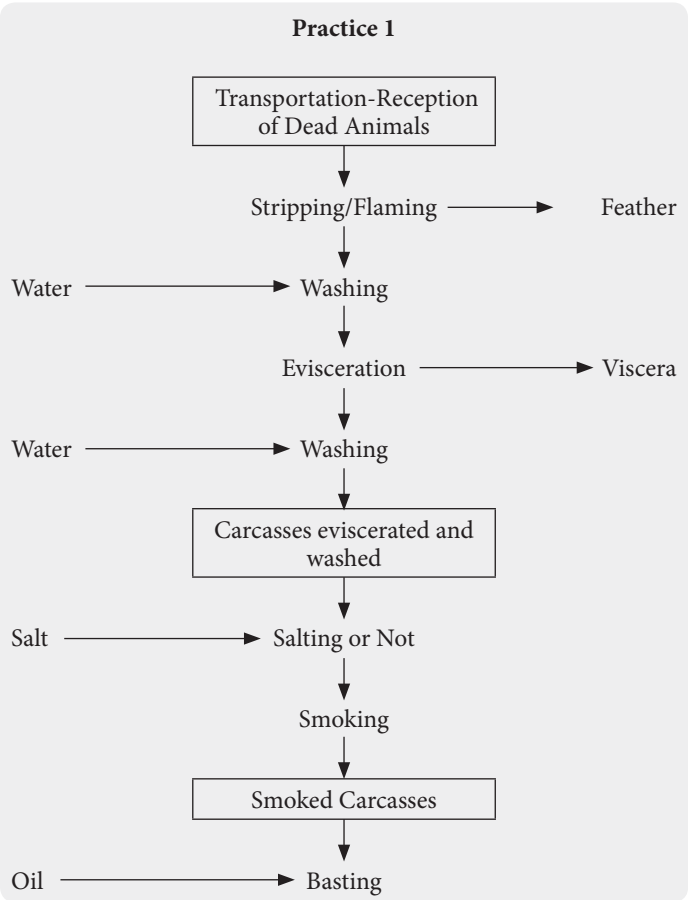

Figure 3. Production diagram for smoked carcasses of *Pternitis* sp, *Guttera pucherani* and *Streptopelia semitorquata* (Practice 1)

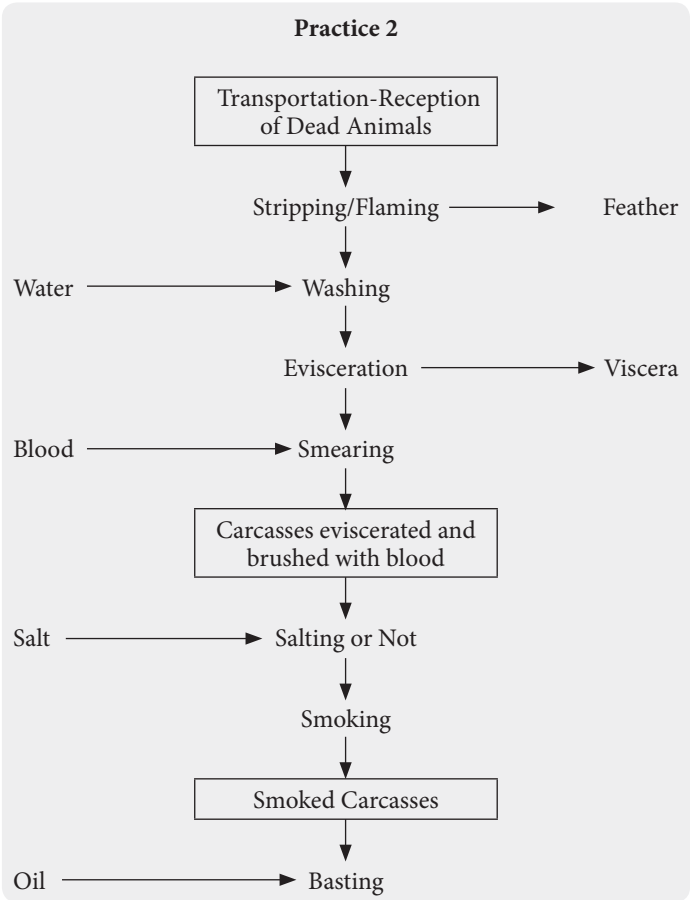

Figure 4. Production diagram for smoked carcasses of *Pternitis* sp, *Guttera pucherani* and *Streptopelia semitorquata* (Practice 2)

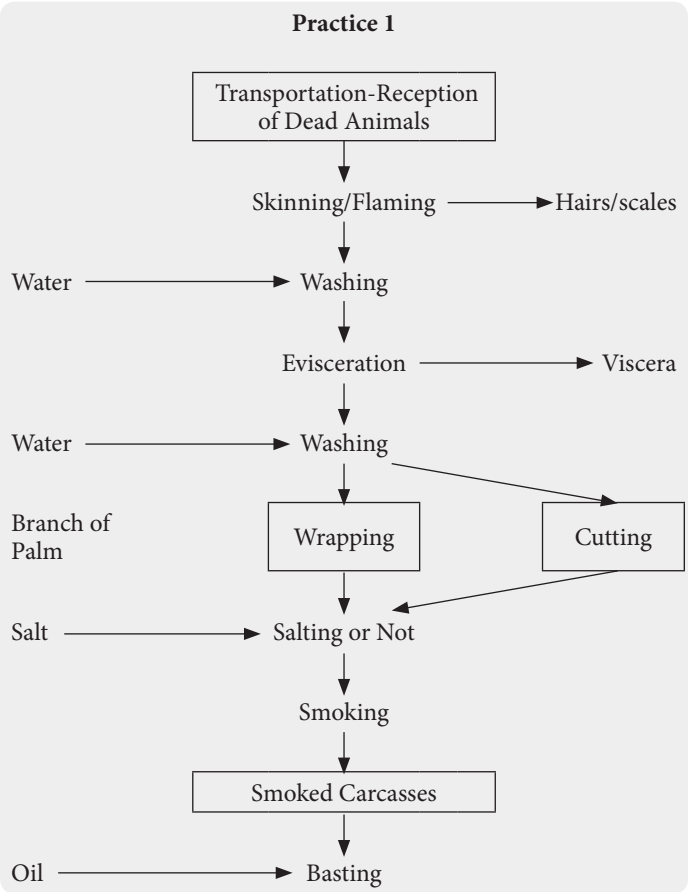

Figure 5. Production diagram for smoked snake carcasses (Practice 1)

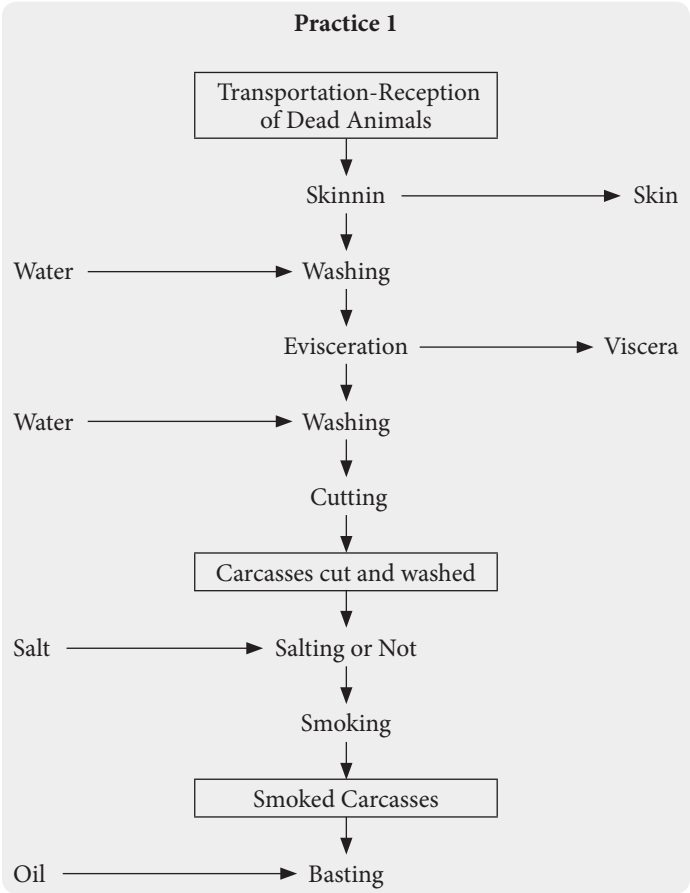

Figure 6. Production diagram for smoked carcasses of *Tragelaphus scriptus* and *Philantomba walteri* (Practice 1)

Evisceration

Evisceration was generally done on cereal bags or on teak leaves on the ground, or occasionally on a wooden board on the ground. An operator removed the viscera through a longitudinal opening in the thorax and abdomen using a knife. All viscera were removed in most cases, but depending on an operator, the kidneys were sometimes left hanging from mammal carcasses (Figure 6).

Spreading carcasses on branches, rolling and cutting

After washing, carcasses of small mammals and monitor lizards were spread with sticks with two pointed ends made of cut and trimmed palm branches which were inserted from one end to the other at the level of carcasses, so that the inside of carcasses was clearly visible. The purpose of this practice is to facilitate smoking and allow good presentation of meat during marketing. However, some carcasses, such as those of snakes, deer and antelope, were not presented in the same way. Carcasses of these species were often cut into easy-to-handle pieces for smoking. Snake carcasses were sometimes coiled on themselves and held by sticks with two pointed ends pushed from one end to the other. These sticks were also made from palm branches. Wild bird carcasses were not spread out on branches. Just curled up and bent in half by the pressure exerted by processors, they were smoked after gutting without further processing. Sometimes the same method was also used for monitor lizards. It was at this step that processors using Practice 2 painted the inside of certain carcasses with blood.

Smoking

Smoking was carried out using charcoal, often from the fire used for flaming.

Carcasses of small mammals and wild birds spread out on a rack sometimes were sprinkled with salt and sometimes were not. The grid containing carcasses was then placed on a hearth held by four blocks of stones. The glowing coals inside the stone blocks produced heat needed to cook meats.

Brushing

After having been smoked, meat was brushed with vegetable oil, and placed in trays to be displayed at the side of the road for sale to passers-by and travelers. Since bushmeat was not weighed, its price was estimated based on its quantity and quality.

Description of bushmeat processing

Certain operator practices that could lead to meat contamination have thus been identified.

Raw materials

Wild animals brought to different processing sites were often dead for more than 12 hours. The skin of these animals was mainly covered with blood or gastric or in-

testinal contents through openings created by rifle bullets or snares.

Tools used and state of cleanliness

Tools used by different operators consisted of a knife, a machete, a basin, a wooden cutting board, a bag of cereals, a container of water, trays iron and a smoking grill. Knives and axes were often not cleaned before processing meat or were only cleaned with water immediately after operations. Bowls, cutting boards and cereal bags were almost never washed. Cans and basins as well as bags of cereals, trays and cutting boards were also left on processing sites, either on the ground or on the roofs. In the absence of a water source at the workplace, operators used drums for water storage. Well or tap water was generally used and collected from the nearest village.

Labor Force

Processors had no qualifications. The work was often done in dirty house clothes or sometimes shirtless. None of the operators interviewed wore gloves, mufflers or appropriate footwear. They lay down, ate and received visitors in the place of processing.

Method of working

None of the animals processed during the investigation period underwent bleeding, so all other steps of the production schemes were carried out without evacuation of the animal's blood. Evisceration and butchering were carried out by one person. The knives used for these operations were left on the ground and the same knives could be used for two or more different animals without washing or disinfection between carcasses. When butchering game by flaming, in the absence of support, processors carried out the operation on the ground. This was followed by washing with dirty water to remove dirt caused by smoke and fire. Washing carried out after evisceration remained incomplete in most cases observed. Water used was the same for all animals treated in the same period. Blood cleaning was absent among some operators who left blood on meat for reasons of protection against flies and aesthetics of meat.

Work places

All five different meat processing stations recorded in this study had a shed with a tin roof except one station which had a shed with a straw roof, with a bare floor and a traditional brick-based hearth. The game was treated in the open air, on wooden cutting boards or very often, in most cases, on wool bags placed on the ground. Although regular sweeping was carried out every morning to remove remains of raw meat, remains of intestines, intestine contents and dead leaves were found around the sheds. Water used for washing carcasses was thrown away near the workplace. Sometimes, there were smells of putrefaction. We also noted the presence of domestic animals (dogs, chickens, roosters) and flies on the processing sites.

Cleaning and disinfection of bushmeat transformation sites

All participants surveyed did not have a drainage system for washing carcasses, hands or work tools. Sharp tools (knives and cutters) used by operators in general were easy to clean, but not very resistant to corrosion and often poorly maintained. In total, 68.75% of respondents used unclean cutters and 31.25% used dirty cutters (Table 2). Similarly, 31.25% used unclean wood to display carcasses, 9.38% used dirty wood and 59.38% of the respondents did not even use wood. Concerning basins, 25% of respondents used basins that were not very clean, compared to 43.75% who used dirty basins and 31.25% who did not use them (Table 2). No specific storage locations or closets existed to store materials for all respondents. There was also a complete absence of scales and water boats.

Table 2. Hygiene of processing sites and tools

Variable	Percentage (N = 32)	CI
STATE OF HYGIENE OF THE SITES		
Not clean	68.75[a]	8.68
Dirty	31.25[b]	12.87
BASIN HYGIENE CONDITION		
Non-existent	31.25[a]	16.23
Not clean (blood residue)	25[a]	16.94
Dirty	43.75[a]	14.69
STATE OF HYGIENE OF SUSPENSION WOOD		
Non-existent	59.38[a]	12.50
Not clean (blood residue)	31.25[b]	16.23
Dirty	9.38[c]	18.57

N: number; CI: Confidence interval, Intra-class percentages followed by different letters are significantly different at the 5% level

Personnel hygiene and bushmeat production operations

No qualified personnel was observed in all the stations surveyed, those responsible for meat production were for the majority of villagers without any notion of hygiene or good practices. All processors did not wear mufflers, clean gloves, clean clothes and suitable shoes. Some processors worked shirtless or in generally dirty house or street clothes, while others carried out meat processing operations barefoot or in street shoes. None of the respondents practiced disinfection. Bleeding at slaughter was completely absent and strict compliance with the slaughter process remained unsatisfactory in all positions encountered. The work was done out of order. Concerning washing carcasses, 3.16% of respondents did not do it, 46.88% did it poorly (with already contaminated water) and 50% did it with a small quantity of water. As for cleaning blood from carcasses, 3.13% of respondents did not do it, 40.63% did it poorly with dirty water and 56.25% did it unsufficiently with a little water (Table 3).

Table 3. Operators' working method

Variable	Percentage (N = 32)	CI
BLOOD CLEANSING		
Non-existent (not done)	3.13[b]	6.03
Badly done (with dirty water)	40.63[a]	17.02
Yes (with a little wather)	56.25[a]	17.19
WASHING		
Non-existent (not done)	3.16[b]	6.06
Badly done (with dirty water)	46.88[a]	17.29
Yes, but poor (barely rinsed)	50[a]	17.32

N: number; CI: Confidence interval, Intra-class percentages followed by different letters are significantly different at the 5% level

Discussion

Analysis of the stages of bushmeat production

The survey carried out showed that grasscutter was the most hunted species and grasscutter meat was the most consumed bushmeat. The same observation was done by Djagoun et al. [27] in South Benin. Hunters' preference for this species was linked to consumer demand. The enthusiasm of the Beninese population for grasscutter meat is not new and to meet the needs of consumers, the Beninese government initiated its breeding and the promotion of this breeding in 1943 [28]. Other hunted species such as snakes *(Python sebae, Bitis arietans*, *Naja nigricolis)*, birds (*Pternitis* sp, and *Streptopelia semitorquata,* etc.) and mammals (*Xerus erythropus*, *Cricetomys gambianus*, *Lepus crawshayi,* etc.) have already been reported by hunters in Benin [14,29]. These hunted species are sold to processors, passers-by and restaurateurs [27,30,43]. These meats were processed using two practices which were similar with the exception of the application of blood to carcasses which was absent in one practice. The transportation and reception steps were carried out under the same conditions by all respondents, regardless of the animal species hunted. Most of the time, dead animals were transported in dirty bags causing contamination of carcasses. This observation has already been done by Nganga et al. [31] in Congo where bushmeat is transported in inappropriate packaging, which affects its quality. In addition, the conditions of bushmeat transportation can lead to meat contamination [32]. The second step, skinning or flaming, was also the same in both practices for various species captured and different from the procedures used for farm animals in slaughterhouses where, after the steps of transportation and reception of living animals, there are several steps (*ante-mortem* inspection, water diet, bleeding) before skinning or flaming [33,34]. The absence of these steps in bushmeat processing is due to the method of slaughter. Indeed, game is often killed with guns, dogs or traps [3,35,36] and it would be very difficult to carry out ante-mortem inspection [37–39] and bleeding. Failure to follow these steps can not only expose consumers to zoonoses, but pose a threat to biodiversity because pregnant females are sometimes slaughtered [40]. The skinning technique reported in small animals in this study was burning because of the small size of these species which would not allow their skin to be easily removed. Unlike small mammals, large mammals like *Tragelaphus scriptus* and *Philantomba walteri* are skinned because their skin is easy to remove and is used for various purposes such as traditional medicine, making drums, quivers, bags, etc. [41–43]. After burning, carcasses were washed so as not to contaminate meat with waste from the hair burned after evisceration. Evisceration common to both practices was carried out late with a lack of water, which could lead to deterioration in the quality of meat on two levels. Firstly, late evisceration leads to deterioration in the quality of a carcass because germs present in the viscera, particularly the abdominal ones, can easily pass on to a carcass. Secondly, washing with insufficient water does not allow processors to rid meat of exogenous contaminants and stomach contents, which exposes meat to high contamination [44]. Indeed, the evisceration process plays an important role in contamination, because feces contain high quantities of coliforms [45]. The absence of cleaning-disinfection of the equipment used leads to heavy contamination of a carcass by microorganisms [45]. After evisceration, a *post-mortem* inspection would normally remove meat unfit for human consumption. Unfortunately, this inspection was not carried out, which could expose consumers to zoonoses. Zoonoses that consumers can contract by consuming uncontrolled meat are Ebola virus disease, Lassa fever, coronavirus disease, monkeypox, etc. [46–49]. However, the presence of some of these diseases has not been reported in Benin and the most recorded zoonoses are Lassa fever and coronavirus disease [21,50–52]. Unlike processors using Practice 1, processors using Practice 2 brushed carcasses with blood before smoking instead of washing after evisceration and this attitude exposed such carcasses to additional contamination because blood is a rich medium for microorganisms [44]. The penultimate step was smoking common to operators of both practices. Smoking considerably reduces the microbial load of carcasses, which results in meat that is more or less suitable for consumption. However, exposure of smoked carcasses to dust during marketing could constitute a new source of contamination. Indeed, the exposure of food products to dust and flies promotes their contamination by pathogenic microorganisms [53].

Hygiene of bushmeat preparation and general services

Most of processed and sold animals were killed by firearms. Consumption of meat from these animals exposes consumers to lead poisoning, as consumption of animal meat killed by lead ammunition has been reported as a high risk factor for lead in blood [4]. Game, once hunted, was processed by merchants who did not have buildings meeting the standards required for slaughtering and processing of meat. Processing carried out in the open air with

an unsanitary environment due to the presence of garbage around the premises and in the premises of almost all operators, promotes contamination of meat. Compliance with hygienic rules in the design and construction of premises, appropriate location and adequate facilities are necessary to enable effective risk control [54]. The viscera thrown away not far from the workstations by almost all operators justifies the high rate of the presence of undesirable odors around the premises, and therefore, the presence of flies, insects and rodents. All these nuisances as well as air pollution are sources of external contamination of bushmeat. Bushmeat can be contaminated with *Escherichia coli, Salmonella enterica, Staphylococcus aureus, Clostridium botulinum, Clostridium perfringens, Bacillus cereus* [34]. The lack of changing rooms, toilets and taps for most operators indicates that meat may be contaminated by workers' dirty hands. As all activities were carried out without separation of clean and dirty circuits, meat intended for consumption was subject to cross-contamination. More than half of processors used insufficient well water. In addition to the dirtiness of this water, it was not enough to carry out washing correctly and this led to further contamination of meat. For operators who used tap water, this contamination was reduced because tap water was clean but nevertheless it remained insufficient. Various tools used (knives, cutters, cutting boards and wooden hangers) were generally dirty or poorly cleaned and constituted sources of carcass contamination. This observation was also made for the evisceration step, which was poorly done, thus leading to contamination of a carcass not only by fecal contaminants from the same carcass during processing, but also by contaminants from previously treated carcasses because the same tools were used without any cleaning [55]. This absence of general hygiene and the non-compliance with hygienic requirements at different steps of meat processing demonstrate the lack of qualification and total ignorance of processors regarding hygiene measures linked to meat processing [56]. Showering before bleeding was absent because there was no bleeding, leading to contamination of meat with germs from the skin. The often long period between the death of an animal and its sale remains a favorable factor for the multiplication of germs, which leads to meat spoilage.

Failure to wear face coverings and clean gloves exposes operators to fluids and blood from hunted animals, leading to cases of zoonoses [34]. In fact, an operator injured in the hand can contract a disease from an animal and, in turn, he can contaminate a carcass [57]. This is confirmed by [58], which states that disease transmission was noted at a higher risk when slaughtering an animal, although disease transmission can occur during manual transportation of animals. Dirty clothes are also a source of meat contamination. Poor washing by most operators exposed meat to a multiplication of germs transmitted by blood. Similar problems are shown in [31], which revealed the total absence of hygiene and non-compliance with good production practices by operators processing bushmeat from the beginning to the end of the chain in Congo.

Conclusion

The assessment of good hygiene practices and slaughtering processes for bushmeat production in southern Benin made it possible to identify two types of bushmeat production practices among operators. The operators using Practice 1 washed game carcasses twice, before and after evisceration. On the other hand, the operators using Practice 2 only washed carcasses once before evisceration, then brushed carcasses with blood after evisceration. Transportation and reception were carried out in poor conditions by all operators and meat processing was done in the open air because there were no real buildings. Sheds were open to dust and nuisances of all kinds, which made the environment unsatisfactory for obtaining meat of acceptable quality. Operations such as bleeding, ante-mortem and post-mortem inspection were not carried out at processing sites. Other operations, such as washing and evisceration, were mostly poorly done, and smoked bushmeat was exposed to poor conditions during marketing. In general, bushmeat processing conditions in southern Benin are not satisfactory and operators need to be made more aware of slaughter hygiene.

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DEVELOPMENT OF AN APPROACH TO PREDICTING THE BIOAVAILABILITY OF ENTERAL NUTRITION PRODUCTS

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Keywords: *bioavailability vector, enteral nutrition, statistical analysis, nutritional supplementation*

Abstract
One of the key factors while developing nutritional supplements is their bioavailability. To determine it, expensive and time-consuming clinical studies of developed products are necessary. Using in silico methods may speed up and reduce the costs of such clinical studies. The purpose of this study is to develop an approach to predicting the integral bioavailability of enteral nutrition products (ENPs) based on a comprehensive analysis of the matrices of components and indicators. The includes a comprehensive empirical study based on a comparative statistical analysis of the matrix of studied ENPs components. Available information on the composition and indicators of 52 commercial ENPs was used as a research object. This information was compiled into a matrix of components and indicators, marked according to the intended purposes of the products. The set of products included in the matrix was divided into 2 subsets: ENPs corresponding to a given intended purpose and other ENPs. This made it possible to separate statistically significant components and indicators that define the intended purpose of the product with a given threshold of the maximum error probability for inequality of mean values. Using Harrington's desirability principle in relation to the identified components and indicators made it possible to obtain an integral estimate of desirability for a given intended purpose. A vector characterizing the distance from the integral estimate to the ideal value was introduced as equivalent predicted bioavailability. The upper limit of the optimal range is 0.37, the upper limit of the acceptable range is 0.63. The predicted bioavailability vector scale is the inverse of the integral desirability scale. In contrast to Harrington scaling, the lower the predicted bioavailability value, the more preferable it is. Analysis of the introduced indicator allowed us to establish significant variability in commercial ENPs with respect to predicted bioavailability for diabetes mellitus and thermal injury. Based on the proposed predicted bioavailability vector, a principle has been developed for the evolutionary development of a statistical approach to predicting bioavailability when designing ENPs. This principle is a universal addition to the principle of food combinatorics while developing meat, dairy and plant-based ENPs.

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Introduction

One of the main characteristics of food products is their nutritional value, i. e. the content of proteins, carbohydrates, fats, vitamins and mineral elements [1,2,3]. However, eating nutrient-rich foods does not guarantee that the body will absorb and use them optimally. All other factors being equal, the absorption during digestion of two products with similar nutritional value may vary greatly [4,5]. This depends on the form of nutrients, food processing methods [6], individual physiological characteristics of the body, etc. [7]. Therefore, nutritional value cannot act as a comprehensive characteristic of a product as a factor of nutrient supply [8,9]. In this regard, the issue of nutritional value should be considered more deeply, i. e. from the perspective of the digestibility of the product's nutrients. In this case, the bioavailability of nutrients inevitably acts as a corrective factor that determines the nutritional properties of food products [10,11]. This term refers to the total proportion of nutrients in the food matrix absorbed by the human body during metabolism [12]. All other factors being equal, the higher the indicator value, the better [13]. High bioavailability of food products for healthy people is certainly important for ensuring the normal functioning of the body [14,15]. However, in the case of functional products, especially enteral nutrition (EN), bioavailability is critical [16,17]. In nutritional supplementation, ENPs are a priori nutrient-compensating products providing the person with a particular pathology with the required amount of energy and essential components [18,19]. The bioavailability indicator is very informative, but the procedure for determining it involves expensive *in vitro* [20,21] and *in vivo* testing, as well as clinical studies [22]. This indicator is determined based on the analysis of final and intermediate products of metabolism [23]. As a result, a number of objective consequences inevitably arise:

- bioavailability analysis is carried out *post factum*, which means the need to obtain a set of preliminary and basic preclinical and clinical studies [24]. Moreover, the product itself must have already been produced in some quantity and consumed by laboratory animals and focus-group patients [13,23];
- the bioavailability indicator is discrete, which is inextricably linked with the previous consequence and is characterized by a strict link to the composition and form factor of the product without the possibility of considering the dynamics of mass fractions for one or another component in the composition;
- high resource consumption because preliminary and basic clinical studies are quite expensive and time-consuming and may last for up to a year or more [25,26];
- not applicable to ENPs due to the need for a dynamic design approach. This is due to the fact that the core of any design algorithm is combinatorics [27,28]. For its successful implementation, it is necessary to be able to dynamically change the mass fractions of individual components within certain limits. The process itself must occur before the physical production of the designed product with the identification of a certain set of solutions that satisfy the basic set of criteria [29,30,31].

Thus, each subsequent consequence accumulates all the previous ones. In this regard, there is a need to develop flexible approaches to *in silico* predicting the bioavailability for an arbitrary matrix of food products even at the design stage.

The work of many scientific groups from leading research centers in the world based on more or less significant samples of food product sets with clearly established bioavailability values has determined that for individual nutrients this kind of predicting may be very successful. Currently, such models for predicting bioavailability exist as Hallberg and Hulthén model [31] for iron cations, as well as Miller, Krebs and Hambidge model [32] for zinc cations. These models make it possible to predict the bioavailability, taking into account the possible synergistic, antagonistic or additive influence of associated components in the food matrix (macro- and micronutrients of organic and mineral nature).

Unfortunately, for most nutrients, such models have not yet been developed, despite the fact that the demand of food science in this regard is continuously increasing (especially in the field of therapeutic nutrition). Thus, the main approach to predicting the bioavailability of nutrients is *in silico* simulation, which should be based on the existing understanding of the kinetics of metabolic processes in the human body. However, this approach requires operating with adequate databases [33]. Similarly, modern methods for simulation of digestibility processes operate with pharmacokinetics, i. e. the metabolism of individual pharmacological components. This fundamentally distinguishes this approach from ideas about the bioavailability of nutrients from multicomponent food matrices, where simultaneous multiple-vector metabolism occurs. In addition, this approach raises a number of questions: Peters and Dolgos [34] point out the problems of non-identifiability of pharmacokinetic model parameters, and Le Feunteun, Mackie and Dupont [35] put in question the possibility for detailed simulation of nutrient absorption due to the limited understanding of the metabolic process. Cacace et al. [36] propose moving from a physiological pharmacokinetics model to an intestinal physiology model. The necessity and promising outlook of developing fundamentally new approaches to simulation of bioavailability are also stated by Pompa et al. [37] and Sugano [38]. The authors [39] agree with them, emphasizing that existing *in silico* models are not able to provide a comprehensive understanding for the kinetics of human metabolic system interaction with the nutrients in the food matrix of the product.

Thus, there is an objective need to develop an integral empirical approach to predict bioavailability.

The purpose of the study is to develop an approach to indirectly predicting the integral bioavailability of ENPs, including meat and dairy ENPs, based on a comprehensive analysis of their components and indicators through a synthetic parameter, i. e. a vector.

Objects and methods

A set of open access data on the composition, nutritional value, glycemic index, osmolality and appropriateness of ENPs for certain pathologies was used as initial data. We analyzed 52 foreign and domestic commercial ENPs currently present on the Russian market:

- manufactured by Fresenius Kabi Deutschland GmbH (Germany): DIBEN® (1), Fresubin® VB Energy (2), Fresubin® Original (3), Reconvan® (4), Supportan® (5), Fresubin® Energy dietary fiber (6) and Intestamin® (7);
- manufactured by Nestlé S. A. (Switzerland): Impact® Enteral (8), Peptamen® AF (9), Resource® Diabet Plus (10);
- manufactured by Nutricia (Netherlands): Nutrison® 1.0 (11), Nutrison® Advanced Diason (12), Nutridrink Nutrison® Advanced (13), Nutrison® Advanced Cubison (14), Nutrison® Protein Intense (15), Nutrison® Protein Advance (16), Nutrison® Energy (17), Nutrison® Energy Multi Fibre (18), Nutrison® Advanced Peptisorb (19), Nutrison® Diason Energy HP (20), Nutrison® Multi Fibre (21), Nutridrink® (22), Nutridrink® Compact Protein (23), Nutridrink® Compact Fibre (24), Renilon® (25), Forticare® (26), Nutrilis® Powder (27), Nutrilis® Clear (28);
- manufactured by B. Braun SE (Germany): Nutricomp® Hepa Liquid (29), Nutricomp® Diabet Liquid neutral (30), Nutricomp® Energy Fiber Liquid (31), Nutricomp® Peptid Liquid (32), Nutricomp® Intensive Liquid (33), Nutricomp® Standard Liquid (34), Nutricomp® Fiber Liquid (35), Nutricomp® Energy Liquid (36), Nutricomp® Drink Plus (37), Nutricomp® Drink Plus Fiber (38), Nutricomp® Chicken Soup (39), Nutricomp® Vegetable Soup (40), Nutricomp® Drink Renal (41), Nutricomp® Immun Liquid (42), Nutricomp® Enbrace Active (43);

- manufactured by InfaPrim (Russia): Nutrien® standard (44), Nutrien® standard fiber (45), Nutrien® energy (46), Nutrien® diabet (47), Nutrien® hepa (48), Nutrien® nephro (49), Nutrien® pulmo (50), Nutrien® fort (51), Nutrien® elemental (52).

The development is based on a comprehensive empirical study based on a comparative statistical analysis of component matrix of the studied ENPs.

Mathematical processing was carried out using Microsoft Excel 2010 spreadsheet processor (Microsoft Ink.) with the "Solution Search" add-on installed. The search for a solution was carried out using the simplex method, as the most universal method, with automatic scaling and accuracy limitation of 10^{-8}.

Results and discussion

Systematization of commercial ENPs

Commercial ENPs of foreign and domestic production presented on the Russian market are of great variability both in manufacturing companies, ingredients and composition, quality indicators and intended purpose. Hereinafter, the term "ingredients" refers to the elements of the formulation and the term "components" refers to the micronutrients and macronutrients. Intended purpose refers to a pathology for which the manufacturer formally declares the applicability of a specific ENP. As a result of systematization of the source data set, ENP applicability matrix by intended purpose (Table 1) and components/indicators matrix (supplemental file) were generated.

Analysis of the applicability matrix showed that, in terms of occurrence, the intended purposes of the studied commercial ENPs significantly differ (Table 2). In the studied set, no more than six products were noted to be used for hypercatabolism or cardiovascular diseases. At the same time, 38 products have been identified as being used for oncological diseases.

For example, in the ENP set, the occurrence of intended purposes belonging to the first quartile is more than four times higher than that for the fourth quartile. This distribution is presumably related to the current distribution of nutrient supply demand regarding ENPs. At the same time, there is some probability of mistake in this assumption due to the initial limitation of the sample. The use of quartiles I and IV for comparison was assumed due to the fact that the extreme quartiles of almost any data set that includes different numerical values are poles in the range, i. e. they have the maximum possible differences.

Determining the maximum error probabilities for inequality of mean values

The design of ENPs within the framework of formal criteria for meeting a certain intended purpose involves the resulting set of formulations. However, the multiplicity of results underlying the combinatorial approach *a priori* implies some variability. It concerns both the composition

Table 1. ENP applicability matrix by intended purpose

Intended purpose	ENP codes *
Diabetes mellitus	1, 10, 12, 20, 22, 30, 47
Preparing for surgery	2 to 4, 6 to 8, 11 to 13, 16, 17, 20 to 23, 26, 28 to 30, 33, 34, 37 to 40, 43, 51
Postoperative period	2 to 4, 6 to 14, 16 to 17, 19 to 23, 26 to 30, 32 to 40, 42, 43, 51, 52
Thermal injury	2, 4, 6, 7, 9, 11, 12, 14 to 21, 23, 30, 31, 33, 35, 37, 42, 44 to 47, 50
Sepsis	2, 6, 8, 9, 11, 12, 15 to 21, 30, 33, 35, 37, 42
Multiple injuries	4, 6 to 12, 14 to 21, 23, 31, 34 to 36, 42
Gastrointestinal diseases	3, 6, 7, 9, 11 to 24, 32, 34 to 40, 43 to 45, 47, 52
Eating disorders, dysphagia	4, 6, 9, 11 to 14, 16 to 23, 27, 28, 30, 33 to 35, 37 to 40, 43, 52
Oncological diseases	2, 3, 5, 6, 8, 11 to 13, 15 to 24, 26 to 28, 30 to 40, 43 to 47, 51, 52
Cardiovascular diseases	2, 6, 8, 9, 11, 13, 18 to 20
Cardiopulmonary diseases	2, 6, 17, 18, 21, 33, 50
Mental illness	9, 11 to 14, 17 to 23, 29, 30, 34 to 40, 43, 44
Neurological disorders	3, 5, 6, 12 to 24, 28, 30, 31, 34 to 40, 43
Geriatrics	34, 35, 37, 44
Cystic fibrosis	2, 13, 20, 23, 44 to 47
Liver diseases	2, 8, 9, 11, 13, 17 to 23, 29, 30, 34, 35, 41, 48
Kidney failure	26, 41, 49
Coma	3, 6, 31, 34 to 36, 50
AIDS **	2, 5, 11 to 13, 17 to 23, 31, 36 to 40, 42
Cachexia	2, 3, 5, 6, 8, 9, 11, 16 to 18, 20, 29 to 31, 34 to 38, 41 to 43, 51
Multiple organ failure	6 to 9, 16, 18, 36, 42
Palliative conditions	44 to 47, 50
Critical conditions	44 to 47
Covid-19	22, 33, 50

* Product codes are determined in "Objects and methods" section.

** AIDS, acquired immunodeficiency syndrome.

Table 2. Occurrence of intended purposes in the set of studied ENPs*

Intended purpose	Occurrence	
	Frequency	Quartile
Oncological diseases	38	I
Postoperative period	37	
Thermal injury	29	
Eating disorders, dysphagia	28	
Neurological disorders	26	II
Preparing for surgery	26	
Gastrointestinal diseases	23	
Multiple injuries	22	
Cachexia	22	
AIDS	19	III
Sepsis	18	
Liver diseases	16	
Mental illness	16	
Cystic fibrosis	9	
Diabetes mellitus	7	IV
Coma	7	
Cardiopulmonary diseases	7	
Hypercatabolism	6	
Cardiovascular diseases	6	

* The table does not include intended purposes with an occurrence of 4 or less.

itself and its potential effect on the human body with pathology corresponding to the intended purpose of ENP. Existing methods for assessing this effect determine the nutrient bioavailability of the product. Due to their *a posteriori* nature, such methods are effective as a final control, but not in the process of actively generating many optimal formulations. To increase the efficiency of searching for an optimal set of solutions, let us postulate that the functional ENPs on the market have fairly high bioavailability. Let us also postulate that, other factors being equal, the bioavailability of an ENP specialized in relation to pathology is determined by the key components in its composition, which have statistical differentiation from the general set of other ENPs. Consequently, at the product design stage, there is a possibility of predictive assessment of its bioavailability. At the same time, taking into account the above postulates, to determine the predicted bioavailability, let us use a comparative statistical analysis of data from the matrix of components and main indicators of commercial ENPs. As an example, let us consider the determination of the predicted bioavailability of commercial ENPs for intended purposes, related to quartiles I and IV in Table 2. Let us take diabetes mellitus and thermal injury as such intended purposes.

For each intended purpose, from the considered set of products D, we will generate two non-overlapping subsets D_1 and D_0 (Figure 1).

Subset D_1 will include the set of all ENPs for the given intended purpose. Subset D_0 will include all the other products of set D that are not intended for the given intended purpose. In this case, the main condition for the existence of set D and subsets D_1 and D_0 is the following:

$$D_1 \subset D \wedge D_0 \subset D \wedge D_1 \cup D_0 = D \wedge D_1 \cap D_0 = \varnothing. \quad (1)$$

Furthermore, within set D and subsets D_1 и D_0, we will leave only those components and indicators whose number of numerical values both within subset D_1 and subset D_0 is at least two. Thus, both for subsets D_1 and D_0 and for set D, the value of z will be the same. As a result, each of the remaining components and indicators a_j will correspond to a certain number of numerical values both within subset D_1 and subset D_0. Thus, within each of the subsets, statistical processing [40] may be carried out in relation to each of these components and indicators to find the mean value ($\overline{du}_j$ and $\overline{eu}_j$, respectively) and the value of the variation interval ($d\Delta_j$ and $e\Delta_j$, respectively):

- for subset D_1

$$\overline{du}_j = \frac{\sum_{i=1}^{dk_j} du_{ij}}{dk_j}, \quad (2)$$

$$\pm d\Delta_j = t_{(d\alpha,\, dk_j - 1)} \cdot \frac{SD_j}{\sqrt{dk_j}}, \quad (3)$$

- for subset D_0

$$\overline{eu}_j = \frac{\sum_{i=1}^{ek_j} eu_{ij}}{ek_j}, \quad (4)$$

$$\pm e\Delta_j = t_{(e\alpha,\, ek_j - 1)} \cdot \frac{SD_j}{\sqrt{ek_j}}, \quad (5)$$

where du_{ij} and eu_{ij} are i-th numerical value of j-th component or indicator of subsets D_1 and D_0, respectively; SD is standard deviation; t is Student's test; dk_j and ek_j are the number of numerical values of j-th component or indicator of subsets D_1 and D_0, respectively; $d\alpha$ and $e\alpha$ are error probability value for subsets D_1 and D_0, respectively.

Let us assume that the values of $d\alpha$ and $e\alpha$ are equal. Due to the physical meaning of Student's test, the smaller the values of $d\alpha$ and $e\alpha$, the greater the values of $d\Delta_j$ and $e\Delta_j$, and vice versa. Therefore, when $\overline{du}_j \neq \overline{eu}_j$, there inevitably exists a value of $d\alpha$ and $e\alpha$ (let us denote it as α_m), at which the following condition will be true

$$\begin{cases} \dfrac{\overline{du}_j - d\Delta_j}{\overline{eu}_j + e\Delta_j} = 1, \overline{du}_j > \overline{eu}_j \\ \dfrac{\overline{du}_j + d\Delta_j}{\overline{eu}_j - e\Delta_j} = 1, \overline{du}_j < \overline{eu}_j \end{cases}. \quad (6)$$

When α_m is exceeded (if $\alpha_m < 1$), the variation intervals formed by the mean values of similar components or indicators of two subsets D_1 and D_0 and by the corresponding variation intervals do not overlap. In other words, α_m represents the maximum error probability for inequality of mean values. Due to the limited nature of the general set of ENPs under consideration, let us assume a value of α_m within 0.2 as a factor for the statistical acceptability of differences in mean values.

Components/ indicators	Products			
	1	2	…	k
a_1	u_{11}	u_{12}	…	u_{1k}
a_2	u_{21}	u_{22}	…	u_{2k}
a_3	u_{31}	u_{32}	…	u_{3k}
…	…	…	…	…
a_z	u_{z1}	u_{z2}	…	u_{zk}

set D

=

Components/ indicators	Products		
	d_1	…	d_k
a_1	du_{11}	…	du_{1dk}
a_2	du_{21}	…	du_{2dk}
a_3	du_{31}	…	du_{3dk}
…	…	…	…
a_z	du_{z1}	…	du_{zdk}

subset D_1

+

Components/ indicators	Products		
	e_1	…	e_k
a_1	eu_{11}	…	eu_{1ek}
a_2	eu_{21}	…	eu_{2ek}
a_3	eu_{31}	…	eu_{3ek}
…	…	…	…
a_z	eu_{z1}	…	eu_{zek}

subset D_0

Figure 1. Generation of subsets D_1 and D_0 from set D

Table 3. Maximum error probability matrix for inequality of α_m mean values

Diabetes mellitus		Thermal injury	
components/indicators	α_m	components/indicators	α_m
components		*components*	
saturated fatty acids	0.01449*	taurine	0.15066
monounsaturated fatty acid	0.03319	medium-chain triglycerides	0.05634
carbohydrates	0.11769	docosahexaenoic acid	0.05385
pantothenic acid B5	0.19965	carbohydrates	0.13299
pyridoxine B6	0.15404	sugars	0.12888
biotin	0.04297	lactose	0.19027
indicators		biotin	0.04294
glycemic index	0.02698	*indicators*	
		dietary fibers	0.16446
		caloric value	0.14725
		osmolarity	0.14228

* Highlighted values are within $\alpha \le 0.05$

Carrying out statistical processing for each similar component and indicator of both subsets made it possible to identify those for which ENPs with given intended purpose are statistically acceptable different from the rest. The results of α_m determination for diabetes mellitus and thermal injury are presented in Table 3. The values of α_m were calculated by numerical methods in the environment of Microsoft Excel 2010 spreadsheet processor (Microsoft Ink.), using the method of searching for solutions to problems by the simplex method with automatic scaling and accuracy limitation of 10^{-8}.

The calculation results made it possible to identify six components and one indicator as acceptable in the case of diabetes mellitus. In the case of thermal injury, there are already seven components and three indicators. It is worth noting that, due to their nature, acceptable indicators for differentiating subsets indirectly include a set of components not included in the final tables and do not increase their dimensions.

It is particularly remarkable that for individual components and indicators, the value of the maximum error probability for inequality of mean values is in the range of 0.05 to 0.06. Among the components, both for diabetes mellitus and for thermal injury, these include fatty acids or triglycerides, and biotin.

Determination of the predicted bioavailability vector

Due to natural statistical variability, all values of each of z components and indicators within subset D_1 are in the interval between a certain minimum $du_{j(\min)}$ and maximum $du_{j(\max)}$. Consequently, in contrast to the arithmetic mean, the most typical value will correspond to the median $du_{j(\text{med})}$. Thus, the general condition for the existence of a statistically acceptable component or indicator a_j of ENP with given intended purpose is as follows:

$$\forall a_j\Big|_{\alpha_m \le 0.2} : \quad a_j\left[du_{j(\min)}; du_{j(\max)}; du_{j(med)}\right]. \quad (7)$$

Therefore, the closer the numerical value of a component or indicator is to $du_{j(\text{med})}$, the more typical it will be for the given intended purpose. Whereas extreme values will be on the border of acceptability. In this case, for an impersonal assessment of the optimality of the component or indicator value, we can use Harrington's two-way desirability function [40,41]:

$$d_j = \exp\left(-\left|q_j\right|^{n_j}\right), \quad (8)$$

where d_j is a particular value of the desirability function;

$q_j = \dfrac{2 \cdot du_j - \left(du_{j(\max)} + du_{j(\min)}\right)}{du_{j(\max)} - du_{j(\min)}}$ is an argument of the desirability function;

n_j is exponent.

Due to the specific feature of this type of Harrington desirability function, extreme values are *a priori* assumed to be equal to $1/e$ (approximately 0.37), where e is the base of the natural logarithm. Intermediate may be assigned a value of 0.63 or 0.8 [41,42]. Since, in this case, we postulated that the median value of each partial sample obtained was close to the ideal bioavailability value for the selected product range, we chose a value of 0.8. Then, in accordance with the methodology for finding the parameter n_j of Harrington's two-way desirability function, we compare the reference value of desirability $q_{j(\text{mid})}$ equal to 0.8 to intermediate (in our case, median $du_{j(\text{med})}$) values of the argument. Then, in accordance with [41], we can determine n_j using the formula:

$$n_j = \frac{\ln\left\{-\ln\left[d_{j(mid)}\right]\right\}}{\ln\left[q_{j(mid)}\right]}. \quad (9)$$

The calculation results are presented in Table 4.

As the table data shows, the values of n_j vary within fairly wide limits. However, it is not possible to give an unambiguous analytical assessment of these circumstances due to the highly empirical nature of the indicator.

Specific feature of Harrington's particular desirability functions is the possibility of involving their arbitrary population in the integral estimate of the desirability indicator in relation to the object being studied. The integral value of

Table 4. Matrix of exponents n_j

Diabetes mellitus		Thermal injury	
components/indicators	n_j	components/indicators	n_j
components		*components*	
saturated fatty acids	0.9320	taurine	18.7392
monounsaturated fatty acid	0.9320	medium-chain triglycerides	4.4578
carbohydrates	1.3653	docosahexaenoic acid	1.2726
pantothenic acid B5	1.0820	carbohydrates	0.6133
pyridoxine B6	1.3653	sugars	9.9676
biotin	0.3257	lactose	12.1716
indicators		biotin	3.7753
glycemic index	0.9927	*indicators*	
		dietary fibers	1.7703
		caloric value	0.9922
		osmolarity	1.5434

Table 5. Predicted bioavailability vectors of ENPs for diabetes mellitus

Item No.	Product ID	d	$\vec{v}_{BA}$
1	10	0.649872	0.350128*
2	30	0.592027	0.407973
3	47	0.569146	0.430854
4	20	0.507267	0.492733
5	1	0.494916	0.505084
6	12	0.474662	0.525338
7	22	0.388335	0.611665

* Treatments with high predicted bioavailability ($\vec{v}_{BA} \le 0.37$)

Table 6. Predicted bioavailability vectors of ENPs for thermal injury

Item No.	Product ID	d	$\vec{v}_{BA}$	Item No.	Product ID	d	$\vec{v}_{BA}$
1	18	0.719421	0.280579*	16	47	0.557450	0.442550
2	20	0.680163	0.319837*	17	15	0.556581	0.443419
3	17	0.653087	0.346913*	18	2	0.548933	0.451067
4	42	0.641100	0.358900*	19	45	0.545660	0.454340
5	16	0.617214	0.382786	20	9	0.538522	0.461478
6	14	0.610536	0.389464	21	11	0.533331	0.466669
7	19	0.608547	0.391453	22	12	0.526965	0.473035
8	31	0.604305	0.395695	23	50	0.482528	0.517472
9	36	0.601881	0.398119	24	30	0.477161	0.522839
10	46	0.600013	0.399987	25	44	0.473995	0.526005
11	37	0.590680	0.409320	26	34	0.472923	0.527077
12	33	0.585353	0.414647	27	6	0.469957	0.530043
13	21	0.579623	0.420377	28	7	0.389956	0.610044
14	35	0.575514	0.424486	29	23	0.367879	0.632121
15	4	0.565899	0.434101				

* Treatments with high predicted bioavailability ($\vec{v}_{BA} \le 0.37$)

desirability (d) may be defined as the geometric mean for the values of particular desirability functions for components and indicators within each analyzed product [41,42]:

$$d = \sqrt[z]{\prod_{j=1}^{z} d_j}\ , \qquad (10)$$

Ideally, the integral desirability values should tend to be equal to 1. However, due to the orientation of the scale, direct use of the integral desirability value to indirectly assess predicted bioavailability is quite inconvenient. In this regard, we introduce a synthetic indicator, predicted bioavailability vector, which is equal to the distance in the metric of integral desirability values to the value of one:

$$\vec{v}_{BA} = 1 - d, \qquad (11)$$

In other words, the lower the value of $\vec{v}_{BA}$, the higher the predicted bioavailability, and vice versa. In this case, the upper limit of the optimal value of the predicted bioavailability vector is 0.37, and the upper limit of the acceptable value is 0.63. Then the maximum predicted bioavailability will correspond to the predicted bioavailability vector value equal to zero.

The results of $\vec{v}_{BA}$ determination for the studied ENPs according to their intended purpose are presented in Tables 5 and 6. The data were ranked in order of predicted bioavailability decreasing.

Analysis of the data obtained showed that ENPs specialized for their intended purpose form a fairly wide range of potential bioavailability. Moreover, among the ENPs specialized for diabetes mellitus, only one corresponded to the optimum area of predicted bioavailability. Among products specialized for thermal injury, there were already four of such nearly optimal ones. At the same time, in the subsets of both intended purposes, there were ENPs for which the predicted bioavailability vector was in the upper limit of the acceptability area. In the subset of products for diabetes mellitus, there was one such product, and in the subset of products for thermal injury, there were two such

products. Such products are formally in the acceptability area, but in relation to the intended purpose, they need to be adjusted in composition to reduce $\vec{v}_{BA}$.

The use of the predicted bioavailability vector in enteral nutrition products based on a comparative statistical analysis of the components/indicators matrix for commercial products allows us to neutralize the entire series of consequences arising from the nature of the currently used bioavailability indicator. At the same time, the need for clinical studies of developed products is not completely eliminated. But these studies move into the category of validation of a new product within the target significance. They are not involved in the design process. Instead, this function falls on the predicted bioavailability vector.

Moreover, with the practical application of the predicted bioavailability vector, the potential for its evolutionary development arises, the principle of which is presented in Figure 2.

This principle assumes that during each application of the predicted bioavailability vector, components and indicators of the designed new ENP are included in the components/indicators matrix after its clinical validation. At the same time, due to the increase in the number of products in the matrix and in the subset of products with the given intended purpose, a statistical refinement of intervals, medians and the maximum error probabilities for inequality of mean values will inevitably occur. This in turn will lead to clarification of exponents and, as a consequence, the values of the predicted bioavailability vector. As a result, the development of each new product will become the next iteration of evolutionary development. In this case, the statistics will work according to the "black box" principle, gradually improving the result without the need for a large-scale simulation of individual component influence on the bioavailability, taking into account the disturbing influence of associated components.

Conclusion

In contrast to direct simulation of the bioavailability for individual nutrients, a comparative statistical analysis of the existing commercial ENPs preliminary differentiated by intended purpose may be a significant tool for predicting bioavailability. Dividing a set of products included in the original matrix into a subset corresponding to a given intended purpose and a subset of the remaining ENPs made it possible to isolate statistically significant components and indicators that identify the products with the intended purpose for a given threshold of the maximum error probability for inequality of mean values. The use of Harrington's desirability principle in relation to the identified components and indicators made it possible to obtain an integral estimate of desirability for a given intended purpose. A vector that characterizes the distance from the integral estimate to the ideal value was introduced fs the equivalent predicted bioavailability (1). The smaller the vector values, the higher the predicted bioavailability. The upper limit of the optimal range is 0.37, and the upper limit of the acceptable range is 0.63. This indicator allowed us to establish significant variability in commercial ENPs with respect to predicted bioavailability for diabetes mellitus and thermal injury. Based on the proposed predicted bioavailability vector, a principle has been developed for the evolutionary development of a statistical approach to determining predicted bioavailability when designing ENPs. This principle is a universal addition to the principle of food combinatorics when developing meat, dairy and plant-based ENPs.

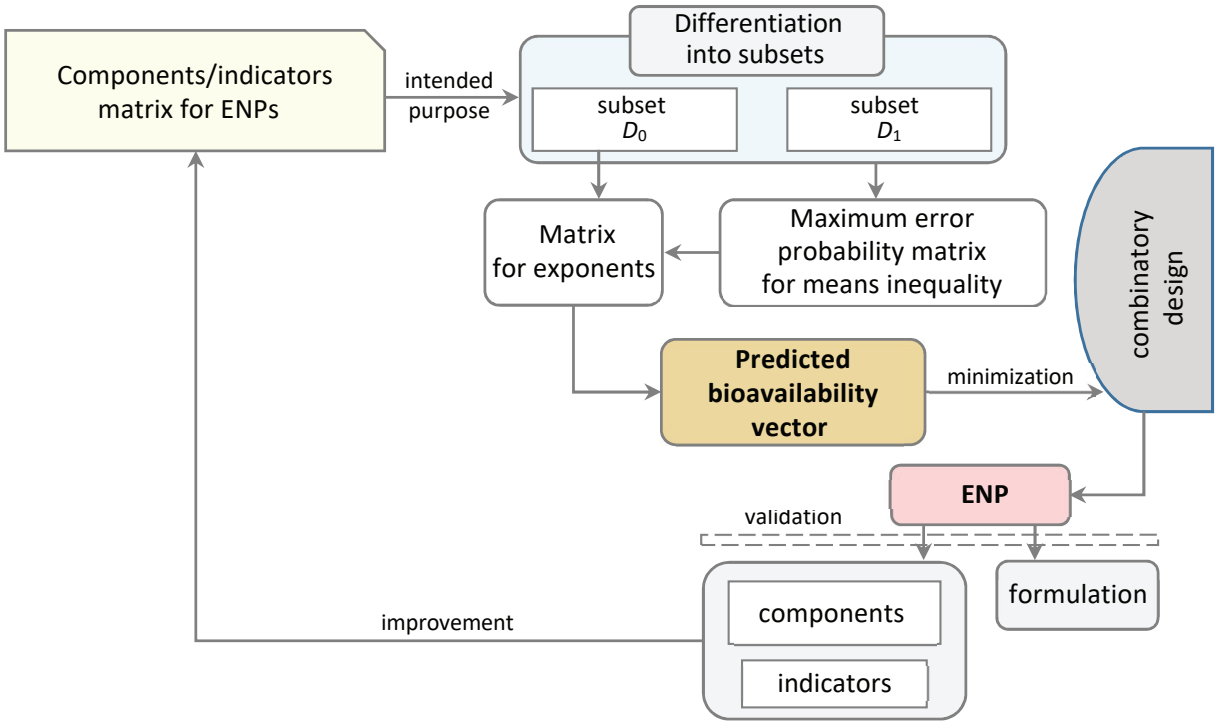

Figure 2. Evolutionary development principle of the statistical approach to determining the predicted bioavailability vector for the design of ENPs

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FOOD SAFETY KNOWLEDGE, ATTITUDE AND PRACTICES OF MEAT HANDLERS IN KHULNA CITY, BANGLADESH

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Keywords: *cross contamination, foodborne disease, food safety, meat handler, raw meat*

Abstract

The research was conducted to identify the present status of the food safety knowledge, attitude and practices of meat handlers in retail meat shops of Khulna City, Bangladesh. The research was performed through face-to-face interviews of randomly selected 65 meat handlers in six areas of Khulna City. Socio-demographic information of all respondents was collected. The illiteracy rate was 15.38%, while the majority (42.9%) of the respondents had secondary education. The highest proportion (43.07%) of meat handlers was low experienced in meat handling. Among the respondents, 58.46% were sellers, while no respondents had any training on butchery and food safety. All respondents worked at least three days a week and meat handling was the main occupation for all respondents. Most of the respondents (50.76%) had low media contact. The highest proportion (74.3%) of the respondents had medium (scores of 11 to 20) food safety knowledge. About 62.9% of the respondents had moderately favorable food safety attitude (scores of 28 to 54), while 60.0% of the respondents had medium food safety practices (scores of 15 to 28). The mean score of the food safety knowledge, attitude and practices was 18.65 ± 3.81, 50.71 ± 9.49 and 27.20 ± 3.22, respectively. This study shows that there is an identified gap in knowledge and correct practices among meat handlers and that there is a need to raise awareness about food safety through education programs regarding food safety and safe food handling practices. These findings can help public health professionals in developing initiatives to improve food safety knowledge and practices of meat handlers and prevent foodborne diseases (FBDs). The government should pay special attention for improving knowledge and ensuring proper food safety practices to avoid the transmission of FBDs in Khulna City.

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Introduction

Foodborne diseases (FBDs) occur both in developed and in developing countries, and each year 10% of the worlds' population falls ill and 420,000 people die after eating contaminated food [1]. In Bangladesh, FBDs are prevalent due to poor food handling practices, inadequate sanitation facilities, insufficient food safety legislation, weak regulatory systems, lack of financial resources to invest in food safety equipment, and lack of food handlers' education and knowledge. Microbiological agents causing infections, biotoxins and chemical pollutants in food pose significant health risks to millions of people [2,3]. Most FBDs are caused by animal-based foods. Worldwide, food safety is a great public health concern, especially when food is handled in a highly contaminated environment [3,4]. Food handlers should have solid food safety knowledge to prevent FBDs. Good knowledge and a positive attitude among food handlers, and proper food handling practices can help control FBDs [2,4]. In Bangladesh, food handlers' knowledge, attitude, and practice (KAP) concerning food safety and FBDs are essential in promoting food safety and safeguarding humans from FBDs. Red meat, which is rich in nutrients, is an appropriate substrate for the growth of a wide variety of microorganisms [5,6]. Food contamination is mostly caused by a poor food handler's health and hygienic practice, according to [7]. A KAP study is a representative study of a single community that uses questionnaires to collect data on what people know, believe, and do about a certain topic. In Bangladesh, there has been little research on vendors' awareness, attitudes, and experience regarding the presence of hazardous bacteria, such as *E. coli*, *Salmonella* sp., and *Staphylococcus aureus* in red meat, which can lead to food poisoning and spreading FBDs to humans [4,8,9,10,11]. Evidence from [3,12,13,14] shows that meat handlers with a greater understanding of food safety and suitable food handling methods have better food safety practices. However, there is also evidence of variations in food safety knowledge and practices among meat handlers. In a study similar to [13] meat handlers' educational level and professional training were positively associated with their knowledge and procedures pertaining to food safety [3]. Food handlers' training has been connected to food safety knowledge and practices, as well as sanitary and hygienic status, and product microbiological quality [15,16]. There are gaps in regular food safety training of handlers, notably training of under-supervised meat

handlers in butcher shops according to Bangladesh Food Safety Act 2013, which emphasizes knowledge-based food safety management systems. However, few studies have been conducted on the essential criteria that determine red meat handlers' KAP regarding food safety. These findings will support a better understanding of how the food safety KAP of handlers interacts across the country, as the study's purpose is to examine factors related to food safety KAP of red meat handlers in the Khulna City Corporation area, Bangladesh. These factors could hinder governments' abilities to accurately apply measures to address food contamination problems that affect public health. Therefore, this study was designed to assess food safety KAPs in meat handling in the Khulna City Corporation area, Bangladesh.

Material and Methods

Study area

Khulna is the third-largest city in Bangladesh, after Dhaka and Chittagong. It is the administrative center of Khulna District and Khulna Division. Khulna's economy is the third-largest in Bangladesh. In the 2011 census, the city had a population of 770,498 (male 423,496, female 347,002). The Khulna City Corporation area is 40.79 sq km, located between 24°45' and 24°54' north latitudes and between 89°28' and 89°35' east longitudes.

Sampling technique and data collection

A cross-sectional survey using a random sampling technique was conducted among 65 meat handlers from retail meat shops in Khulna City, Bangladesh (Figure 1). An interview schedule was developed in English for data collection. To obtain data on the identified variables, the interview schedule included both easy and straightforward questions. Before the final data collection, the interview schedule was pre-tested. Based on pre-test results, the necessary corrections, additions, changes, and re-arrangements were made in the interview schedule. The interview schedule was then finalized and multiplied to collect data.

An interview schedule was structured into four distinct parts including socio-demographic information including gender, age, level of education, year of experience, training on food safety/butchery, monthly income, family size, media contact/communication/organizational participation. For measuring a level of media contact of the respondents, 13 media types (radio, television, newspapers, online agricultural apps, CIG, clubs, tours, office call (ULO), office call (ADLO/DLO), mobiles, friends, relatives, neighbors) were incorporated in the interview schedule and the respondents were asked to indicate their level of media contact (regularly, often, occasionally, rarely and not at all) against 13 media types. Scores of 4, 3, 2, 1, and 0 were assigned to a level of media contact. Similarly, three places (houses of friends, relatives and others, other upazilla headquarters, other district towns) were incorporated for measuring the level of communication and six organizations (Govt. mentioned organization, NGO, Bazar committee, Masjid/Madrasha/Mandir, Co-operative society/society, CIG of DLS) were incorporated for measuring the level of organizational participation. Scores of 2, 1, and 0 were assigned for an executive member, general member and no membership, respectively.

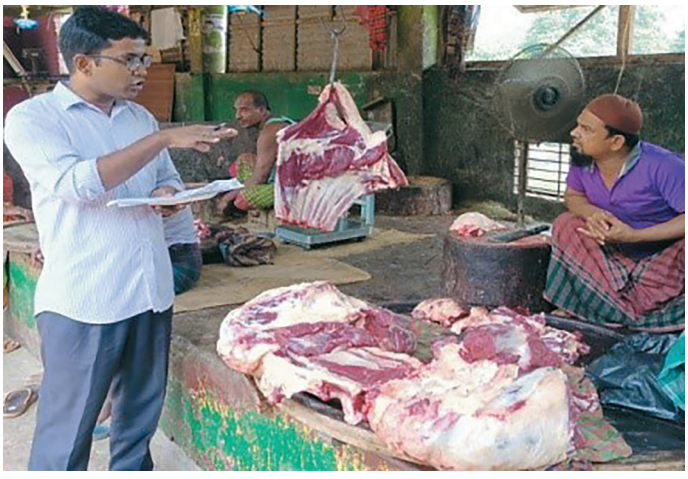

Figure 1. Face-to-face interview of a meat handler

The second section was about food safety knowledge. Questions on food safety knowledge referred to their personal hygiene, symptoms of foodborne diseases, time-temperature control etc. For measuring the knowledge of the respondents, 15 questions were incorporated into the interview schedule and each question had three optional answers ("full", "half" and "don't know"). Scores of 2, 1and 0 were assigned to "full", "half" and "don't know", respectively. The knowledge score of a respondent was calculated by summing up all scores for 15 selected questions. The knowledge score could range from 0 to 30, where 0 indicates the lowest knowledge and 30 indicates the highest knowledge. Meat handlers that got an overall score of ≤10 points were considered to have low knowledge, those scored 11 to 20 points medium knowledge and ≥21 points high knowledge about food safety.

The third section was about food safety attitudes. For measuring an attitude of the respondents, 16 statements (8 +ve and 8 -ve) were incorporated in the interview schedule. A Likert Type 5-point rating scale (strongly agree, agree, undecided, disagree and strongly disagree) was employed against 16 statements. Scores of 5, 4, 3, 2 and 1 were assigned to positive statements and opposite scores (1, 2, 3, 4 and 5) were assigned to negative statements, respectively. An attitude score of a respondent was calculated by summing up all scores against 16 selected statements. The attitude score could range from 16 to 80 where ≤27 points indicate a less favorable attitude, 28 to 54 points indicate a moderately favorable attitude and ≥55 points indicate a highly favorable attitude.

The fourth section was about food safety practices. To measure the score of food safety practices of the respondents, 14 practices were included. The respondents were asked to indicate their level of food safety practices (regularly, occasionally, rarely and not at all) against 14 practices. Scores of 3, 2, 1 and 0 were assigned to a level of food

safety practices. The practice score of the respondents was calculated by adding all scores for 14 practices. The food safety practices score could be in a range from 0 (lowest scores) to 42 (highest scores) where ≤14 points indicate low food safety practices,15 to 28 points indicate medium food safety practices and ≥29 points indicate high food safety practices.

Statistical analysis

After collection, data were analyzed according to the objectives following the SPSS version 20. Statistical measures, such as number, percentage (%), range, standard deviation, and mean, were used to interpret the data.

Results

Sociodemographic characteristics of the respondents

The sociodemographic profiles of the respondents are summarized in Table 1. All respondents (100.0%; N = 65) were males. The mean age of the respondents was 36.22 ± 8.34, ranging between the ages of 19 to 53 years. Most of the respondents (42.9%) had secondary education, followed by a considerable amount of illiterate (22.0%) and primary education (22.0%). More than half (54.3%) of the respondents were sellers. The majority of the respondents (57.1%) had a monthly income of 13,000 BDT to 25,000 BDT. A greater number (42.9%) of respondents had working experience of up to 10 years with an average length of 16.00 ± 9.65 years. All respondents (100.0%; N=65) worked at least three times a week. No respondents had training in both food safety and butchery. Although most of the studies have shown that training may contribute to upgrading the food safety knowledge of food handlers, this does not always translate into a positive change in food handling behavior and attitudes [11,17]. Approximately 51.4% of the meat handlers had 5–6 family members with an average number of family members equal to 5.02 ± 2.06.

Media contact/communication/organizational participation

Media contact/ communication /organizational participation of the respondents are summarized in Table 2. Most of the respondents (54.3%) had low media contact

Table 1. Sociodemographic characteristics of the respondents

Characteristics	Categories	Number(N)	Percentage (%)	Mean ± SD	Range
Gender	Male	65	100		
	Female	0	0.0		
Age (Years)					
(Up to 35)	Young aged	33	50.76	36.22 ± 8.34	19–53
(36–50)	Middle aged	28	43.07		
(>50)	Old aged	4	6.15		
Level of education (Year of schooling)					
(0)	Illiterate	10	15.38		
(1–5)	Primary	17	26.15	6.14 ± 4.66	0–17
(6–10)	Secondary	29	44.61		
(11–12)	Higher Secondary	5	7.69		
(>12)	Above Higher Secondary	4	6.15		
Field of Duty	Helper	4	6.15		
	Seller	38	58.46		
	Entrepreneur	1	1.53		
	Entrepreneur and Seller	22	33.84		
	Butcher	0	0.0		
Monthly income					
Up to 12,000 BDT	Low	15	23.07	19.02 ± 7.50	12–40
13,000–25,000 BDT	Medium	37	56.92		
>25,000 BDT	High	13	20.0		
Year of experience					
Up to 10	Low	28	43.07	6.00 ± 9.65	1–38
11–20	Medium	19	29.23		
>21	High	18	27.69		
Working day per week					
1–2	One or two times a week	0	0.0		
3–6	At least three times a week	65	100.0		
7	Daily	0	0.0		
Food Safety Training	Yes	0	0.0		
	No	65	100.0		
Training on Butchery	Yes	0	0.0		
	No	65	100.0		
Family size					
Up to 4	Small	15	42.9	5.02 ± 2.06	2–15
5–6	Medium	18	51.4		
>6	Large	2	5.7		

followed by 45.7% of the respondents who had medium media contact. The mean score of the media contact was 19.37 ± 4.98 and the maximum score was 35. There was a medium communication among most of the respondents. The mean score of the communication was 4.29 ± 1.20 and the maximum score was 7. About 68.6% of respondents had low organizational participation and the mean score of the organizational participation was 4.29 ± 1.20. The maximum score was 8.

Table 2. Media contact/communication/organizational participation

	Scores	Categories	Number (N)	Percentage (%)	Mean ± SD	Range
Media contact	≤17 18–35 ≥36	Low Medium High	33 32 0	50.76 49.23 0.0	19.37 ± 4.98	13–35
Communication	≤3 4–6 ≥7	Low Medium High	22 39 4	33.84 60.00 6.15	4.29 ± 1.20	3–7
Organizational participation	≤4 5–8 ≥9	Low Medium High	45 20 0	69.23 30.76 0.0	4.69 ± 1.32	3–8

Food safety knowledge

The overall food safety knowledge of the respondents is summarized in Table 3. About 74.3% of respondents had a medium level of food safety knowledge with a mean score of 18.65 ± 3.81. Only 10.47% of respondents could name two health problems arising due to improper handling of meat. Most of the respondents (63.07%) knew what cleaning agents are used for washing hands. About 60.0% of the respondents knew how long meat remains safe without using preservatives. Among the respondents, 55.38% knew that diarrhea can be transmitted through food and they also knew how to prevent it. Respondents had the least knowledge about storage time without using preservatives (53.84%), the fact that irregular washing of hands before and after handling of meat causes health problems (30.76%), disinfectants used for disinfecting working surfaces and types of equipment (15.38%), main practices to keep food safe (38.46%), the fact that uncovered meat is more unsafe than covered meat (36.92%), food adulteration and commonly used adulterants in food (35.38%), health problems associated with food adulteration (26.15%), protective equipment used during meat processing (23.07%). Most of the respondents had good knowledge about safe water for washing meat (76.92%), changes in deteriorated meat (92.30%), and a refrigerator as an ideal place to store meat (55.38%).

Attitude about food safety

The overall food safety attitude of the respondents is summarized in Table 4. About 62.9% of respondents had a moderately favorable attitude toward food safety with a mean score of 50.71 ± 9.49. The highest score was 26 and the lowest score was 69. About 29.0% and 52.0% of respondents strongly agreed and agreed with the statement that people with open skin injury, gastroenteritis, and ear or throat diseases should not be allowed to touch/ handle meat. Only 3.07% of respondents disagreed that regular training can improve meat safety and hygiene practices. Only 10.76% of respondents agreed with the statement that wearing watches, earrings, and rings will increase the risk of meat contamination, whereas 26.15% of respondents strongly agreed that regular waste disposal reduces the risk of contamination. About 29.0% and 57.0% of respondents strongly agreed and agreed that keeping working surfaces and utensils clean reduces the risk of illness. Only 23.07% of respondents thought that knives, hooks and cutting boards can be a source of food contamination. An extremely small

Table 3. Food safety knowledge of the respondents

Knowledge	Percentage (Number)			
	Don't know	Half	Full	Total
Can you name two health problems arising due to improper handling of meat?	20.0(13)	69.23(45)	10.47(7)	100.0(65)
What are cleaning agents used for washing hands?	0.0(0)	36.92(24)	63.07(41)	100.0(65)
How long does your meat remain safe in an open place without using any preservative?	12.30(8)	26.15(17)	61.53(40)	100.0(65)
Is diarrhea transmitted through food? If yes, how to prevent it?	7.69(5)	36.92(24)	55.38(36)	100.0(65)
Is it possible to preserve meat for a long time without using any preservative? If yes, how will you do this?	3.07(2)	43.07(28)	53.84(35)	100.0(65)
Does irregular washing of hands before and after handling of meat cause any health problems? If yes what are the health problems?	3.07(2)	66.15(43)	30.76(20)	100.0(65)
What are the disinfectants used for disinfecting working surface and equipment?	23.07(15)	61.53(40)	15.38(10)	100.0(65)
Can you name two items of protective equipment used during meat processing?	46.15(30)	30.76(20)	23.07(15)	100.0(65)
Is pond water safe for washing meat? If not, what is the source of safe water?	4.61(3)	18.46(12)	76.92(50)	100.0(65)
Can you name three main practices to keep food safe?	3.07(2)	58.46(38)	38.46(25)	100.0(65)
Is uncovered meat more unsafe than covered meat? If yes what is the reason?	4.61(3)	58.46(38)	36.92(24)	100.0(65)
Is there any change in deteriorated meat? If yes, what type of changes occurs?	0.0(0)	7.69(5)	92.30(60)	100.0(65)
What is food adulteration? Can you name one commonly used adulterant in food?	18.46(12)	46.15(30)	35.38(23)	100.0(65)
What are the health problems associated with food adulteration?	12.30(8)	61.53(40)	26.15(17)	100.0(65)
Is a refrigerator an ideal place to store raw meat? If yes, what is the reason?	6.15(4)	38.46(25)	55.38(36)	100.0(65)
Mean score	18.65 ± 3.81			

percentage of respondents strongly agreed (6.15%) and agreed (7.69%) that drinking or eating in the workplace increases the risk of contamination. About 31.0% of respondents were undecided whether insects and pests can be a source of raw meat contamination. Most of respondents (58.0%) disagreed with the statement that wearing protective clothing and shoes does not improve work safety and hygiene practices. About 11.0% and 52.0% of respondents strongly agreed and agreed that the use of potable water to wash working surfaces and cutting tools is important.

About 28.0% of respondents strongly disagreed that working surfaces and equipment should not be cleaned before re-using for meat processing, whereas 16.92% of respondents strongly disagreed that covering the nose or mouth when sneezing or coughing cannot reduce contamination. A very low percentage (15.38%) of respondents agreed that inspecting meat for freshness and wholesomeness is not valuable. Only 7.69% of the respondents strongly agreed that high temperature or freezing is unsafe for meat preservation whereas only 3.07% of respondents strongly agreed that we should use non-potable water for meat processing. Almost none of the respondents disagreed that smoking in the workplace does not increase the risk of contamination (3.07%).

Food safety practices

Table 5 shows the food safety practices of the meat handlers. About 60.0% of the respondents had medium food safety practices with a mean score of 27.20 ± 3.22 where the lowest and highest scores were 19 and 3, respectively. It was found that 50.76% of the respondents washed their hands regularly before and after handling meat. About 85.0% of the respondents washed their hands regularly after handling waste/garbage and disposed waste regularly after working. Most of the respondents covered their meat (69.23%), used potable water to process meat or to wash working surfaces and cutting tools (100.0%), used a sanitizer when washing service utensils (knives, hooks and cutting boards) (53.84%), avoided meat processing when they were ill especially due to gastroenteritis, cough or skin diseases (58.46%), used soaps or detergents after using toilet (93.84%) regularly. Concerning using washing agents, 50.76% of respondents used washing agents such as soap or detergent when washing hands occasionally. Most of the respondents did not remove their jewelry materials while handling meat (53.84%), did not avoid smoking in the workplace (58.46%), and did not replace knives after each meat processing (55.38%). About 48.0% and 35.0% of the respondents checked their meat for freshness and wholesomeness regularly and occasionally, respectively. Concerning hand washing after sneezing or coughing, none of the respondents washed their hands regularly, whereas 30.76% and 38.46% of the respondents washed their hands occasionally and rarely, respectively.

Discussion

Unlike other food processing, males are most likely involved in meat processing [16,18,19]. This is also true for our finding. The mean age of the respondents in this study (36.22 ± 8.34) is lower than that in the studies conducted

Table 4. Food safety attitude of the respondents

Statement	Percentage (Number)					
	Strongly agree	Agree	Undecided	Disagree	Strongly disagree	Total
People with open skin injury, gastroenteritis, and ear or throat diseases should not be allowed to touch/ handle meat.	29.23(19)	52.30(34)	12.30(8)	6.15(4)	0.0(0)	100.0(65)
Regular training can improve meat safety and hygiene practices.	10.76(7)	58.46(38)	27.69(18)	3.07(2)	0.0(0)	100.0(65)
Wearing watches, earrings and rings will increase the risk of meat contamination.	0.0(0)	10.76(7)	58.46(38)	7.69(5)	23.07(15)	100.0(65)
Regular waste disposal reduces the risk of contamination.	26.15(17)	55.38(36)	7.69(5)	10.76(7)	0.0(0)	100.0(65)
Keeping working surfaces and utensils clean reduces the risk of illness.	29.23(19)	56.92(37)	6.15(4)	7.69(5)	0.0(0)	100.0(65)
Knives, hooks and cutting boards can be a source of food contamination.	0.0(0)	23.07(15)	30.76(20)	32.30(21)	13.84(9)	100.0(65)
Drinking or eating in the workplace increases the risk of contamination.	6.15(4)	7.69(5)	38.46(25)	24.61(16)	23.07(15)	100.0(65)
Insects and pests can be a source of raw meat contamination.	12.30(8)	21.53(14)	30.76(20)	29.30(19)	6.15(4)	100.0(65)
Wearing protective clothing and shoes does not improve work safety and hygiene practices.	3.07(2)	18.46(12)	20.0(13)	58.46(38)	0.0(0)	100.0(65)
It is not important to use potable water to wash working surfaces and cutting tools.	13.84(9)	7.69(5)	15.38(10)	52.30(34)	10.76(7)	100.0(65)
Covering the nose or mouth when sneezing or coughing cannot reduce contamination.	7.69(5)	13.84(9)	10.76(7)	50.76(33)	16.92(11)	100.0(65)
Working surfaces and equipment should not be cleaned before re-using for meat processing.	6.15(4)	9.23(6)	6.15(4)	50.76(33)	27.69(18)	100.0(65)
Inspecting meat for freshness and wholesomeness is not valuable.	0.0(0)	15.38(10)	33.84(22)	46.15(30)	4.61(3)	100.0(65)
High temperature or freezing is unsafe for meat preservation.	7.69(5)	9.23(6)	23.07(15)	53.84(35)	6.15(4)	100.0(65)
We should use non-potable water for meat processing.	3.07(2)	7.69(5)	15.38(10)	61.53(40)	12.30(8)	100.0(65)
Smoking in the workplace does not increase the risk of contamination.	35.38(23)	30.76(20)	30.76(20)	3.07(2)	0.0(0)	100.0(65)
Mean score	50.71 ± 9.49					

Table 5. Food safety practices of the respondents

Practices	Percentage (Number)				
	Regularly	Occasionally	Rarely	Not at all	Total
Do you wash your hands before and after handling of meat?	50.76(33)	49.23(32)	0.0(0)	0.0(0)	100.0(65)
Do you wash hands after handling waste/garbage?	84.61(55)	15.38(10)	0.0(0)	0.0(0)	100.0(65)
Do you dispose waste after working?	83.07(54)	16.92(11)	0.0(0)	0.0(0)	100.0(65)
Do you use washing agents such as soap or detergent when washing hands?	43.07(28)	50.76(33)	6.15(4)	0.0(0)	100.0(65)
Do you remove your jewelry materials while handling meat?	20.00(13)	15.38(10)	10.76(7)	53.84(35)	100.0(65)
Do you cover your meat?	69.23(45)	6.15(4)	3.07(2)	21.53(14)	100.0(65)
Do you check your meat for freshness and wholesomeness?	47.69(31)	35.38(23)	15.38(10)	3.07(2)	100.0(65)
Do you wash your hands after sneezing or coughing?	0.0(0)	30.76(20)	38.46(25)	30.76(20)	100.0(65)
Do you use potable water to process meat or to wash working surfaces and cutting tools?	100.0(65)	0.0(0)	0.0(0)	0.0(0)	100.0(65)
Do you avoid smoking in the workplace?	20.0(13)	13.84(9)	7.69(5)	58.46(38)	100.0(65)
Do you use a sanitizer when washing service utensils (knives, hooks and cutting boards)?	53.84(35)	30.76(20)	15.38(10)	0.0(0)	100.0(65)
Do you replace knives after each meat processing?	0.0(0)	0.0(0)	44.61(29)	55.38(36)	100.0(65)
Do you avoid meat processing when you are ill especially due to gastroenteritis, cough or skin diseases?	58.46(38)	18.46(12)	12.30(8)	10.76(7)	100.0(65)
Do you use soaps or detergents after using toilet?	93.84(61)	6.15(4)	0.0(0)	0.0(0)	100.0(65)
Mean score	27.20 ± 3.22				

by [20] (41.5 ± 9.5), [21] (43.9 ± 8.4), and [7] (43.9 ± 8.4) but higher than that in [22] (25.1 ± 9.6). In our study, the literacy rate of food handlers was higher than that in [19], but lower than the findings of other studies [20,23]. It has been found that none of the respondents attended training on food safety and butchery. Lack of training among food handlers has a negative consequence on performing behaviors [24]. Several studies mentioned that food safety trainings should be provided to improve the knowledge, attitude and safety practices of food handlers [20,25]. Our study has found that respondents had a medium level of food safety knowledge with a mean score of 18.65 ± 3.81, although this is higher than the findings of other studies [15,19]. Previous studies showed that food safety training increased knowledge regarding food safety issues [26]. Training and education may be effective tools to increase food safety knowledge among food handlers and thus improve food safety practices [17]. It is necessary to know the importance of proper meat handling, proper hand washing and other important hygienic procedures by meat handlers since meat handlers can serve as vehicles for cross-contamination and spread of foodborne pathogens [17]. According to [27], proper hand washing among meat handlers has a significant impact on reducing the threat of diarrheal disease transmission.

An attitude of meat handlers plays a key role influencing food safety practices that help to decrease the chance of foodborne disease outbreaks. The study carried out by [20], showed a strong linkage between positive attitudes and maintaining safe food handling practices. About 29.0% and 52.0% of meat handlers strongly agreed and agreed that a person with open skin injury, gastroenteritis, and ear or throat diseases should not be allowed to touch/ handle meat. Our findings are lower than those in other studies [13,19,28], in which 82.0%, 85.0% and 98.9% of respondents were aware of the risk of touching or handling meat by persons with open skin injury, gastroenteritis, and ear or throat diseases, respectively. About 69.0% and 63.0% of respondents thought that regular training can improve meat safety and hygiene practices, and it is important to use potable water to wash working surfaces and cutting tools. Our findings are lower than those in [19].

Food safety practices play a vital role in ensuring food safety and safeguarding a consumer from foodborne infection and intoxication. A higher percentage (93.84%) of the respondents in this study said that they used soaps or detergents regularly after using the toilet. In [19], about 86.8% of the meat handlers reported that they washed their hands after using the toilet.

Conclusion

The purpose of the study was to investigate the present status of knowledge, attitude and practices of the meat handlers regarding food safety in Khulna City. Meat handlers had unsatisfactory knowledge and practices with respect to food safety. It may be due to low media contact and communication, lack of training and low level of experience among the meat handlers in the study area. They need to improve their expertise, gain more work experience, increase training, increase media contact and communication. These factors are linked to better food safety awareness among meat handlers in Bangladesh. Training programs must be institutionalized with specific guidelines that cover food safety and meat hygiene topics to educate meat handlers better. Meat handlers play an important role in preventing food contamination that can develop into foodborne disease outbreaks. Meat handlers in Khulna City must handle meat properly to avoid food contamination. Finally, to reduce foodborne infections and diseases

in Bangladesh, intervention and longitudinal studies including large, diverse samples of Bangladeshi meat handlers, are needed to investigate characteristics associated with their food safety knowledge and practices. The findings of this study can help public health professionals in developing initiatives to improve food safety knowledge and practices of meat handlers and prevent FBDs. The government should pay special attention to improving knowledge and ensuring proper food safety practices to avoid the transmission of FBDs in Khulna City.

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RABBIT MEAT AS A POTENTIAL SOURCE OF MULTIDRUG-RESISTANT AND ENTEROTOXIGENIC *STAPHYLOCOCCUS AUREUS* STRAINS

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Abstract
Staphylococcus aureus in rabbit meat is a consequence of insufficient hygienic handling and improper processing posing a major health hazard. This study was conducted to assess rabbit meat as a potential source of Staphylococcus species, particularly Staphylococcus aureus (S. aureus). Furthermore, the identified S. aureus isolates were tested for the detection of the mecA virulence gene of methicillin-resistant Staphylococcus aureus (MRSA) and enterotoxin encoding genes (Sea, Seb, Sec, and Sed). A total of 80 samples of different rabbit meat cuts represented by shoulder, ribs, loin, and thigh (20 of each) were collected from various markets of different sanitation levels. The results obtained revealed that the mean counts of Staphylococcus species were 7.40×10^5, 7.58×10^5, 7.60×10^5 and 8.29×10^5 CFU/g in the examined shoulder, ribs, loin and thigh samples, respectively. Out of 17 identified S. aureus isolates, 5 (29.4%) strains were characterized by the presence of the mecA gene. A large proportion of the isolates obtained were resistant to at least three antibiotics. Enterotoxins were evaluated by ELISA. The results showed that three strains isolated from shoulder produced Sea, Seb, and Sec enterotoxins, the strains isolated from ribs failed to produce enterotoxins, while two strains isolated from loin and thigh produced Sea enterotoxin. The presence of S. aureus, especially MRSA strains, in the examined rabbit meat indicates the necessity of enforced application of strict hygienic measurements.

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Introduction

Consumer awareness of and demand for efficient protein sources have increased in recent decades as consumer understanding of the relationship between nutrition and a healthy lifestyle has developed [1,2]. The rabbit industry has gained much more interest due to the fact that rabbit meat has various advantages, which qualify it as one of the most beneficial healthy foods.

Rabbit meat is a popular culinary product and one of the most consumed meats throughout the world. Its use has recently grown in a number of the Middle Eastern countries, notably Egypt [3–5]. It is recognized as an excellent source of easily digestible animal protein, polyunsaturated fatty acids (PUFAs), vitamins, and minerals (such as calcium, magnesium, and zinc), while being low in fat, sodium, and cholesterol [6]. Rabbit meat, on the other hand, is very prone to deterioration and food poisoning bacteria due to its high protein and moisture content. This has been related to the spread of microbial contamination that may have originated from the animal itself, personnel, equipment, or the environment throughout various stages of slaughter and processing [7,8].

Staphylococcus aureus is a spherical Gram-positive bacterium that is present in nearly one-third of the world population and causes staphylococcal food-borne intoxication, as some of its pathogenic strains are able to produce heat-stable enterotoxins [9]. Staphylococcal food poisoning (SFP) is one of the most prevalent food-borne illnesses in the world. It results from the ingestion of staphylococcal enterotoxins produced by enterotoxigenic strains of coagulase-positive staphylococci in food, mainly *S. aureus* and usually occurs within 30 minutes to 8 hours resulting in several symptoms that include vomiting, nausea, abdominal cramping, diarrhea, chills, and sweating [10]. Staphylococcal food poisoning (SFP) is generally self-limiting and resolves typically within 24–48 h after beginning based on the quantity of contaminated food consumed, the amount of the ingested toxin in food, and the general health of patients [11]. Occasionally, it can be serious enough to warrant hospitalization, especially when it comes to children, the elderly, or debilitated people.

Staphylococcus aureus produces numerous toxins including staphylococcal enterotoxins (SEs; SEA to SEE, SEG to SEI, and SER to SET) with the emetic activity. The detection of SE-encoding genes allows the identification of potentially enterotoxigenic *S. aureus*, regardless of whether a strain produces the toxin or not [12]. SEs are a significant contributor to food poisoning, which typically happens after ingestion of various foods, especially processed meat and dairy products that have been exposed to *S. aureus* through improper handling and subsequent storage at high temperatures [13].

Despite the low prevalence of MRSA in raw food, there is still a chance that it could spread through the food supply, particularly in uncooked meat. In fact, MRSA-related foodborne disease outbreaks have been documented [14]. Moreover, food handlers who handle contaminated food may also be at risk for health problems. Foods can be contaminated during processing by MRSA-colonized food handlers, and carcasses from MRSA-infected animals can become contaminated during slaughter [15].

The incidence of antibiotic resistance in food-associated pathogenic bacteria, including *S. aureus*, has been a growing issue over the last few decades due to the intensive use of antibiotics in public health and animal husbandry, and the risk of transfer of antibiotic resistance determinants [16]. Lack of adequate hygienic measures during food preparation is one of the main causes of contamination as food handlers themselves can carry the pathogenic bacterium. Besides that, *S. aureus* can withstand a broad variety of temperature, pH, and salinity [17]. In addition, most of the nosocomial *S. aureus* infections are triggered by methicillin-resistant *S. aureus* (MRSA) strains and have become a world-wide recognized cause of morbidity and mortality [18]. Methicillin-resistant *S. aureus* (MRSA) strains that are resistant to quinolones or multi-resistant to other antibiotics have emerged, which leaves a restricted option for their control [19]. Therefore, the current study was designed to determine the incidence of enterotoxigenic and methicillin-resistant *S. aureus* (MRSA) strains in rabbit meat cuts (shoulder, ribs, loin, and thigh) retailed in Zagazig city, Sharkia governorate, Egypt, as well as to investigate the antimicrobial susceptibility profile and the major staphylococcal enterotoxins (SEs) among the isolated *S. aureus* strains.

Objects and methods

Samples collection and preparation

The objects of the study were the rabbit meat samples from Zagazig City, Sharkia province, Egypt. Eighty random samples of rabbit meat (20 each of shoulder, ribs, loin, and thigh) were collected from various locations with different levels of sanitation. All collected samples were promptly transferred in an icebox under complete aseptic conditions to the laboratory for bacteriological examination without delay. Twenty-five grams of each rabbit meat cut were homogenized aseptically for 1 min with 225 mL of 0.1% sterile buffered peptone water (HIMEDIA, M614, Mumbai, India) in a stomacher (Colworth, model 400, UK) to prepare a homogenate of 10^{-1} (as an initial dilution) and allowed to stay for 5 min. Quantity of 1 ml of the homogenate was transferred into a sterile test tube containing 9 ml of 0.1% BPW and then serially diluted tenfold in the same diluent [20].

Determination of Staphylococcus count

The *Staphylococcus* species count in the samples was determined through bacteriological analysis using Baird Parker agar (BP) supplemented with egg yolk tellurite emulsion following ISO 6888–1:2021 with slight modifications [21]. Briefly, 0.1 mL from each prepared dilution was spread onto duplicate plates of Baird-Parker agar (HIMEDIA, M043–100G, Mumbai, India) supplemented with egg yolk tellurite emulsion (50 mL/L, Oxoid SR54, UK) and then incubated at 37 °C for 24–48 hours. After incubation, presumptive colonies (black, shiny, convex, 1–1.5 mm in diameter, surrounded by a clear halo zone) and/or atypical colonies (black colonies with no zones) were observed and counted. The grown colonies were subsequently confirmed as *Staphylococcus* and identified as belonging to the genus *Staphylococcus* through gram staining, as they typically appear as gram-positive cocci arranged in clusters.

Isolation and identification of Staphylococcus aureus

For *S. aureus* isolation, up to five suspected colonies were picked up and cultured on slope agar for further identification. Isolated purified strains were morphologically identified using Gram's stain and further confirmed as *S. aureus* by biochemical tests (catalase, mannitol fermentation, coagulase, and DNase tests) according to MacFaddin [22].

Genomic DNA Extraction and PCR Analysis

Genomic DNA extraction from each coagulase-positive *S. aureus* isolate was performed using the QIAamp DNA kit (Qiagen, Germany, GmbH) following the manufacturer's instructions. Identification of coagulase-positive isolates was carried out through a species-specific PCR assay. The PCR analysis included the detection of species-specific (nuc) and methicillin resistance (mecA) genes in *S. aureus* isolates. The oligonucleotide primer sequences (Applichem GmbH, Germany) used in PCR reactions for the amplification of the target genes of *S. aureus* and the sizes of amplified products are detailed in Table 1. The DNA amplification process was carried out using a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). PCR protocols for both (nuc and mecA) virulence genes were carried out according to Cho et al. [23]. Amplified DNA fragments were analyzed using 1.5% agarose gel electrophoresis in 1X TBE buffer stained with ethidium bromide (Applichem, Germany, GmbH), captured, and visualized using a UV transilluminator.

Detection and typing of staphylococcal enterotoxins

The incidence of enterotoxins was evaluated by ELISA. According to Shingaki et al. [24], the clear culture supernatant fluid was tested serologically by Reverse Passive Latex Agglutination technique (RPLA) using kits for the detection of staphylococcal enterotoxins A, B, C and D (SET-RPLA, Denka Sekeu LTD, Japan). The sensitivity of this test kit in detecting enterotoxins is 0.5 ng/ml of test extract. The test was conducted in a V-type microtiter plate, with each row containing 8 wells. Each test sample required the use of 5 rows of wells. Initially, 25 µl of diluent was dispensed into each well using a micropipette. Then, the sample was mixed simultaneously with 5 diluents (25 µl each). Two-fold dilutions of the test sample were performed across the 5 rows, with the last well in each row containing only 25 µl of diluent. Quantities of 25 µl of latex suspensions sensitized separately with anti-enterotoxin A, B, C, and D were added to the wells of the 1st, 2nd, 3rd, and 4th rows of the plate, respectively. Additionally, 25 µl of control latex was added to each well in the fifth row, followed by thorough mixing. The plate was covered and left undisturbed at room temperature for 24 hours. Subsequently, each well in every row was examined for agglutination.

Table 1. Oligonucleotide primers of PCR reactions for the amplification of the target genes of *S. aureus*

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
nuc (F)	5′ GCGATTGATGGTGATACGGTT ′3	270	[25]
nuc (R)	5′ AGCCAAGCCTTGACGAACTAAAGC ′3		
mecA (F)	5′ TAGAAATGACTGAAC GTCCG ′3	533	[26]
mecA (R)	5′ TTGCGATCA ATGTTACCGTAG ′3		

nuc: thermonuclease and mecA: methicillin-resistant *S. aureus* (MRSA) virulence genes.

Demonstration of antimicrobial susceptibility profile of S. aureus isolates

Antibiotic susceptibility testing of *S. aureus* strains was performed using a single diffusion assay against 16 antibiotic discs of varying concentrations [27]. The antimicrobial discs, such as kanamycin (K), cephalexin (CE), oxacillin (OX), penicillin G (P), tetracycline (T), nalidixic acid (NA), cephalothin (CN), ampicillin (AM), sulphamethoxazole (SXT), cefotaxime (CF), clindamycin (CL), erythromycin (E), ciprofloxacin (CP), gentamicin (G), linezolid (LZ), and amikacin (AK), were used to perform antibiogram analysis. Each strain was streaked on Mueller–Hinton agar (Himedia, Mumbai, India), and drug-impregnated discs were placed on the agar medium surface.

The Multiple Antibiotic Resistance (MAR) index was calculated using the formula: MAR index = a ÷ b, where (a) represents the number of antibiotics, to which the isolates were resistant, and (b) is the total number of tested antibiotics.

Statistical analysis

Data were analyzed with one-way ANOVA test using the Statistical Package for Social Sciences software for Windows (SPSS-14; Chicago, IL, USA) using post hoc tukey-kramer honestly correction to estimate the differences in microbial counts. *P*-value of <0.05 was considered statistically significant.

Results and discussion

Prevalence and count of Staphylococcus species in the examined rabbit meat samples

As shown in Table 2, the prevalence and mean *Staphylococcus* count in the examined samples were recorded. All investigated rabbit meat cuts (shoulder, ribs, loin, and thigh) were positive for *Staphylococcus* (100%). *Staphylococcus* species count of shoulder samples ranged from 2.3×10^4 to 2.0×10^6 with a mean value of $7.40\times10^5\pm1.21\times10^5$ CFU/g, while ribs samples recorded *Staphylococcus* count varied from 3.2×10^4 to 1.6×10^6 with a mean count of $7.58\times10^5\pm0.83\times10^5$ CFU/g. Besides, loin samples and thigh samples had *Staphylococcus* count of $7.60\times10^5\pm0.82\times10^5$ and $8.29\times10^5\pm0.85\times10^5$ CFU/g, respectively.

Table 2. *Staphylococcus* species count (CFU/g) and prevalence in the examined rabbit meat samples

Samples	Positive samples	*Staphylococcus* species count (CFU/g)		
	No (%)	Minimum	Maximum	Mean ± SE
Shoulder	20 (100%)	2.3×10^4	2.0×10^6	$7.40\times10^5\pm1.21\times10^5$
Ribs	20 (100%)	3.2×10^4	1.6×10^6	$7.58\times10^5\pm0.83\times10^5$
Loin	20 (100%)	2.5×10^4	1.3×10^6	$7.60\times10^5\pm0.82\times10^5$
Thigh	20 (100%)	3.2×10^4	1.4×10^6	$8.29\times10^5\pm0.85\times10^5$

Means are not significantly different at *P*> 0.05; No (%): number and percentage of positive samples; CFU/g: Colony Forming Units per gram.

Rabbit meat and offal are unique sources of high-quality animal protein that also have a high nutritive value for other nutrients. However, rabbit meat is also regarded as a potential source of spoilage and food poisoning organisms, which can cause a variety of negative health effects and shorten the shelf life of rabbit meat [4]. *Staphylococcus* contamination of food results from inadequate hygienic handling and processing, which could be hazardous to human health [28]. Regarding *Staphylococcus* count, Morshdy et al. [8] recorded an initially higher count of 1.34×10^4 CFU/g in freshly untreated rabbit meat samples from Egypt. However, lower results of *Staphylococcus* count were reported by Khalafalla [29] in freshly slaughtered and processed rabbit samples obtained from grocery stores in Beni-Suef city, Egypt, with mean values of 10^2 and 4×10^3 CFU/g, respectively. The high counts of staphylococci could be associated with improper personal hygiene of untrained employees and cross contamination from skin and utensils.

As concerns the incidence of *S. aureus*, 17 (85%) out of 20 *Staphylococcus* isolates in the present study were serologically identified as *S. aureus*. Lower results were obtained by

Kpodékon et al. [30] who detected staphylococci isolated from 30 frozen rabbit carcasses in Benin with a prevalence of 26%, while Kohler et al. [31] documented staphylococci prevalence in rabbit samples from Switzerland with a percentage of 30.6%. Furthermore, Rodriguez-Calleja et al. [32] investigated prevalence of *S. aureus* isolated from rabbit carcasses in Spain with a percentage of 52.9%. Additionally, Bello et al. [33] demonstrated *S. aureus* prevalence from rabbit meat in Nigeria with a percentage of 30.3%. Moreover, Khalafalla [29] isolated *S. aureus* from freshly slaughtered and processed rabbit samples obtained from grocery stores in Beni-Suef, Egypt with a prevalence of 5% and 10%, respectively. The variations of the results may be attributed to how the samples were handled and unsanitary practices observed during data collection. The sharing of environments, facilities, and equipment for the processing of rabbits and poultry, as well as the maintenance of such environments, facilities, and equipment, and the effectiveness of hygienic practices, are critical factors that may have a significant impact on the microbiological profile of the final product [29,34].

Detection of enterotoxigenic and methicillin-resistant S. aureus

The data presented in Table 3 indicate that only five strains were enterotoxigenic. Among six tested shoulder isolates, only one multitoxigenic strain carried three virulence genes (Sea, Seb, and Sec) with each gene accounting for 16.6% (1/6). Similarly, one strain out of five tested loin isolates carried only one gene (Sea gene) with a percentage of 20% (1/5). Furthermore, one strain out of four tested thigh isolates carried only one gene (Sea gene) with a percentage of 25% (1/4). However, *S. aureus* isolates from ribs did not produce any type of enterotoxins.

The results obtained in Figure 1 indicate that all isolates of *S. aureus* were positive for the species-specific (nuc) gene, while the methicillin resistance (mecA) gene was detected in only 5 strains. These strains were classified as methicillin-resistant *S. aureus* (MRSA), accounting for a percentage of 29.4%. This distribution included two isolates from the shoulder (2/6=33.3%), two isolates from the loin (2/5=40%), and one isolate from the thigh (1/4=25%), while the ribs tested negative (Table 3).

Table 3. Incidence of enterotoxins and *mecA* virulence genes among the isolated *S. aureus* strains

Samples	*S. aureus*	SEA	SEB	SEC	SED	mecA
Shoulder	6 (30%)	1 (16.6%)	1(16.6%)	1(16.6%)	0	2 (33.3%)
Ribs	2 (10%)	0	0	0	0	0
Loin	5 (25%)	1 (20%)	0	0	0	2 (40%)
Thigh	4 (20%)	1 (25%)	0	0	0	1 (25%)
Total	17 (85%)	3 (17.6%)	1 (5.8%)	1 (5.8%)	0	5 (29.4%)

SEA: *S. aureus* enterotoxin A; SEB: *S. aureus* enterotoxin B; SEC: *S. aureus* enterotoxin C; and SED: *S. aureus* enterotoxin D. mecA: MRSA gene.

Staphylococcus aureus produces an extracellular thermostable nuclease, which is encoded by the *nuc* gene and is one of the most distinctive and useful traits that could be used to differentiate *S. aureus* from other *Staphylococcus* species [35]. Similarly, Manukumar and Umesha [36] demonstrated the *nuc* gene in all *S. aureus* strains isolated from different food samples in India. Also, Maktabi et al. [37] detected the *nuc* gene in all 150 *S. aureus* isolates obtained from different raw meat samples in Iran.

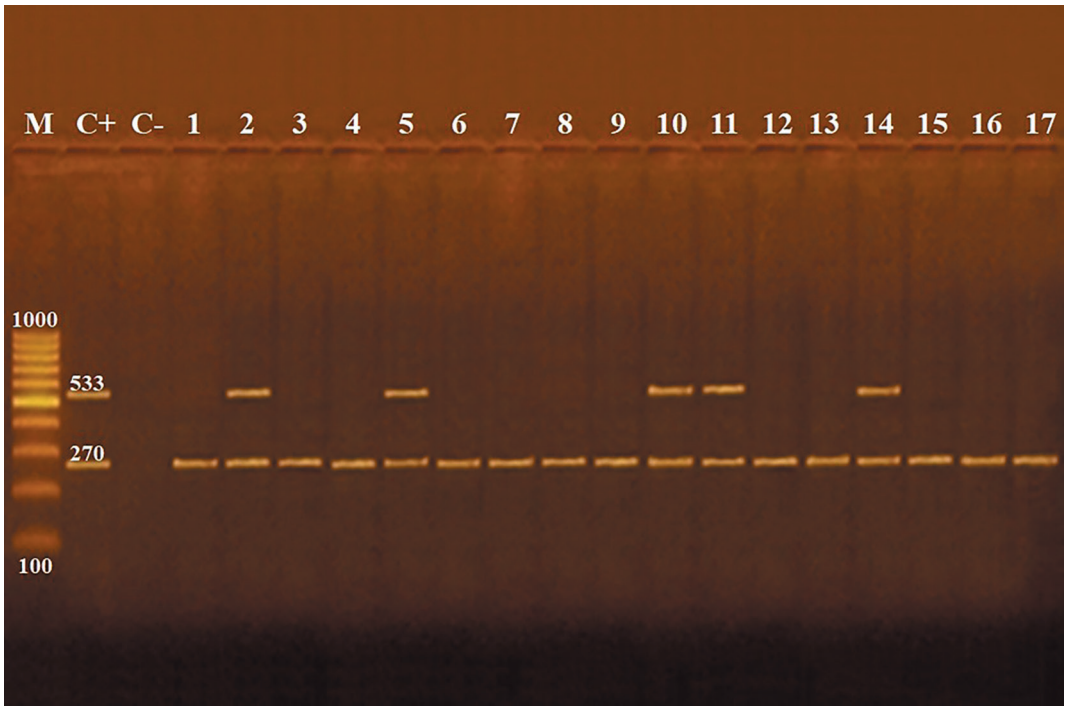

Figure 1. Agarose gel electrophoresis of PCR amplification products of species-specific (nuc) and methicillin resistance (mecA) genes in *S. aureus* isolates. **Lane M**: 100 bp ladder as a molecular size DNA marker. **Lane C+**: Positive control for nuc (270 bp) and mecA (533 bp) genes in *S. aureus* isolates. **Lane C-**: Negative control. **Lanes 2, 5, 10, 11, and 14**: Positive *S. aureus* strains for the mecA gene. **Lanes 1, 3, 4, 6, 7, 8, 9, 12, 13, 15, 16, and 17**: Negative *S. aureus* strains for the mecA gene. The nuc gene, specific to *S. aureus* with a molecular size of 270, was positive for all 17 isolates

The isolates of *S. aureus* were tested for demonstration of enterotoxins and the *mecA* virulence gene of MRSA. In a Spanish study, among 27 *S. aureus* isolates from rabbit samples, Rodriguez-Calleja et al. [32] detected two harbored genes for staphylococcal enterotoxin B (*Seb*), and two harbored genes for staphylococcal enterotoxin C (*Sec*), while the remaining isolates were negative for *Sea*, *Seb*, *Sec*, *Sed*, and *See*. Besides, Kohler et al. [31] identified 102 (67.5%) staphylococcal strains carrying enterotoxin genes from rabbit samples in Switzerland. On the other hand, all 281 *S. aureus* isolates from rabbit samples in Fujian, China, detected by Wang et al. [38] were negative for *Sea* and *Seb* virulence genes. *S. aureus* toxins were not detected in rabbit meat samples in Slovakia [39]. According to Le Loir et al. [40], most *S. aureus* strains isolated from food do not produce SEs. Moreover, other species of *Staphylococcus* can produce SEs, but are not looked for in routine testing.

The isolates were also tested for presence of the *mecA* gene. Moreno-Grúa et al. [41] identified 30 methicillin-resistant *S. aureus* with a percentage of 12.5%, while the methicillin-resistant *mec*A gene was detected in 27 isolates with a percentage of 11.25% in the studied isolates from commercial rabbitries in the Iberian Peninsula. Besides, MRSA was found in 48% (11/23) of the rabbits carrying *S. aureus* in Italy by Agnoletti et al. [42]. Furthermore, Lozano et al. [14] identified MRSA in 5 out of 318 (1.6%) food samples (pork, chicken, rabbit, veal, and wild boar) in Spain. On the contrary, Kohler et al. [31] failed to detect the *mec*A gene from the investigated *Staphylococcus* isolates obtained from rabbit samples in Switzerland.

The results of the present study highlight that rabbit meat may constitute a risk for consumers and especially for immunocompromised individuals. In immunocompromised persons, the specific and non-specific immune responses are not able to act as barriers to prevent colonization of the gastrointestinal tract, and ingestion of food contaminated by MRSA may sometimes lead to lethal diseases [43].

Antimicrobial susceptibility profile of S. aureus isolates

The isolates of *S. aureus* (n=17) were tested for antimicrobial susceptibility as depicted in Table 4. The highest resistance was recorded against kanamycin, cephalexin, oxacillin, penicillin G, tetracycline, and nalidixic acid with a percentage of 100%, 76.5%, 64.7%, 58.8%, 52.9%, and 47.1%, respectively, while the most effective antimicrobials were amikacin, linezolid, gentamicin, ciprofloxacin, clindamycin, cefotaxime, erythromycin with a percentage of 94.1%, 88.2%, 88.2%, 82.4%,76.5%, 70.6%, and 70.6%, respectively. The isolates' MAR index ranged from 0.063 to 1 with an average of 0.389 (Table 5).

Antibiotic susceptibility testing was performed on all 17 *S. aureus* isolates. A total of sixteen antimicrobial drugs from various antibiotic classes were employed. Some were chosen because research revealed that a substantial percentage of bacteria were resistant to them [44,45]. Antibiotics with veterinary and human health implications were also considered. The high prevalence of multidrug-resistant strains found in this study is consistent with previous findings in intensively raised rabbits in the Iberian Peninsula [38]. The obtained results were in parallel with Attili et al. [46] who documented high tetracycline resistance (95.8%), but low penicillin resistance (3.1%) of 96 *S. aureus* strains isolated from rabbit samples in central Italy was observed. Also, Wang et al. [37] detected resistance of *S. aureus* strains isolated from rabbit samples in Fujian Province, China, to kanamycin and penicillin with a percentage of 19.57% and 11.03%, respectively. In accordance with the results, Simonová et al. [39] revealed high resistance among *S. aureus* isolates obtained from rabbit meat samples in Slovakia to penicillin (100%). Also, high resistance to erythromycin and gentamycin (64% for each) was recorded. In agreement with the current study, Rodriguez-Calleja et al. [47] found high resistance of *S. aureus* strains isolated from rabbit meat in Spain to tetracycline (61.5%), but in difference with the detected results, low penicillin resistance (26.9%) was reported.

Table 4. Antimicrobial resistance profile of *S. aureus* isolates (n=17)

Antimicrobial agents	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Kanamycin (K)	—	—	—	—	17	100
Cephalexin (CE)	3	17.6	1	5.9	13	76.5
Oxacillin (OX)	4	23.5	2	11.8	11	64.7
Penicillin G (P)	5	29.4	2	11.8	10	58.8
Tetracycline (T)	7	41.2	1	5.9	9	52.9
Nalidixic acid (NA)	6	35.3	3	17.6	8	47.1
Cephalothin (CN)	9	52.9	—	—	8	47.1
Ampicillin (AM)	9	52.9	1	5.9	7	41.2
Sulphamethoxazol (SXT)	10	58.8	1	5.9	6	35.3
Cefotaxime (CF)	12	70.6	1	5.9	4	23.5
Clindamycin (CL)	13	76.5	—	—	4	23.5
Erythromycin (E)	12	70.6	2	11.8	3	17.6
Ciprofloxacin (CP)	14	82.4	1	5.9	2	11.8
Gentamicin (G)	15	88.2	—	—	2	11.8
Linezolid (LZ)	15	88.2	1	5.9	1	5.9
Amikacin (AK)	16	94.1	—	—	1	5.9

n: Number of *S. aureus* isolates. No.: Number of sensitive, intermediate or resistant *S. aureus* isolates.%: Percentage of sensitive, intermediate or resistant *S. aureus*.

High penicillin resistance is not surprising because of its widespread use for treatment in humans and animals. Although the European Union regulates the use of antibiotics as growth promoters, the existence of resistant organisms is still found, confirming their intensive use in therapy [48]. High susceptibility to erythromycin in this study may be attributed to the fact that this antibiotic is not used in rabbits due to its toxicity [49]. More resistant strains are thought to have the best chances of survival; thus, their prevalence increased as they filled the space left by those who did not survive the antibiotic treatment. This finding

Table 5. Resistance profile of multi-drug resistant *S. aureus* isolates (n=17)

Pattern	Resistance profile	Number of antibiotics	Number of isolates (%)	MAR
I	K, CE, OX, P, T, NA, CN, AM, SXT, CF, CL, E, CP, G, LZ, AK	16	1 (5.88%)	1
II	K, CE, OX, P, T, NA, CN, AM, SXT, CF, CL, E, CP, G	14	1 (5.88%)	0.875
III	K, CE, OX, P, T, NA, CN, AM, SXT, CF, CL, E	12	1 (5.88%)	0.75
IV	K, CE, OX, P, T, NA, CN, AM, SXT, CF, CL	11	1 (5.88%)	0.688
V	K, CE, OX, P, T, NA, CN, AM, SXT	9	2 (11.76%)	0.563
VI	K, CE, OX, P, T, NA, CN, AM	8	1 (5.88%)	0.500
VII	K, CE, OX, P, T, NA, CN	7	1 (5.88%)	0.438
VIII	K, CE, OX, P, T	5	1 (5.88%)	0.313
XI	K, CE, OX, P	4	1 (5.88%)	0.250
X	K, CE, OX	3	1 (5.88%)	0.188
XI	K, CE	2	2 (11.76%)	0.125
XII	K	1	4 (23.5%)	0.063
Average				0.389

MAR: Multiple Antibiotic Resistance index
K: Kanamycin **CE: Cephalexin** **OX: Oxacillin** **P: Penicillin G**
T: Tetracycline **NA: Nalidixic acid** **CN: Cephalothin** **AM: Ampicillin**
SXT: Sulphamethoxazol **CF: Cefotaxime** **CL: Clindamycin** **E: Erythromycin**
CP: Ciprofloxacin **G: Gentamicin** **LZ: Linezolid** **AK: Amikacin**

suggests that long-living rabbits play an important role in maintaining resistant strains and spreading them to newly introduced and newborn individuals.

Conclusion

Generally, the current study identified multidrug-resistant and multitoxigenic *S. aureus* in rabbit meat, highlighting its potential as a source for transmitting foodborne pathogens. The data obtained confirms that rabbit meat can cause staphylococcal intoxication in consumers, with the majority of *Staphylococcus* isolates being *S. aureus*, and some testing positive for MRSA and enterotoxin virulence genes. The high *Staphylococcus* count in raw retail rabbit meat in the Egyptian market suggests a risk of common foodborne diseases. The assessment of antibiotic resistance and pathogenicity revealed severe issues for food industrial applications and quality control as many isolates showed resistance to at least three antibiotics. Thus, initiatives are needed to enhance sanitary standards in Egyptian markets, especially in traditional markets with higher contamination rates. Health agency regulations should be disseminated to all workers, and safety programs for slaughtering and meat preparation outlined by international organizations and national authorities must be followed. Effective preventive measures must be authorized and implemented to safeguard consumer health.

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THE ROLE OF ENZYMES IN THE FORMATION OF MEAT AND MEAT PRODUCTS

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Keywords: *enzyme, protease, meat tenderness, meat industry, cross-linked meats*

Abstract

The meat industry is one of the most dynamic and competitive sectors of the food industry. As the global population keeps on growing and the demand for protein does the same, the consumers define ever higher standards of quality for the meat producers. One of the key quality criteria is the tenderness and juiciness of meat, which directly affects its taste and texture characteristics. In order to satisfy the expectations of the modern consumers and to ensure the stable quality of the meat product, meat processing enterprises actively introduce the innovative technologies. In recent decades, proteolytic enzymes have been increasingly used to improve the quality characteristics of the meat products, which is a more progressive method in comparison with to mechanical methods of processing due to less impact on other consumer properties. This article overviews the role and importance of enzymes in the meat industry. We will consider various aspects of the application of these enzymes for the meat products, their effect on the level of tenderness, juiciness and other characteristics of meat, as well as prospects for the further development of their using.

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Introduction

The meat industry is one of the biggest sectors of the food industry, and the quality of meat products plays an important role in the consumers' satisfaction [1]. Tenderness and juiciness of meat are considered to be the key characteristics that determine its taste and quality [2,3]. It has been shown that tenderness, defined as "the ease, perceived by consumers, with which meat structure is disorganized during mastication" [4], plays the largest role in making decision on the purchase of the particular product [5,6].

It is not an exaggeration to say that the meat obtained from each animal is the "unique meat" with its own evolution [7]. The meat industry must ensure that quality standards for the meat it produces are complied with. The meat industry has developed a variety of approaches to achieve this purpose, including both traditional methods of curing or aging, as well as the new technologies [8]. In modern conditions the methods of mechanical processing with the needles and blades or by massaging are widely used as preparation of the raw meat for its subsequent sale [9,10]. The main effect is aimed to the softening of the meat product during such processing. So the mechanical processing softens the muscle component of the product, which results in loosening of the meat surface, rupture of muscle fibers and release of myofibrillar proteins [11]. An alternative to mechanical processing is tenderization with proteolytic enzymes [12], which is a more progressive method compared to mechanical ones due to less impact on other consumer properties [13]. Many enzymes that make changes to the connective tissue structure or myofibril integrity were extracted from plant, bacterial, and fungal sources and were thoroughly studied (Figure 1).

The special feature of this review is the comparative characteristics of enzymes involved in the formation of meat and meat products. The results of these enzymes application are illustrated in the article with examples taken from the publications of the recent years, including our paper too. The analysis presented in this review will undoubtedly be useful for proper orientation in the development of the modern approach to improving the food industry processes, in particular improving the consumer qualities of the meat products.

Objects and methods

The developments of domestic and foreign scientists on application of enzymes for meat products, presented in the articles, are the object of the study. The search for the data was run in the databases ScienceDirect, PubMed, Google Scholar, eLibrary and other open electronic sources. Combinations of keywords such as enzyme, protease, meat tenderness, meat industry, fermented sausages, microbial transglutaminase (applied for cross-linked meats) were used. Keywords were used in English and Russian.

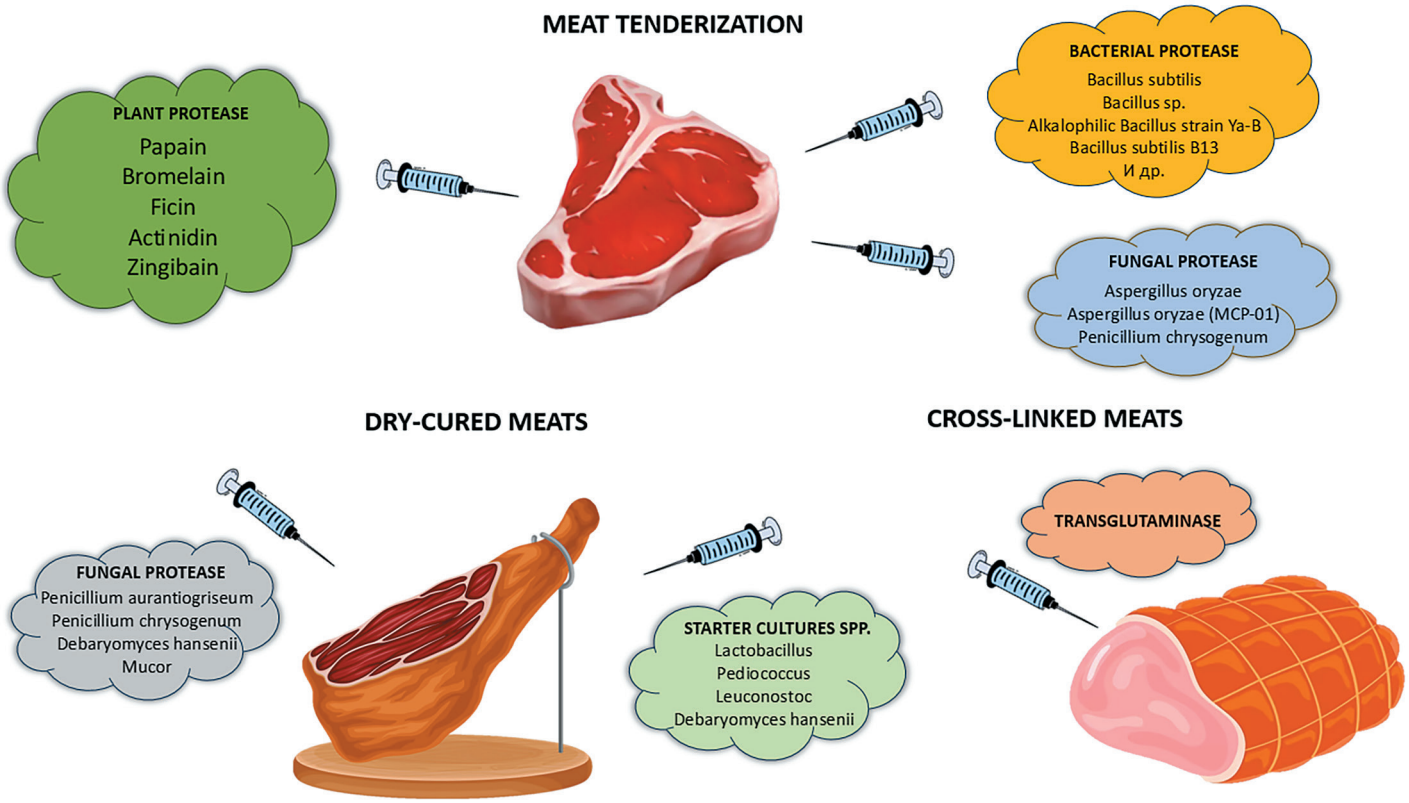

Figure 1. Enzymes used to cure steaks and other meat products

In addition, thematically similar articles were searched for using the citation chains. Non-peer-reviewed, uninformative and duplicate sources were excluded from the search results, as well as the sources included in the search patterns that were not related or were just indirectly related to the topic of the research.

Proteolysis during post-mortem aging

Quite soon after the slaughter of the animal the homeostasis gets lost, and biochemical changes and a number of physicochemical reactions in the muscles start [14]. To provide the required amount of ATP without the oxygen, glycogen is decomposed in the muscles through anaerobic glycolysis. This leads to the formation of lactic acid with a simultaneous decrease in pH from approximately 7.2 in a live muscle down to approximately 5.5 in the slaughtered meat. Stress or the pre-slaughter treatment are the key factors that influence the process of post-mortem glycolysis, which in its turn determines the final level of pH and therefore the quality of meat [15]. Decrease of pH and temperature leads to a decrease of the glycolytic enzymes activity, which, in turn, leads to a gradual decrease of ATP levels [16]. As a result, the main contractile proteins — actin and myosin — become irreversibly linked to each other, thus leading to a phenomenon known as *rigor mortis*, which heightens the meat toughness through a phase of sarcomere shortening. Meat with very short sarcomeres tends to be tougher [17].

Animal muscles have fibrous structure necessary to ensure their strength, contractibility and stretchability. As a result, even in case of exposure to intensive tenderization, the inherent fibrous texture of the meat is still preserved. The conversion of muscle to meat is shown schematically in the recent articles [7,15]. Decreasing of the raw meat toughness during its storage after slaughter are generally recognized as enzymatic process, it involves intracellular proteolytic systems capable of post-mortem proteolysis [18]. Several endogenous proteolytic systems found in meat, including calpains, cathepsins and lysosomal proteases, are capable to degrade the myofibrillar and cytoskeletal proteins [19].

Calpains

The calpain system, responsible for the proteolysis of cytoskeletal proteins (titin and nebulin) and intermediate filaments (desmin), as part of the cysteine protease system, is now widely recognized as critical factors promoting muscle tenderization during postmortem maturation [20]. The calpain system includes endogenous proteases (calpains), which are considered the main candidates for the muscle degradation that starts in the first 24 hours after slaughter, and their inhibitor — calpastatin [21]. Studies show that the degree of tenderization in the meat of different animal species is inversely related to the calpastatin: calpain ratio [22]. These enzymes have been reported to be capable of degrading specific muscle proteins, including intermediate filament proteins like desmin, and structural proteins like titin [21] with minimal effects on myosin and actin. Postmortem proteolysis, as measured by loss of intact desmin and troponin-T, was limited in transgenic mice expressing calpastatin [23]. These results were later confirmed by a similar study in calpain knockout mice [24]. More than

30 years ago, it was found that the administration of calcium chloride (CaCl2), which activates calpains, accelerates postmortem proteolysis in the muscles [25]. On the contrary, the introduction of calpain inhibitors prevents postmortem proteolysis and, accordingly, prevents the tenderization of meat. Numerous studies have again confirmed the crucially important role of calpains in the proteolysis and tenderization of lamb, beef and pork [20].

Cathepsins — caspases

Calpains are not the only proteases in muscle, and there is evidence that other proteases are either directly involved in the meat maturation, or interact with calpain. Cathepsins are proteases that digest cellular components in lysosomes and that are active at the acidic pH of meat [26]. However, they gain the access to myofibrils only under conditions of lysosomal membranes rupture. Cathepsins also proteolyze actin and myosin, and during the meat maturation only very limited destruction of these proteins is observed [27]. Becila et al.'s work views early maturation as a process of apoptosis or controlled cell death [28]. Caspases are key cysteine-dependent aspartate-targeting enzymes, activated during apoptosis. There is evidence that caspase 3 is able to reproduce many of the characteristics of postmortem proteolysis in the myofibers [16]. The work examined the application of human recombinant caspase 3 (rC3), which was expressed in *Escherichia coli* bacteria, in order to degrade myofibrillar proteins in pork muscle after a pig slaughter. The work showed that recombinant caspase is capable of degrading a variety of proteins, including alpha-actin, troponin T, myosin, myofibrillar proteins, desmin and troponin I [16]. Caspases were also suggested to be the target of serine peptidase inhibitors, also called as SERPINs, which correlate with beef strength [29].

There are lot of evidences that caspases interact with calpains and are able to proteolyze the calpastatin inhibitor [30]. Thus, the current view of postmortem maturation of meat focuses on calpains, but also includes their interactions with several other groups of proteases. Cathepsins are more thermostable than calpains [31], as the latter are reported to be completely inactivated at the temperatures above 55 °C. While cathepsins, especially B and L, remain active even after 24 hours of exposure to the temperature of 55 °C [32]. Recent studies have shown that mitochondria-mediated apoptosis also affects post-mortem muscle tenderization by promoting the degradation of myofibril proteins and cytoskeletal structures. In result mitochondria provide their influence on the formation of color, tenderness and taste of meat through the effects on oxidative stress, changes in the redox state of myoglobin, glycolysis and apoptosis [33].

Enzymes as texture and tenderness modifiers

Meat is primarily muscle protein, present as bundles of protein fibers clustered into blocks surrounded by connective tissue. This connective tissue also contains structural proteins including collagen and elastin. In order to give the meat a more tender structure, it is possible to run preliminary splitting of connective tissue proteins and some muscle fiber proteins. Given the structural features of muscle and fibrous tissue that affect the tenderness of meat, it is necessary to strive for an integrated approach that affects both muscle and connective tissue.

The tenderness of meat is the result of combination of several factors that can be considered in certain sequence [34]. "Background toughness" is determined by the characteristics of the muscle, in particular the connective tissue (collagen), its quantity and type [35]. This in its turn depends on factors such as species, age of the animal, nutritional status, sex and muscle type. Different types of muscle fibers differ in their collagen content, and therefore muscles with different fiber compositions also vary in level of their tenderness [36]. There is also a correlation between the tenderness and the diameter of a fiber. Small-fiber muscles are considered more tender in cattle and pigs [37]. There is also data on correlation of tenderness with total content of lipids, intramuscular fat levels [38] and content of water in the cell [39]. Differences in tenderness still exist even when controlling for the above-listed parameters, and this has proven to be still a very difficult aspect to predict and control. The introduction of exogenous enzymes for meat tenderization has recently gained the increasing interest among food technologists and scientists (Table 1). This promotes consistent production of tender meat and increases the nutritional value of lower quality meat cuts.

The toughness of meat is determined by two structural components. The first structural component is connective tissue in meat, mainly composed of structural proteins that provide support to muscles at different levels. Structural aspects and the contribution of connective tissue to the tenderness of raw and cooked meat are considered in the work [58]. The contribution of connective tissue to the toughness of meat depends on the structure and/or amount of various collagens and elastin in the meat. This part of meat toughness is mainly influenced by "on-farm" factors like breed, sex, age, physical activity, and so on. Processing and post-mortem handling have a negligible effect on "background toughness". The second structural component that influences the toughness of meat after slaughter is changes in the contractile apparatus of the muscle (sarcomere), which becomes shorter (the phase of *rigor mortis* development). The stiffness caused by muscle contraction is primarily dependent on the conditions of the processing. Thus, by changing processing conditions, a significant improvement in meat tenderness can be achieved [15].

Plant proteases have already been proven to improve meat tenderness through a proteolytic degradation mechanism. Common exogenous plant proteases used for the processing of the meat are bromelain extracted from pineapple (*Ananas comosus*), papain from papaya (*Carica papaya*), ficin from figs (*Ficus carica*), actinidin from kiwi (*Actinidia Lindl*) [59] and zingybaine from ginger root (*Zingiber offi-*

Table 1. Application of enzymes for meat tenderisation

Application	Source	Optimal conditions	Active agents group	Type of enzyme
Hydrolysis of connective tissue and myofibrillar proteins, beef (tough meat)	Papaya (*Carica papaya*)	4.0–8.0 pH 50–70 °C	Sulfhydryl group of cysteine	Papain [15]
Hydrolysis of myofibrillar proteins, beef (tough meat) and beef steak	Pineapple (*Ananas comosus*)	3.0–7.0 pH 35–70 °C	Sulfhydryl group of cysteine	Bromelain [15,40,41]
Hydrolysis of myofibrillar proteins, beef steak	Fig (*Ficus carica*)	5.0–8.0 pH 45–60 °C	Thiol group	Ficin [42]
Hydrolysis of myofibrillar proteins, beef steak	Kiwi (*Actinidia Lindl*)	7.5 pH 2–45 °C	Thiol group	Actinidin [43–46]
Hydrolysis of connective tissue and myofibrillar proteins, beef (tough meat)	Ginger root (*Zingiber officinale*)	6.0 pH 60 °C	Proline	ZIngibain [15,47]
Hydrolysis of myofibrillar proteins and collagen, beef steak	*Bacillus subtilis*	7.0 pH 60 °C	Subtilisin and neutral protease	Bacterial (*Bacillus subtilis*) [48]
Hydrolysis of collagen and myofibrillar proteins, beef tenderloin	*Bacillus* sp. (EL31410)	5.5–6.0 pH 10–50 °C	Alkaline elastase	Bacterial (*Bacillus* sp. (EL31410)) [49]
Hydrolysis of elastin and collagen, beef (tough meat)	*Alkalophilic Bacillus strain Ya-B*	5.5–6.0 pH 50 °C	elastase	Bacterial (*Alkalophilic Bacillus strain Ya-B*) [50]
Hydrolysis of elastin and collagen, beef (tough meat)	*Bacillus subtilis B13*	7.5 pH 60 °C	Serine/metalloprotease	Bacterial (*Bacillus subtilis* B13) [51]
Hydrolysis of elastin and collagen, beef (tough meat)	*B. siamemsis S6*	7.5 pH 60 °C	Serine/metalloprotease	Bacterial (*B. siamemsis S6*) [51]
Collagen hydrolysis, beef steak	*Clostridium histolyticum*	6.0 pH 60 °C	Collagenase	Bacterial (*Clostridium histolyticum*) [52]
Collagen hydrolysis, synthetic substrate	*Pseudoalteromona* ssp. (SM9913)	9.0 pH 40 °C	Collagenolytic protease (MCP-01) (serine protease)	Bacterial (*Pseudoalteromonas* sp. (SM9913)) [53]
Hydrolysis of elastin and collagen, beef (tough meat)	*Aeromonas salmonicida*	6.0 pH 4–30 °C	Metallopeptidase, M9	Recombinant metallopeptidase (*Aeromonas salmonicida*) [54]
Hydrolysis of myofibrillar proteins, beef (tough meat)	*Aspergillus oryzae*	6–10 pH 50 °C	Protease	Fungal (*Aspergillus oryzae*) [55]
Hydrolysis of myofibrillar proteins and collagen, beef steak	*Aspergillus oryzae* (MCP-01)	2.5–6.0 pH 75 °C	Aspartic protease	Fungal (*Aspergillus oryzae* (MCP-01)) [48]
Hydrolysis of myofibrillar proteins and collagen, muscle of pork loin	*Penicillium chrysogenum* (EPg222)	3.0–5.0 pH 30–60 °C	Serine protease	Fungal (*Penicillium chrysogenum* (EPg222)) [56,57]

cinale) [60]. Thus, plant proteases can be obtained from a wide range of untaped plant resources via suitable extraction technologies. These proteases decompose the connective tissue proteins of the muscle by hydrolyzing peptide bonds in proteins into peptides, and finally into amino acids, thereby reducing meat toughness [59,61]. The classification of plant proteases is detailed in the chapter "Use of Plant Proteolytic Enzymes for Meat Processing" [62].

In practice the mixture of plant enzymes and bacterial collagenase is often used [59], or a combination of two or more plant proteases is used. The use of more than one protease has a synergistic effect. The proteolytic effect of exogenous plant proteases on muscle protein increases the nutritional value of the product due to the availability of essential amino acids, improving digestibility and taste [63,64].

Controlling the plant proteases action plays an important role, because the excessive exposure of meat under uncontrolled conditions leads to loss of consumer qualities and spoils of the product. This affects texture and flavor, increases bitterness due to the formation of basic amines and some amino acids. In order to be able to control the process of meat tenderization, it is necessary to determine their enzymatic kinetics and characteristics, as well as an understanding of the influence of their surrounding conditions (pH, temperature) on enzyme function. This will create the optimal conditions for tenderization of the fresh meat and eliminate or reduce any negative impact on the other quality characteristics.

Enzyme activity is influenced by processing conditions like combination of duration and temperature, pH, and the contact area of the enzyme with the surface. In the meat industry, there is an acute demand for proper regulation of enzyme activity, diffusion of enzymes in the meat matrix to obtain the desired effect on tenderness, texture, color, flavor and juiciness of meat [65]. The cooking method also contributes to achievement of the required tenderness of the dishes. For example, studies have shown that slow cooking of meat increases protease activity, and improves tenderness [66]. Thus, slow cooking requires lower concentrations of papain and bromelain than fast cooking to achieve the same level of the meat tenderness.

It is possible to highlight the main advantages of plant enzymes application:

- Plant proteases are produced naturally and are found in most plant sources, in contrast to microbial proteases, which primarily are the by-products of microbial fermentation. In addition, the hydrolytic activity of plant

proteases on meat myofibrillar proteins is more profound than the same of bacterial proteases.
- Consumers prefer to use plant-based proteases over animal-based ones due to the potential risk of disease transmission, environmental concerns, sustainability, lower cost, religious and ethical concerns. Also, in some countries, enzymes obtained by recombinant technology are not allowed into the food industry.
- Plants are also used as a natural preservative in the meat industry.
- Plant extracts are a rich source of polyphenols, essential oils, minerals and other biologically active compounds. Thus, the use of plant extracts may provide more advantages than the commercial preparations of enzymes, like higher nutritional value, wider product variety, and positive effects on the organoleptic properties of the meat products.

The main disadvantages of using plant enzymes are as follows:
- Plant proteases typically lack substrate specificity for the meat proteins, and extensive and non-selective hydrolysis of myofibrillar and connective tissue proteins results in a soft texture and "unpleasant" sensory notes such as "grainy" texture and "bitter" taste. Microbial proteases, in its turn, exhibit more specific activity towards meat substrates;
- There might be allergic reactions caused by the use of or exposure to papain, ficin, bromelain and actinidin, as well as thaumate-like protein extracted from kiwi;
- Issues related to composition, stability and control of enzymes after their processing. Many of these issues are related to the fact that commercial protease preparations contain many complex proteins and proteases that feature variable and uncontrollable hydrolytic activity and can lead to excessive tenderization;
- Another issue with plant protease extracts is that they may have some inherent flavor that may be acceptable to some kinds of meat and unacceptable to others, like ginger extracts containing zingibaine;
- Tenderization of meat with the help of plant proteases is quite time-consuming process; thus, there is a possibility of deterioration of the meat color. The use of new technologies such as high pressure treatment, ultrasound, shock waves, hydrodynamic pressure, pulsed electric field, etc. can increase the efficiency of enzymes by improving the enzyme-substrate ratio due to their better penetration/diffusion of the enzyme within the meat tissue.

New meat processing technologies such as ultrasonication, high pressure processing, reverse phase micellar processing, shock waves and pulsed electric field are increasingly being used today [15]. These methods help to increase the efficiency and activity of plant proteases due to deep penetration, and also increase the efficiency and reactivity of plant proteases due to better enzyme-substrate interaction [67].

The optimal and controlled tenderization of meat with plant proteases will help reduce batch-to-batch variability in meat and meat products and will improve their acceptability for the consumers. The significant reduction in meat hardness by plant proteases makes these products suitable for consumption by older people (who are not able for the full energy chewing), as meat is necessary for a complete balanced diet and the provision of quality proteins.

At the current moment in the profile literature there is no sufficient information on the effect of exogenous proteases on the tenderization rate, depending on different periods of time elapsed after slaughter and on cooking methods (speed of cooking, final temperature of cooking), which requires further research to find the optimal mode of these proteases application. The method of treatment with proteases (coating, pickling, injection or tumbling) can also have a significant impact on the degree of tenderization.

Enzymes of fungal and bacterial origin

In addition to plant proteases, microbial enzymes can serve as sources for meat tenderization as they cause degradation of connective tissue and induce changes in the muscle fibers of tough meat [55,68]. The chemicals like organic acids and phosphates, used in tenderizing meat, can greatly affect the health of the consumers. The acute necessity of their replacement with environmentally friendly substances to improve food safety urges the scientists to find ways to use various proteases, especially obtained from microbial sources, in order to decompose and tenderize meat in the meat industry (Table 1).

Bacterial proteases are reported to be more effective for degrading meat connective tissue than papain enzyme. They can be targeted and used to tenderize meats with a high number of connective veins [69]. Proteases of bacterial and fungal origin are widely used in the food industry and biotechnology. They have a lot of advantages in comparison with proteases of the plant origin. Microorganisms can be cultured comparatively quickly under stringent conditions that provide better control of protease production. Protease expression and activity can be managed with the help of modifying the conditions of production or cloning.

To improve the tenderness of meat, peptidases of fungal (aspartic protease from *Aspergillus oryzae*) and bacterial (neutral protease from *Bacillus subtilis*) origin are used; however, the bacterial peptidase has wider range of application in the meat industry because it has higher specificity and lower temperatures of inactivation. However, in the fungi *Rhizomucor miehei* CAU432 the process of cloning the aspartic protease gene (RmproA) resulted in a protease with the same efficiency as papain, used for pork tenderization [70]. Fungal protease obtained from *Penicillium chrysogenum* (EPg222) is also used to tenderize meat. It has an optimum temperature of 45 °C, it is effective between 30 °C and 60 °C and it is still active at pH below 4.5. EPg222 exhibits better activity in myofibrillar proteins than papain and *A. oryzae* [56].

Microbial proteases are grouped in reference to their acidic or basic properties. They can also be classified based on the present functional groups and the position of the peptide bond [55,71]. Bacterial enzymes such as subtilisin and neutral proteases are produced mainly by *B. subtilis*, *B. licheniformis, B. alcalophilus* and *B. lentus* [72]. Usually, bacterial proteases are specific in their activities. They are dependent on temperature, which makes them the best tool for the meat tenderization due to their ability to self-regulate. Proteases derived from *Bacillus amyloliquefaciens* and *Bacillus subtilis* are denatured at higher temperatures. This property gives them an advantage in catalytic action in the meat hydrolysis, and their activity can be easily controlled.

In this article [69] it was reported that meat products treated with *Aspergillus oryzae* have higher water retention capacity than the meat products treated with papain and bromelain. This means that treatment with *A. oryzae* preserves meat freshness, color characteristics, and shows better tenderizing potential than bromelain and papain. Most fungal proteases provide degradative effects on elastin and collagen, and effect the myofibrillar proteins to a lesser extent [73]. Aspartic protease produced by *A. oryzae* operates optimally at pH 2.5–6.0 and loses 80% of its activity at 75 °C after cooking, it affects collagen, however it provides less impact on meat myofibrillar proteins in comparison with the action of plant proteases [12]. The strain of alkaliphilic rod *Ya-B*, which produces alkaline elastase, dramatically degrades elastin and collagen in comparison with collagenase of *C. histolyticum* [50].

Raw meat is a complex object, where the ratio of muscle and connective tissue of different types can strongly vary depending on the type of meat, physiological and anatomical pecualirities of the animal. Therefore, the specificity of the enzyme preparations action plays an important role. Collagen is an insoluble fibrillar protein that provides strength and elasticity to connective tissue. Collagen is the most abundant protein found in the body of mammals and accounts for 25 to 35% of the total mass of proteins. Meanwhile collagen is difficult to digest, which makes it one of the main factors influencing the toughness of meat [11]. It is known that the wide range of proteases specifically affect the collagen molecules [11,74,75]. The work of Zhao et al researched the softening effect and effect of the cold-adapted collagenolytic enzyme MCP-01 on beef. At 4 °C, meat shear force decreased by 23% and relative myofibril fragmentation index increased by 91.7%, while meat freshness and moisture content remained unchanged [53].

The particular interest is raised in regards to M9 family of metallopeptidases [11] from among the microorganisms *Clostridium histolyticum* [76] and *Vibrio alginolyticus* [77]. It is known that the collagen filament features a specific sequence "G-X-Y", where "G" is glycine, "X" is often proline, and "Y" is hydroxyproline or one of the hydrophobic acids. *Vibrio* and *Clostridium* collagenases have specific ability to digest native triple-helix collagens of I, II and III types into a mixture of small peptides, cutting on the bond "Leu-Gly" under physiological conditions. Since the toughness of meat is partly caused by the presence of collagen, it is possible to use these collagenases to tenderize meat. However, the introduction of such enzymes into the technological process is associated with a number of challenges. Industrial use of microbial collagenases is limited by safety concerns related to their potential toxicity and the other adverse effects [78]. One of possible approaches involves the production of recombinant collagenases [54] in non-pathogenic microorganisms to avoid contamination by associated virulence factors. For example, we previously obtained the recombinant metallopeptidase from *Aeromonas salmonicida* using the transformation of *Pichia pastoris* for subsequent softening of meat. Histological examination of beef shank samples found a pronounced separation of the perimeter from the muscle bundles and the breakdown of collagen fibers, while the muscle fibers remained unchanged [54].

Bacterial and fungal proteases have proven to be more acceptable and beneficial than plant proteases, including their ability to act at a lower temperature, thereby preventing excessive tenderization of meat (myofibrillar and connective tissues) during its fermentation. Their hydrolytic activity depends on the substrate. Meanwhile the cold-active peptidases are isolated from *Chryseobacterium soli* and used to tenderize meat (for fragmentation of myofibrils) [79]. Proteases are also used to separate meat from bones for its further use in animal feed. In this particular case, peptidases must affect primarily the connective tissue, hydrolyzing collagen and elastin. The enzymes of microbial origin are commercially available from non-pathogenic sources. However, some consumers feel uncomfortable with the concept of bacterial or fungal food additives. The good strategy to overcome this negative perception is to use probiotic bacteria as sources of effective proteases that can be used for the dual function of maintaining the intestines health and for meat tenderization [80].

Food proteases are not commonly found in genetically modified animal/plant forms, probably because the universal properties of native proteases are suitable for most food processing temperatures and pH and their mechanism of action is well known (Table 1). This may also be due to the abundant availability of microbial proteases for industrial use. Recent studies of engineered food proteases from *Aspergillus* and *Bacillus* species demonstrated that engineered metalloproteases obtained through site-directed mutagenesis of His224 feature the improved substrate affinity [81]. Acid protease obtained from a mutant strain of A. oryzae was obtained using solid-state fermentation with potato pulp powder with increased glycine release activity [82]. Also, neutral protease from *A. oryzae* had an optimal pH of 8.0 and an optimal temperature of 55 °C, and its enzymatic characteristics showed that it was efficient in producing antihypertensive peptides and elimination of bitterness [83]. The alkaline protease of *Bacillus*

alcalophilus is active at 10 °C and alkali-resistant, but these properties have been improved through directed evolution using error-prone PCR [84] for its use in food processing at lower temperatures. Similarly, an alkaline serine protease obtained from mesophilic *Bacillus pumilus* was created that has increased hydrolytic efficiency at 15 °C without compromising its thermal stability [85].

Dry-cured meats

Dry-cured meats are the uncooked meat products that are being manufactured for more than 8 months, during which period the certain microorganisms multiply on the surface of the meat. When processing dry-cured meat products, sarcoplasmic and myofibrillar proteins undergo the process of proteolysis, which provides the significant effect on the taste of the product. The changes that take place during proteolysis are associated with the action of both endogenous and microbial enzymes. Microbial proteolytic activity is initiated predominantly by the lactic acid bacteria (LAB), mainly belonging to the genera *Lactobacillus*, *Pediococcus* and *Leuconostoc*, and to a lesser extent from micrococci (*Micrococcus/Kocuria spp.*). The main species that develop during the process of natural fermentation are *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum* and *Staphylococcus xylosus*. Amino acids play the decisive role in determining the taste of the food [86,87], Moreover, this effect is clearly enhanced by the activity of proteolytic enzymes. The application of purified proteinases (PrA and PrB) and aminopeptidases (arginyl aminopeptidase and prolyl aminopeptidase) extracted from *Debaryomyces hansenii* CECT 12487 allows obtaining the desired sensory quality. These enzymes catalyze the hydrolysis of sarcoplasmic proteins to form ammonia, increase pH and accelerate the proteolytic pathway [88].

A lot is known about the proteolytic activity of enzymes in the meat products [89], however, there are practically no studies on the proteolysis of the cured ham. The role of molds in dry-cured ham is also important. Various species of Penicillium contain enzymes with proteolytic activity capable to produce peptides and amino acids [56,90]. Certain strains of *Penicillium* and *Mucor* isolated from minced meat have proteolytic activity against meat proteins both in vitro and in processed minced meat [91,92]. *Penicillium aurantiogriseum* has high proteolytic activity against dry-cured meat. The enzyme *Penicillium chrysogenum EPg222* is very active against myofibrillar proteins. At the same time, collagen, being the dominant protein of connective tissue, remains relatively unchanged [56]. An extracellular protease obtained from *Penicillium chrysogenum* isolated from dry-cured ham is also of interest for the taste of dry-cured meat products [93], because it hydrolyzes proteins and reduces processing time.

As far as the yeasts are concerned, strains of *Debaryomyces hansenii* isolated from Iberian ham feature significant aminopeptidase and proteolytic activity [94,95]. Although the activity of these enzymes has mainly been assessed in regards to specific substrates such as myosin [95].

Cross-linked meats

The enzymes for proteins cross-linking like transglutaminases (EC2.3.2.13) are used to improve the texture, flavor and shelf life of the meat products. Transglutaminase is able to adsorb on the surface of meat and, through the acyl-transferase reaction, to cross-link the ε-amino group of the lysine residue with the γ-carboxamide group of the glutamine residue in proteins [96,97]. In industrial production, transglutaminase is predominantly obtained from the bacterium *Streptoverticillium mobaraense* [98]. One of the largest producers of transglutaminase (TG) from *Streptomyces mobaraensis* is the Japanese company Ajinomoto ("ACTIVA" TG) [99]. This enzyme is a monomeric protein with a mass of 38 kDa, that contains 331 amino acids [97]. Since transglutaminase remains active even when refrigerated, it is able to bind raw meat during its storage at temperatures close to 0 °C. For this reason, transglutaminase is widely used in the production of sausages, raw smoked ham and fish products. The effect of TG on meat is significantly increased by the presence of sodium chloride [100]. Adding of salt (more than 2%) increases the solubility of fibrillar muscle proteins, which then become available to TG for their cross-linking. In the last two decades the researches have been run on the use of TG in meat products to improve their properties, such as gelation, water binding, emulsion stability, cleaning losses, cooking losses, etc. The recent review considers the various possibilities for using TG to control the functional characteristics of meat and its products, including the processes of restructuration and value-adding [101].

Cross-linked meats are interesting because it allows using the lower quality cuts of meat, such as trimmings vie their assembly into integral lump meat products, which shape is more appealing to the consumers. However, the process of creating the cross-linked meat often involves its freezing and can cause the product to lose its color, which can make its sale pretty challenging [102]. To avoid consumers' misunderstanding the meat, meat products and meat semi-finished products that may look like a single lump of meat but are actually made up of different pieces mixed together should be labeled as "formed meat" (Annex VI, Part A No.7 Regulation (EU) No 1169/2011[1]). There is some evidence that transglutaminase (TG) in combination with dietary proteins can form proteins that are structurally similar to gluten [103]. This can create problems for people who suffer from celiac disease. Additionally, using parts from different animals in one lump can make it difficult to trace the origin of a product and identify potential sources of diseases outbreaks [104].

Nowadays the sensitive analytical method has been developed to verify the labeling of meat products, including the products restructured with the help of transglutaminase, and to protect the consumers from possible misrep-

[1] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006. Retrieved from https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32011R1169 Accessed January 16, 2024

resentation. High performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) is used to detect TG obtained from *Streptomyces mobaraensis* in the restructured meat products. Six tryptic marker peptides are used for this purpose [100]. Among these six marker peptides two peptides (VTPPAEPLDR and SPFYSALR) are suitable for the detection of all three types of TG in the meat and meat products and have been successfully inter-laboratory validated by the method HPLC–MS/MS [105].

In addition to transglutaminase, the other oxidative enzymes were researched as probable candidates for meat cross-linking, in particular: tyrosinases [106] and laccases [107]. Tyrosinase proved to have advantages in improving water holding capacity in comparison with transglutaminase (TG) [108]. However, none of these enzymes are currently used in the meat processing industry [109]. Microbial transglutaminase is recognized as an effective "protein maker", capable to catalyze the cross-linking reactions between protein and peptide molecules within the meat products, thus improving their functional characteristics [101]. Based on the above, it is obvious that TG can be successfully used to develop new products with improved properties [110]. It can be concluded that in the future TG will play an important role in creation of restructured meat products with increased value.

Identified gaps and future trends

Many researches are devoted to the ability of exogenous enzymes to improve meat tenderness [12,111]. However, the problem and issues with exogenous enzymes are rooted in the regulation of their specific activities. Many kinds of proteases have a tendency to indiscriminately degrade the essential proteins in muscle due to their broad substrate specificity [1,111], which leads to extensive damage to the fiber microstructure and affects the color, taste and moisture content of the meat.

Bromelain, ficin extracted from *Ficus insipida*, papain, and peptidase obtained from *Bacillis subtilis* are currently approved by the Food Safety Inspection Service of the US Department of Agriculture as "generally recognized as safe" and are allowed for their use in improving meat tenderness [112]. However, the excessive tenderization of meat by plant proteases under uncontrolled conditions, due to the non-specificity of their proteolytic activity, still leads to loss of quality characteristics and promote the accelerated spoilage of meat, which still remains a challenge for the meat industry [111]. The use of plant proteases affects the taste, increases bitterness due to the formation of basic amines and amino acids, spoils texture and deteriorates the consumer appeal of the processed meat products. This fact can be explained by the general principles of interaction of the enzymes with the substrate [11]. In raw meat, which consists mainly of muscle tissue, the proteases predominantly affect the soluble globular proteins due to the overwhelmingly larger contact area. Several studies have noted that papain causes a mushy texture and foreign flavors [113]. Increased concentrations of non-plant peptidases may also provide negative effects on the sensory characteristics of meat, in particular making the processed product bitter [49]. To improve the methods of meat tenderization, it is necessary to solve the problem of obtaining safe enzymes with different specificities depending on the target component of the meat raw material, regardless of its being muscle or connective tissue.

However the application of microbial proteases also faces another challenge: native enzymes often have limitations that require certain modification to fit the specific conditions of the food processing. The use of genetic modification of enzymes became the solution. This modification aims to rationally improve enzyme characteristics such as purity, yield, specificity, catalytic efficiency, stability and versatility of functions. The purpose of those modifications is to ensure cost-effective production and sustainable development of the food industry.

On the other hand, the use of enzymes in food production and processing has been a representative example of the practical application of biotechnology for a long time. The reasons for this long-term success are explained by the specificity, rapid action and biocompatible nature of the enzymes. These features of the enzymes enable efficient chemical modification of the substrates and food processing to preserve nutrients along with the compliance with the public health and safety requirements. Most of the applied enzymes are derived from microbial sources, which can be attributed to the large number of transferred catalytic activities, plasticity and the relatively undemanding requirements for its growth along with the high productivity, provided by microorganisms. Moreover, recombinant DNA technology has made it possible to express the enzymes, being the subject of interest, from the higher organisms or from the slow-growing or pathogenic microorganisms in fast-growing microbial strains that comply with health safety regulations. As a result of market limitations that require increasing the production capacity as well as production of the new products, there is a necessity of research to improve the performance of known enzyme catalysts or to find the new ones. In the first case, a better understanding of the catalytic mechanisms and protein configuration at the molecular level allows for a better understanding of the behavior pattern and stability of enzymes. Along with this, a more rational approach to application of enzyme compositions, including immobilization techniques, is required. These strivings in their nature are interdisciplinary, and this trend is expected to keep on going in the future. Screening for the new or more efficient enzymes is increasingly using more and more microorganisms obtained from extreme environmental conditions, as they are likely to provide enzymes capable of operating under the production conditions more suitable for given processes where they typically use the enzymes obtained from mesophilic organisms. This approach has achieved the significant success with recent developments in metagenomics, proteomics, and the identification of efficient systems of expression. Moreover the creation or development of en-

zymes with improved properties has become possible due to the development of directed evolution strategies combined with reliable computational methods. The use of multifunctional catalysts and the development of *de novo* enzymes, capable of performing any assumed chemical reaction, will allow multi-step reactions to be run in one step. All of these new interesting developments are highly likely to be implemented in the nearest future and therefore will improve and expand the role of enzymes application in the food industry.

Conclusion

The use of enzymes in the meat industry is an efficient way to improve the tenderness, juiciness and quality of meat products. These enzymes provide manufacturers with the new tools to create food products that meet the consumers' expectations and comply with the quality requirements. Moreover they help optimize production processes and increase the competitiveness of the industry in the food market.

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ASSESSING THE ROLE OF MEAT CONSUMPTION IN HUMAN EVOLUTIONARY CHANGES. A REVIEW

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Keywords: *animal-source foods, meat eating, human evolution, paleolithic, hominids, hunter-gatherers, food systems, diets*

Abstract

The historical study of changes in food patterns is an integral part of the study of biological and social adaptations during the formation and further development of Homo sapiens species. For quite a long time, diets have been considered the driving force of human evolution. Changes in the type of food consumed and the way it was obtained have been associated with encephalization and the emergence of bipedalism, as well as ecological, social and cultural evolution of hominins[1]. Archaeological and paleontological evidence indicates that at least about 3 million years ago, hominins increased their meat consumption and developed the necessary fabricated stone tools, while their brains and bodies evolved for a novel foraging niche and expanded hunting grounds. Animal-source foods have always been an integral part of the human diet. However, the way they are obtained and processed changed dramatically during human evolution. Meat became a common food source when systematic hunting began using technologies and tools focused on killing animals and meat cutting, which reduced the time and effort spent on chewing food, and later, on its cooking. At some point after this, humans began to hunt together, which made it possible to obtain meat from big game, and as a result, develop the social and altruistic skills to distribute the prey between sexes and ages. The eating habits of our ancestors have been studied using a variety of methods, including anthropometry, the use of archaeological data, and isotope analysis of bones and teeth to determine trophic status. The adaptive biological significance of meat-eating, which played an important role in human evolution, was analyzed, including the "expensive tissue hypothesis" draw attention to the evolutionary forces responsible for the increase in hominin brain size. Furthermore, data on changes in human anatomy, digestion and metabolism are systematized, indicating an evolutionary dependence on and compatibility with significant meat consumption. At the same time, a number of changes in the human body are associated with the skill of using fire in cooking. Heat processing of food stimulated our ancestors to overcome the food specialization intrinsic to animals. The question of what is the right diet for the human species and what are the potential consequences of limiting meat consumption is briefly addressed.

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Introduction

Food affects a variety of aspects in human biology, including the characteristics of behavior. The nutritional factor has always been decisive in the struggle for existence and passing on genes to future generations [1]. Traditions of obtaining food are a fundamental characteristic of the economic and cultural structure of society. "Food is the element of material culture in which traditional features are preserved more than other ones... and at the same time, it more easily and quickly borrows, varies and changes itself" [2]. Nutrition is something special in the life of *Homo sapiens* compared to what it is in other species. The image of the actual human specificity of nutrition phenomenon is formulated in a phrase belonging to Claude Lévi-Strauss: "Humanity starts from cooking" [3].

The nutritional needs of modern people and their diets arose as a result of a multimillion-year evolutionary process in which almost all genetic changes reflected the life circumstances of our ancestors [4].

Human nutrition played a key role in the process of hominization (the process of evolutionary transformation of the human ancestor from anthropoid ape into modern humans) [5]. In the 5-to-7-million-year period since the evolutionary emergence of hominins (bipedal primates within the taxonomic tribe *hominini*), 20 species may have existed [6]. Studies of hunter-gatherers have shown that there was no only universal diet consumed by all extinct

[1] The original meaning of "hominid" term referred only to humans (Homo) and their closest extinct relatives. However, by the 1990s, humans, primates, and their ancestors were considered "hominids." The former restrictive meaning is now largely assigned to "hominin" term, which includes all members of the human clade after the split from chimpanzees. The main difference between hominid and hominin: hominid is the family level that includes humans, whereas hominin is the tribe level that includes humans. (https://translated.turbopages.org/proxy_u/en-ru.ru.e1cacc63–649e6558–1ff4b86b-74722d776562/https/en.wikipedia.org/wiki/Hominidae).

hominin species. Most likely, diet varied depending on geographic location, climate, and specific ecological niche [6]. However, researchers should strive to uncover the dietary flexibility rather than any 'ideal food' that drove hominin adaptation [5].

Archaeological and paleontological evidence indicates that at least about 3 million years ago, hominins increased their meat consumption and developed the necessary fabricated stone tools, while their brains and bodies evolved for a novel foraging niche and expanded hunting grounds. Tools helped in hunting and cutting meat and reduced the time and effort spent on chewing food, and later, on its cooking [7,8].

Meat is one of the most valuable protein sources; also, it contains fats, vitamin B complex, especially vitamin B12 [9] playing an important role in maintaining the nervous system and genetic material of the body through the synthesis of methyl donors, which are necessary for the development and maintenance of methylation patterns in the body, vitamins A and D, large amounts of iron, zinc and other minerals [10]. It contains heme iron, an exclusive animal nutrient that is more easily absorbed than non-heme iron [11]. Although meat consumption is currently associated with a number of diseases, including cardiovascular disease, cancer and diabetes, meat plays an important role not only in maintaining proper growth, development and health, but also in human evolution [9].

Aspects of human anatomy, digestion, and metabolism differ from other primates, indicating an evolutionary dependence on and compatibility with significant meat consumption [12]. Meat consumption has a long history in human evolution, potentially going back to the earliest known anthropomorphic ancestor, who lived 5 to 7 million years ago [13,14]. Primitive stone tools discovered in eastern Africa age-dated back to about 2.5 million years indicate that early humans likely had the ability to cut and process animal meat. For example, cuts made by stone tools on the bones of carnivores found in Kenya and Ethiopia indicate meat consumption [14,15,16]. There is no archaeological evidence of meat consumption before 2.5 million years ago, but the commonality of hunting and meat-eating in our closest common ancestor and chimpanzee suggests that meat-eating has an ancient history, which originated before the anthropomorphic primate evolved, i. e. about 6 to 8 million years ago [17].

Materials and methods

A methodology used the article is an interdisciplinary approach, which combined in the context of the study such areas of anthropology as archaeology, biological and sociocultural anthropology, as well as a number of medical aspects of meat consumption impact on human health.

The advanced search methodology used to conduct the study consisted of two stages. At the first stage, a literature search was conducted to collect representative studies to achieve the purpose of this article; the second stage involved a selection process performed by reviewing the title and abstract of each publication.

To present a global vision of how the scientific community considers methodological problems in assessing the role of meat consumption in human evolutionary changes, a review and analysis of publications on key words and phrases was conducted: eating behavior and hominin adaptations, dietary reconstruction, meat eating and human evolution, encephalization in meat eating, strategies for obtaining food by hominids.

The search was focused on scientific articles and/or book chapters published in English and Russian from 1983 to 2023 in Scopus, PubMed, Google Scholar, Science Direct, eLibrary, and Russian Science Citation Index (RSCI) database.

Inclusion criteria were as follows:

1. Scientific research in the following areas:
   - Human eating behavior and problems of anthropogenesis;
   - Food strategies in prehistoric hunter-fisher-gatherer societies;
   - Anthropological and archaeological evidence of the transition to meat consumption;
   - Climate changes and changes in the diet of hominins;
   - Social role of fire and food innovations of Homo;
   - Nutritional requirements for encephalization.
2. Methods used to prove meat consumption.

Exclusion criteria were as follows:

Scientific articles presenting reviews of human attitudes towards meat-eating based on cravings (positive attitude) and aversion (negative attitude) in a given socio-economic context.

The role of feeding strategies in the evolution of human ancestor

The study of nutrition in past societies is an integral part of the study on biological and social adaptations during the formation and further development of *Homo sapiens*. When describing a species, it is the tradition of biology to give characteristics of the feeding base, food adaptation, and behavioral patterns associated with obtaining, preparing and consuming food. Studies of the behavior of various representatives of the Primates order have shown that, within the entire taxon, the choice of one or another feeding strategy certainly affects the social behavior of individuals within the community. This indicates the extreme importance of variability in nutrition types at various stages of anthropogenesis [18].

As studies of the nutrition in modern representatives of the Primates order have shown, the choice of one or another feeding strategy is associated with the environment and the behavior of animals. Consequently, reconstruction of nutrition types in human ancestors may be used as one of the keys to understanding the anthropogenesis [18]. Diet is related to the behavior, cognitive abilities, life and growth history of each hominin. The adoption of hunting

strategies is hypothesized to have led to improved health in early hominids, which explains the increase in adult height about 2.0 to 1.7 million years ago.

The origin of humans from anthropoid apes is confirmed by the similarity of their anatomy, physiology, ethology, immunology and genetic organization, as well as by the bone remains of intermediate fossil animals (Pithecanthropus) and, in general, is also practically assured in natural science. Obtaining food in anthropoid apes is associated with special forms of "food extraction." The cognitive abilities of anthropoid apes have improved greatly due to these skills. All primates are predominantly herbivorous species, and obtaining meat by hunting provides a small proportion of diet [3].

The corpses of dead and killed animals may be considered an affordable and valuable source of animal protein. An interesting method for determining the source type of animal-source foods was used by R. Brantingham. He compared the taphonomic parameters of prey remains typical of Plio-Pleistocene necrophages (some species of hyenas), predators (wolf) and early australopithecines (Koobi Fora). It was revealed that the set of bones in the layers associated with the activity of early australopithecines is more reminiscent of the remains of wolves' meals than of hyenas' meals. Based on this, the author concludes that Plio-Pleistocene australopithecines were hunters, not scavengers [19]. As evidenced by observations of the hunting behavior of modern primates, hunting contributes to the development of more complex relationships within the hunting group, and also stimulates the use of various types of tools.

Anthropologists discovered the remains of ancient humans age-dated back to about one million years in the regions of Central and Southern Africa, Mediterranean, Java Island. The first archaeological evidence was found in 1892 on the island of Java and was named Pithecanthropus (from Greek "pithekos" — ape, "anthropos" — human), i. e. ape-man. And in 1962, Bernard Campbell introduced the concept of *Homo erectus*, i. e. erect man, synanthrope. Therefore, most anthropologists classified Pithecanthropus and Sinanthropus, who lived 2.5 million to 400 thousand years ago, as the species of ancient humans [18].

During the Middle Paleolithic age, about 250 thousand years ago (with a total duration of about 200 thousand years), a glacier advanced. At this time, intensive adaptation of the human body to harsh environmental conditions occurs. More high-calorie foods (fats, proteins) were required than in previous, warm climatic conditions, the main suppliers of which were meat and animal-source foods. Under the influence of climate, nutrition and social system (the primitive communal system was replaced by the clan system), the human himself changes. In particular, the consumption of meat, which is rich in easily digestible proteins, in addition to saving time for the development of a primitive crafts, contributed to significant changes in the structure of the human higher nervous system. According to many researchers of evolutionary processes, this was a significant step in the formation of *Homo sapiens* as a species. The gradually dying out Pithecanthropus was replaced by the Neanderthal man during the Upper Paleolithic age (lasting about 30 to 36 thousand years). About 150 to 30 thousand years ago, Neanderthal man became the most common type of hominid [18].

Neanderthals inhabited mainly the periglacial zone of Europe. Especially many remains were found by archaeologists in the territories southward of 50° north latitude (France, Belgium, Germany, Italy, Spain, Yugoslavia, Czech Republic, Slovakia, etc.) [18]. Neanderthals knew well how to handle fire, and they also learned to use animal skin as clothing. Unlike *Homo erectus*, Neanderthals hunted constantly, and not from time to time.

The food patterns of Neanderthals are more consistent with the harsh conditions of the Ice Age. Simulating of the energy expenditure of Neanderthals, based on body proportions and reconstructed body mass, showed that these expenditures were very high. Heavy physical activity combined with the moderate body size of Neanderthals required the absorption of large amounts of energy-dense food, therefore, their diet had to include a large number of high-energy and highly nutritious foods [18]. During the late Pleistocene (100,000 years ago), meat consumption by Neanderthals was so regular that animal-source foods were the dominant component in the diet [20].

Neoanthrope ("new human", i. e. the first *Homo sapiens*, Cro-Magnon man) is a generalized name for modern humans who replaced all their predecessors during the period of 40 to 10 thousand years ago. Cro-Magnon men habitat was the territory of modern Africa, Europe, southern part of North America, northern part of South America, European part of Russia. All activities of the Cro-Magnon men were improved compared to their ancestors [18].

Homo sapiens (from Latin "intellectual human") is the only one currently living species of the genus Homo (humans) from the family of hominids in the order of primates. In addition to a number of anatomical features, it differs from modern anthropoids in a significant development of material and non-material culture (including the manufacture and use of tools), the ability for articulate speech and abstract thinking [21].

Since the first Homo, humans have always remained omnivores. The proportion of animal proteins and food of plant origin varied in evolution and history. However, the ability for maximum omnivory more than once was a property that provided humans with the prospect of survival in a variety of conditions, and specialization to any single food source became only a temporary "salvation", which closed the prospect of developing for such a specialized group. This pattern of specialization/omnivory alternating still emerged in later stages of human evolution and history [3].

Findings from paleontological and archaeological research have supported the theory that the inclusion of more animal proteins began with the earliest Homo.

It is assumed that *Homo habilis*, whose appearance is age-dated back to approximately 2.4 million years, obtained meat primarily by scavenging (and a smaller portion by hunting), while for *Homo erectus*, hunting was the predominant method of obtaining animal proteins, and this appears to be an important adaptive shift in human evolution [22].

The adaptive biological significance of meat-eating was summarized by Milton [23], who concluded that "the inclusion of animal-source foods in the diet played an absolutely important role in human evolution", otherwise the arid and seasonal environment that was probably the cradle of hominids would not have provided sufficient amount of protein.

Changes in the diet of early men: biological and cultural evolution

Diet is fundamental to an organism's ecology, and it is not surprising that changes in diet have been perceived as key milestones in human evolution [24].

The first revolution in eating habits occurred with the "discovery" of fire by the modern human ancestor, *Homo erectus*. Cooking and using fire were originally very rare and may have originated with *Homo erectus* in Africa about 1.8 million years ago [25]. Fire must have allowed them and subsequent modern humans (such as *Homo sapiens*) to make better use of food. Cooking improves digestion and can eliminate possible toxins contained in food. Thus, this likely included several foods in the early man's diet. Scientists hypothesize that, in addition to the nutritional aspect, fire facilitated the social gathering for meals. Primitive human beings also used fire apparently to surround their prey during the chase and to scare off predators [26].

The "discovery" of fire by *Homo erectus*, the direct ancestor of *Homo sapiens*, is not accepted by other researchers. Some researchers argue that *Homo erectus* was able to control and maintain fire, but did not generate it [26].

The second revolution occurred approximately 11,000 years ago with the advent of agriculture in Southwest Asia. This not only marked the introduction of grains into the human diet (e. g. oats, barley, rye, wheat, etc.), but also established human populations in certain places. While humans were still hunter-gatherers, populations remained in the same place until migration in search of food became necessary. With the development of agriculture, men no longer needed to be nomads because food could be grown near their habitats. Raising animals has also facilitated human efforts to survive in fixed locations [26].

Omnivory allowed the human species to establish itself throughout the world. If humans were exclusively vegetarians, they would not settle in areas with few plants, such as Alaska. If they were purely carnivores, they would have faced considerable difficulties, mainly because successful hunting was not guaranteed. When some authors argue that the diet of hunter-gatherers during the Paleolithic period was more suitable, one question arises. If people today have no proper food, while diets were better before agriculture, then why are people living longer today? [26]

The use of animal-source foods in the human diet has a long history of at least 5 million years. Meat consumption in human evolution may be divided into four periods: 1) occasional hunting and possibly gathering; 2) the beginning of regular hunting presumably about 2 million years ago; 3) the transition from hunting and gathering to domestic food sources, both animal and plant based, began 10,000 years ago; and 4) sustainable meat consumption, especially after World War II [14].

Hunter-gatherer food obtaining strategies. The role of hunting in meat eating

Analysis of both ethnographic and quantitative dietary data showed that, even at lower latitudes where plant food sources are available year-round, animal-source foods were the preferred source of energy for most hunter-gatherers around the world [27].

Although meat consumption has a long history, it was probably not a common food source until systematic hunting using meat-focused technology began presumably about two million years ago. Hunting large prey by groups of cooperative adults provided humans with regular and predictable access to protein and micronutrients [14].

Traditionally, special attention is paid to the consumption of meat by the first humans, because hunting is followed by the subsequent division of prey. This procedure involves some altruistic behavior towards members of the community who did not participate in the hunt, which is extremely important for human behavior. On the other hand, gathered foods are also distributed among members of society [18]. Hunting strategies are thought to have led to the division of labor and the development of more complex social systems [28].

H. erectus used stone tools [29]. The creation of tools of the Olduvai culture allowed for successful hunting and easier processing of carcasses, as well as increased access to meat, marrow, and bones of the animals [30]. Hunting requires cooperative interaction, which led to pantomime and vocalization, which was a turning point in the development of language [31].

Archaeological evidence indicates that after cutting, prey was transported to the place of distribution, cutting and common eating of food. In the opinion of a number of researchers, such a place is the prototype of home as the center of the economic and social life of society. According to Foley [32], the wider use of meat as the main source of proteins allowed early humans to solve the problem of surviving the unfavorable dry hungry season, which is why it was a decisive adaptation for humans.

Cooperation in hunting and distributing meat was one of the first steps in sociogenesis. Even today, hunting may be a way to escape social tensions in the presence of close friends, while common meat and food consump-

tion remains a bonding mechanism. Additionally, the origins of the art are linked to hunting rituals, and animals that were hunted as prey, such as bison, became the first known subjects of animalistic art during the Upper Paleolithic age. Later, the rituals of hunting and animal sacrifice became an integral part of various religions and were firmly attached to the cultural framework of myths and folk tales [31].

Humans obtaining food are similar to other animals in their natural environments in that they attempt to maximize the ratio of energy intake to energy expenditure when hunting, fishing, or gathering food [33].

An assessment of the energy values while consuming various plant-source and animal-source foods that are components of hunter-gatherer diets shows that animal-source foods provide the greatest energy and that consumption of larger animals tends to provide greater energy than smaller animals. For example, the potential food mass would be the same for one deer weighing 44.8 kg and 1600 mice weighing 28 g each. However, people obtaining food have to use significantly more energy to catch 1600 mice than to catch one deer [33]. Likewise, varying amounts of fat in the edible carcass also determine the ratio of energy from protein and fat [27]. Hunter-gatherers tended to avoid very small or low-fat animals due to their excessive protein content [33]. Historical and ethnographic evidence shows the adverse health effects that occurred when men were forced to consume fat-poor lean wild meat [34]. Excessive consumption of lean protein without sufficient fat or carbohydrates causes a condition that early American researchers called "rabbit fasting", which leads to nausea, diarrhea and ultimately death [34]. For humans obtaining food, preventing the physiological consequences of excess protein in the diet has been an important factor in forming their feeding strategies [34,35]. Thus, lean meat could not be consumed in unlimited quantities, but had to be accompanied by sufficient fat or carbohydrates from plant food sources.

Using archaeological data and isotope analysis to study changes in diet (evidence of meat consumption)

The first cases of meat consumption by early hominins were noted in Africa about 3.4 million years ago in Dikika, Ethiopia [25].

To obtain sufficient data on diet and meat consumption during human evolution, scientists use indirect and direct approaches. The indirect approach is based on evidence of fossil morphology as well as on plant and animal remains found during archeological excavations. The direct approach involves isotope analysis of bones and teeth. The stable isotopes contained in foods are incorporated into the growing teeth and bones of food consumers. These tissues then acquire an isotopic composition that matches that of the original food, which may reveal a lot about the paleo diet [24].

Archaeological evidence

Significant evidence of the consumption of meat and bone marrow are traces of meat cutting found on the bones. About 2 million years ago, an increase in the number of sites with animal remains with cut marks and stone tools was noted in East Africa [25]. The earliest, well-documented evidence of persistent carnivorous feeding behavior for early humans and animal consumption (carnivory) from fossil fauna obtained *in situ* comes from a large concentration of stone tools and numerous bone elements processed by hominins and age-dated back to about 2.0 million years, which are located at the Oldowan site in Kanjera, Kenya [36]. In addition to terrestrial animals, evidence from one site at Koobi Fora suggests that approximately 1.95 million years ago, hominins began to include aquatic animals in their diets, such as turtles, crocodiles and fish [37].

Tool sets may also have been necessary for hominins to cut carcasses, with cutting edges for processing soft tissue, as well as percussion tools for extracting bone marrow. With the onset of the Acheulean period and the emergence of *Homo erectus* populations *sensu lato* in Africa, between 1.9 and 1.7 million years ago, a change in diet was observed with more evidence of carnivorous and predatory behavior [25].

Meat consumption occurred quite early in human evolution, but habitual meat consumption, complex forms of cooperative hunting, entire meat cutting sequences, and the transport of bones and carcasses, which require advanced cognitive skills such as planning and decision-making, appear to have evolved later, not earlier than 1 million years ago, throughout the African continent and the Levant in combination with environmental changes of the early Middle Pleistocene [25]. Later, in Western Europe, a large number of remains of herbivores were discovered at Middle Paleolithic sites (about 400 to 40 thousand years ago) [25]. Results from isotope ratio studies [38,39] and dental wear data [40] show a significant intake of animal proteins in the diet of Neanderthal men. Neanderthals could rely for up to 80% on animal protein and 20% on plant proteins, making them the most emblematic carnivorous and competitive big-game hunters among extinct hominins [25].

Isotope analysis

This approach is based on the analysis and comparison of the presence and ratios for stable isotopes of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$). The relative values for stable isotopes of carbon and nitrogen may be useful for understanding the ratio of animals and plants in the diet.

The use of carbon isotope analysis in paleodietary studies is based on the simple concept that you are what you eat. Isotopic traces of past meals are frozen in tooth enamel, and are recoverable after millions of years, because enamel is essentially prefossilized and therefore, resistant to postmortem isotopic alteration [41].

Isotope studies of Neanderthal and Paleolithic human bones indicate that the dominance of animal-source foods in the human diet has a long history. These studies provide

objective data showing that the diets of hominids living in Europe during the Paleolithic were indistinguishable from top trophic level carnivores such as arctic foxes and wolves [33].

It is believed that early in the evolution of our genus, hominids may have experienced a number of genetic adaptations to animal-source diets similar to those of obligate carnivores such as cats. The carnivore diet reduces evolutionary selection pressure to maintain certain anatomical and physiological characteristics necessary to process and digest large quantities of plant foods. Inclusion of ever increasing quantities of animal-source foods into a diet of hominids led to a decrease in the gut size, as in the case of felines, with a concomitant increase in the brain size and metabolic activity [33].

Until recently, most of our understanding of past diet was based on the remains of plants and animals found at archaeological sites. These data sources provide a complete list of what humans ate, but not their relative proportions. The complete list presents a picture of diet, while proportions reflect nutrition, which is actually what we want to know. New insights into nutrition are provided by the chemical evidence of diet found in the bones and teeth of earlier humans [42]. The intensity of accumulation for stable isotopes of nitrogen ^{15}N and carbon ^{13}C is subject to environmental laws that have now been fairly well studied [43]. The accumulation of these isotopes in the main structural protein of bone tissue, i. e. collagen, follows these general laws. This fact allows for paleoecological reconstructions of the average diets of individuals. Some of the best information comes from stable isotope analysis of carbon [$^{13}C/^{12}C$ ratio ($d^{13}C$)] and nitrogen [$^{15}N/^{14}N$ ratio ($d^{15}N$)] extracted from human bones. Analysis of carbon isotope ratios shows the use of C3 plants versus C4 plants in the diet, since the isotope ratios of these plants are different (and therefore, the tissues of the men consuming these plants are different). C3 plants grow primarily in temperate climates, while C4 plants are tropical ones, such as corn in the Americas, millet in Europe and Asia, and sorghum in Africa.

Stable nitrogen isotope ratios are also used to determine the relative contribution of plant and animal protein sources or the position of the organism in the food chain (trophic level) [20].

There are a number of regions where we currently know a lot about dietary changes. For example, on the southeastern Atlantic coast of the United States (Georgia and Florida), a simultaneous decrease in seafood consumption and an increase in corn consumption have been documented [42]. Overall meat consumption has also likely declined. Although seafood sources have decreased in the Atlantic coastal areas with the development of agriculture, they still play an important role in the nutrition of the population. In Neolithic Greece and some other coastal areas where agriculture became important, seafood sources appear to have been greatly reduced (or even eliminated), at least as identified by stable isotope analysis. In general, the emerging picture indicates that over the last 10,000 years of human evolution, there has been a reduction in animal-source foods in the diet [14].

What did meat consumption give?
Changes in the body of ancient humans associated with meat consumption

Diet and nutrition type significantly affect the formation of the digestive system and other systems of the human body and are one of the most important components of the external environment during the evolutionary development of human. A leap in development, i. e. increasing brain complexity and volume, is directly related to changes in food patterns. And those fossil primates who did not switch to a mixed diet are not associated with the origin of the genus Homo [44].

Archanthropes were the first humans to reliably hunt large animals. A sharp increase in brain volume, body length, body weight, life expectancy, a decrease in sexual dimorphism (anatomical differences between males and females of the same biological species, not counting the genitals) indicate significant changes in food patterns due to an increase in the proportion of meat [18,45].

Hunting and meat eating resulted in increased body size. *Homo erectus/ergaster* males had an average body mass of 66 kg compared to *Homo habilis*, which weighed 37 kg, while body mass of females increased by 53%, from 32 kg for *Homo habilis* to 56 kg for *Homo erectus/ergaster*. The height increased from 131 cm to 180 cm (by 33%) for males and from 100 cm to 160 cm (by 37%) for females.

Bipedalism, which distinguished ancient humans from other apes, appeared in the oldest known species of Australopithecus, who lived in Africa about four million years ago. According to some investigations, 'postural' bipedalism was found in *Australopithecus afarensis*, and locomotor bipedalism did not appear until the emergence of *Homo ergaster* between 1.9 and 1.5 million years ago. Some authors consider that bipedalism in *Homo ergaster* was associated with climate changes in Africa where more open habitat formed and food resources became patchily distributed forcing humans to move in order to find food. Moreover, bipedalism may be considered one of the first strategies in the evolution of human nutrition [22].

As meat became more common in the diet, changes occurred in the digestive tract. Despite the fact that during the evolution of australopithecines, the total area of chewing teeth increased from 460 mm^2 in *Australopithecus afarensis* to 756 mm^2 in *Australopithecus boisei*, in early species from the genus Homo, there was a decrease in the posterior dentition. The surface area of postcanine teeth decreased from 478 mm^2 in *Homo habilis* to 377 mm^2 in early *Homo erectus* [30].

While changes in molar size reduction and anterior teeth strengthening may have been caused by changes in diet associated with tearing and chewing meat, changes in

intestinal morphology reflected the impact of a transition to a high-quality diet. Typically, large primates have a dilated colon, which is necessary to extract additional energy in the form of volatile fatty acids obtained during fermentation of plant fibers. On the other hand, humans have a thinner colon and an enlarged small intestine. These differences in intestinal morphology are the result of adaptation to the easily digestible animal proteins in the human diet.

The genus Homo had significantly smaller molars, chewing muscles, weaker maximum bite force, and relatively smaller intestines than earlier hominids. It is believed that this paradoxical combination of increased energy requirements along with decreased masticatory and digestive abilities became possible by the addition of meat to the diet, mechanical processing of food using stone tools, or cooking. Researchers found that if meat comprised one-third of the diet, the number of chewing cycles per year would have declined by nearly 2 million (a 13% reduction) and total masticatory force required would have declined by 15%. Furthermore, by simply slicing meat and pounding USOs, hominins would have improved their ability to chew meat into smaller particles by 41%, reduced the number of chews per year by another 5%, and decreased masticatory force requirements by an additional 12% [47].

Meat and body height

It is possible that the adoption of hunting strategies led to improved health in early hominids about 2.0 to 1.7 million years ago, which explains the increase in adult height [14]. McHenry and Coffing documented a dramatic increase in body weight by 44% in males (from 37 kg in *Homo habilis* to 66 kg in *Homo erectus/ergaster*) and by 53% in females (from 32 kg in *Homo habilis* to 56 kg in *Homo erectus/ergaster*) [45,46]. This represents an increase in height by 33% for males (from 131 cm to 180 cm) and by 37% for females (from 100 cm to 160 cm) [46].

Numerous studies show that in different eras of *Homo sapiens* existence, periods of increase and decrease in body size periodically replaced each other. The height of the first representatives of the Cro-Magnon men was no less than that of modern Europeans, while by the Middle Ages, the body length of the population in this part of the world had noticeably decreased. The cyclical nature of acceleration and retardation processes (acceleration and deceleration of growth) is typical for various regions of the planet.

At the level of entire nations, differences in height are determined not by ethnicity or race, but by living conditions [48]. The height of certain population representatives may be considered a historical indicator, showing both the quantity and quality of foods consumed in youth, and the living conditions that existed at a given time [49].

Study of archaeological specimens shows variations in the height of modern humans. These variations are associated with an access to high-quality food, which includes meat. In a number of countries around the world, the transition to agriculture has led to a decline in height, reflecting a focusing on the use of domesticated plants, probably less meat consumed, and certainly a reduction in diet in general [14].

A significant amount of anthropometric historical data on height variation is derived from analysis of military and other records. Costa and Steckel [50] analyzed data on the height of recruits in North America from the eighteenth to the twentieth centuries and found a maximum increase of about an inch in height from 1710 to 1830, followed by a decline of two inches during the remainder of the nineteenth century. Since about 1890 and up to the present day, there has been a steady increase in height. Studies conducted in the 20th century also found an association between meat consumption and increased height, for example in Belgium [51].

In other human populations, access to meat appears to improve health as measured by height. The Equestrian Plains tribes of North America, for example, are among the tallest of any native American population. Their access to buffalo and other animals was greatly facilitated by the use of horses for hunting and the subsequent distribution of food [14].

According to measurements taken in 1974, the average height of Russian male city dwellers born between 1916 and 1957 showed an increase from 167.03 cm (born in 1916) to 172.42 cm (born in 1957) respectively [52]. Systematization of research results shows how periods of malnutrition, social cataclysms and wars affected the height of males.

The above results are consistent with research conducted by scientists from the Department of Anthropology of Moscow State University named after M. V. Lomonosov [53], who showed that despite the significant variability in secular changes in body size at different regions of Russia, a common pattern was identified for all populations, which consisted of an increase in definitive body length over the studied period of time. The analysis includes materials on body length for the adult population of 50 territorial subjects of the Russian Federation (34 regions, 9 republics, 6 territories, 1 autonomous district), surveyed from the end of the 19th to the beginning of the 21st century. The average values of secular increases in definitive body length in the Russian population over the entire survey period were about 8 cm in men and over 4.5 cm in women, which is comparable to global values [53].

From the mid-1950s to 1978, meat consumption *per capita* increased from 60.8 kg/y to 98.0 kg/y, representing a 50% increase. At the same time, grain consumption declined and fruit consumption increased. Heights showed dramatic increases. After World War II, this trend was repeated globally in developed and developing countries [14].

Encephalization role in evolution

In hominins, the increase in brain size has occurred primarily over the last 2 to 3 million years. In fact, since the time of *Australopithecus afarensis* about 4 million years ago, brain size has tripled [54,55]. The driving force behind

the dramatic increase in brain size is unknown, although many reasonable hypotheses have been put forward based on socio-ecological factors.

Irrespective of the driving force for encephalization, two critical requirements had to be met: the brain's chemical requirement for long chain (LC) polyunsaturated fatty acids (PUFA), particularly arachidonic acid (AA, 20:4, n-6) and docosahexaenoic acid (DHA, 22:6, n-3) and the increased metabolic requirements of a larger brain. The fatty acids mentioned are the major structurally significant and biochemically active components of the brain gray matter in all mammalian species. The availability of these particular fatty acids may have provided a selective pressure acting to increase brain size, by simply supplying adequate dietary substrate to allow formation of brain tissue [55].

It should be noted that in the gray matter of the human brain, the ratio of major lipids is approximately the following: 25% is DHA, 25% is stearic acid, 14% is AA and 12% is oleic acid. In the photoreceptors of the retina, DHA content exceeds 59% of the total fatty acids, since high photosensitivity must be combined with high membrane fluidity [56].

Along with the body size, brain size also increased from 400 cm^3 in the earliest australopithecines to 1300–1400 cm^3 in modern humans, although similar changes in brain size were not found during regular periods of evolution. The greatest level of encephalization was found in *Homo erectus*, which had a larger brain relative to body mass than any other primate during evolution. The brain size of *H. erectus* reached 800–900 cm^3, which is approximately 200 to 300 cm^3 larger than the brain size of *Homo habilis* [30].

Larger brains were required for complex feeding behavior and tool use [31]. It is hypothesized that the practice of cooking using fire was critical for the development and maintenance of the enlarged hominin brains that began to evolve around 1.9 million years ago [57].

The human brain is a metabolically high-demand organ. On the basis of *in vivo* determinations, the mass-specific metabolic rate of the brain is approximately 11.2 W/kg (watts per kilogram). This is over 22 times the mass-specific metabolic rate of skeletal muscle (0.4 W/kg) [58].

Due to the process of "encephalization" in humans, the brain size is larger than it would be expected for their body size. To support an extremely large brain, energetic compensation was required during hominin evolution. When examining individual organs, excess brain mass (and its energy requirements) is closely balanced by a decrease in size (and correspondingly reduced energy requirements) of the gastrointestinal tract. This is not surprising given that the intestine is the only organ whose size may vary sufficiently to counterbalance the metabolic costs of a larger brain. This process required a transition from a diet high in low digestibility plants (requiring large fermentation compartments such as rumen or cecum, or large colon), to a higher quality diet where foods are higher in calories and require less digestive processing [12].

In 1995, Aiello L. C. and Wheeler P. developed the "expensive-tissue hypothesis" to explain how our huge brains evolved without causing a huge increase in our metabolic rate. They suggested that the energy demands of the large brain could be counterbalanced by a decrease in the size of the liver and gastrointestinal tract; these organs, like brain, have metabolically "expensive" tissues [54].

By considerably lowering the energetic cost for basal metabolism, a reduced intestinal mass may permit disposal of sufficient energy to cover the extra-expenditure required by a larger brain. Aiello and Wheeler based their argument on the relationship between body mass and Basal Metabolic Rate (BMR): the Kleiber line characterizing relationship between BMR and body size is identical for all mammals, including humans [12]. Because intestine and brain tissue are equally costly to maintain, Aiello and Wheeler proposed that intestine decrease led to the emergence of large brains in hominids. They argue that without the significant number of calories provided by eating meat, the human brain simply would not have been able to develop to its current size [54].

The role of fire. Eating raw or cooked meat: what are the energy effects?

The beginning of the active evolutionary development of mankind is associated with the use of fire, which occurred approximately 700 thousand years ago. In addition to the numerous functions of fire, one of the most important is the ability to process food, which led to the development of culture and, one can safely say, contributed to the "humanization" of a creature that at the dawn of history was only a project of what we now call man. Not raw, but cooked food became the most important milestone, a revolutionary anthropological turn that oriented human to the difficult process of evolutionary development and expansion of cultural forms. The process of food heat treatment stimulated a new quality of life for early humans, who began to evolve and formed their human identity overcoming the food specialization characteristic of animals [59].

In 1999, a process of hominization was hypothesized, which became possible by the early use of fire for cooking. It was assumed that the early archanthropes, who appeared about 1.9 million years ago, already knew how to cook food over fire, which made it possible to sharply reduce the metabolic cost of digestion [60]. The hypothesis was based on indirect data. For example, on the fact that in early archanthropes, not only the brain increased, but also the overall body size did. In addition, their teeth became smaller. This mean that they had to work less with their jaws. By comparison, chimpanzees spend an average of 5 hours a day for chewing, while modern hunter-gatherers who cook over fire spend only one hour for chewing.

Human is the only species in the world that cook over controlled fire, and there is archaeological evidence that at least some hominins were cooking over fire as early as 1.5 million years ago [61]. Interestingly, there are other scientists who suggest that hominin cooking behavior must

have originated around 2 million years ago. This logic is based on the basic nutritional benefits of cooking. Specifically, the cooking process breaks down nutrients and softens food, making it easier to digest, thereby providing more energy. Cooking also allows to eat a wider variety of foods and acts as a short-term food preservative. Additionally, approximately at the same time, hominin teeth began to decrease in size [62], possibly because larger teeth were no longer needed for chewing softened food.

Controlling fire and using it for cooking marks an important shift in nutrition. Despite the apparent importance of meat consumption in human evolution, some studies [63] have shown that raw food diets provide insufficient energy to maintain body weight, suggesting that food processing and cooking are very important. Indeed, when carbohydrates or proteins are cooked, they become more tender, better digestible, easier to chew (chewing time is reduced), metabolic cost is reduced, and energy extraction per mass unit increases [25]. Cooking also kills foodborne pathogens. However, the content of iron and some vitamins, such as vitamins B12 and C (which primates cannot synthesize), decreases at high temperatures and long cooking times. Cooking may be quite an expensive process in terms of the energy required to gather fuel, time required to start and maintain a fire, and requires some cognitive skills, e. g. for gathering fuel (selecting wood, bones, stones, etc.) and fireplace organization [64].

According to some authors [25], the increased energy requirements of a larger brain are compensated by a decrease in mass-specific metabolic rates in other tissues, such as gastrointestinal tract. Their studies in humans and nonhuman primates suggest coevolution between brain and intestine size, which is dependent on energy intake and may be determined by the dietary quality. They believe that hominins, especially after 2 million years ago (such as early *Homo erectus sensu lato*), had small intestines and would have required the use of fire and cooking to efficiently process lowly digestible foods such as meat. However, some authors believe that this compromise scenario appears to be insufficient, and they emphasize that brain size does not correlate with other energetically expensive organs or with the mass of the digestive tract, and that encephalization and fat accumulation in primates is a strategy to control calorie deficit [25].

Thus, cooking played a special role in the development of human diet. Primitive food cooking, as it was among early humans, went through a long and difficult path of development before it turned into the refined culinary art, which included the achievements of modern science [44].

Potential consequences of limiting meat consumption. What is the proper diet for the human species?

This question has been of concern to scientists for a long time, and current discussions and controversies are not resolved yet. In addition to scientific aspects, many other factors influence our eating habits, such as religion, tradition and socio-political situation, etc. [6,23,26,29,33,44,55].

It is not easy to answer this question. Each advocate of the eating habits hypothesis has his own arguments, often based on scientific evidence, but not always. Some scientific "evidence" changes very quickly. To better understand the question above, it is necessary to understand how the eating habits and behavior of our species evolved. It is also necessary to compare human eating behavior today and at the time when our species appeared [26].

From an anatomical point of view, several studies have shown that modern humans are unchanged from their Paleolithic ancestors. This leads to the conclusion that our diet must correspond to the standards of that period, because our species established itself on Earth exactly during that time [33].

In Western diets, increased consumption of meat (especially red and processed meat) is positively correlated with mortality from cardiovascular disease. However, a number of researchers provide additional evidence confirming the dominant role of animal-source foods in the diets of hunter-gatherers, and show how these dietary patterns do not necessarily contribute to the development of atherosclerosis and cardiovascular disease [33].

An analysis of the Ethnographic Atlas data by Cordain et al. (2002) showed that most foods in the diets of most hunter-gatherers were obtained from animal food sources [33]. The majority (73%) of the world's hunter-gatherers obtained >50% of their diet from consuming animal-source foods, and only 14% of hunter-gatherers obtained >50% of their diet from plants, respectively. For the 229 hunter-gatherer communities studied, the average dependence on animal-source foods was 66–75%. On the contrary, the average dependence on gathered plant-based foods was 26–35%. Re-analysis of data from Lee's original subsample (n¼58) of the Ethnographic Atlas showed the results almost identical to the above studies [27]. Dependence on animal-source foods obtained by hunting and fishing was 66–75% (median value), while the median value of gathered plants was 26–35%.

Scientists estimate that the food of ancient humans contained much less saturated fatty acids than the food of current people. Moreover, ancient foods contained almost equal amounts of n-6 and n-3 acids (about 1.5:1 ratio) and fewer trans fatty acids than modern foods. During our evolution, the total fat content of food was slightly more than 20%. In the middle of the 19th century, the situation began to change in the direction of increasing this indicator, and currently the fat content reaches almost 40% [65].

Humans also exhibit a range of specific adaptations indicative of extensive reliance on animal-source foods in the diet. Similar to obligate carnivores, humans have an inefficient ability to elongate 18carbon fatty acids from plant into the 20- and 22carbon polyunsaturated fatty acids (PUFA) essential for cell membrane and brain tissue function, hence requiring direct consumption from animal tissue. Likewise, humans have inherited a decreased ability to synthesize the amino sulfonic acid, taurine, which is involved in numerous essential physiological functions.

Studies on vegans reveal a plasma level of taurine 78% lower than in omnivores [55].

In addition, animal-source foods (especially red meat) are a well-known source of heme iron, which is absorbed more efficiently than non-heme iron found in plants [55].

There are potential nutritional benefits and risks associated with limiting meat consumption that vary depending on context, population, life cycle phase, and substitute foods. In many low- and middle-income countries, especially in sub-Saharan Africa and South Asia, meat consumption is very low and rates of malnutrition are high [66]. These populations could benefit from increasing, rather than decreasing, meat consumption. Livestock and animal-source foods are vital to sustainable development as they play a critical role in improving nutrition, reducing poverty, increasing gender equality, improving living conditions, increasing food security and improving health [67]. At certain stages of life, nutrient-dense and bioavailable foods are needed to meet all requirements: women of reproductive age, pregnant and lactating women, infants and young children, and the elderly. Diet changes in high-income countries, which tend to involve a decrease in red meat consumption, have been accompanied by a simultaneous increase in iron deficiency. For women of reproductive age, meeting iron requirements in any diet may be challenging, but limiting consumption of ruminant meat, one of the richest sources of bioavailable iron, makes it difficult to address this problem in the absence of iron-fortified foods or supplements [12].

Recently, research has shown that particular problems arise with brain functionality when a person's diet lacks animal foods, and this primarily manifests itself in children and the elderly [55].

An assessment of the Western diet shows that it distorts the finely tuned metabolism that has evolved over the long period of human evolution, leading to increased oxidative stress, immunopathological processes, chronic inflammation and hyperinsulinemia. These mechanisms create a single pathophysiological platform for the development of many chronic non-infectious diseases. Epigenetic modifications play an important role in the transmission of civilization diseases to subsequent generations. Returning to eating pattern close to the ancestral diet may be beneficial, but is associated with significant challenges. In general, the influence of nutrition on health reflects the well-known postulate that everything is good in moderation [68].

Conclusion

It is increasingly recognized that some of the fundamental changes in diet and habits that have occurred since the Neolithic Revolution, and especially since the Industrial Revolution and in the modern times, have occurred too recently on an evolutionary time scale for the human genome to fully adapt to them.

Meat is undoubtedly an important factor in human evolution. Changing dietary strategies was one of the triggers for brain evolution. In this sense, improving the quality of a meat-dominated diet allowed overcoming the limitations on brain growth.

Currently, various trends influence the choice to eat or not eat meat. The nutritional composition of meat makes an important contribution to the human diet, influencing proper growth, physical and cognitive development. However, awareness of animal welfare, environmental pollution and some of the disorders and diseases associated with meat production and consumption have created a meat-free trend. On the other hand, millions of people, mostly children, in a number of developing countries are starving due to a lack of animal protein.

A summary of the results of a number of studies shows that there is no historical or scientifically proven argument for eliminating lean meat from the human diet. There are a significant number of reasons to suggest that it should be the basis of a well-balanced diet.

The spread of a Western-style diet, which differs from the evolutionary dietary pattern, is significantly correlated with an increase in the incidence of chronic noninfectious diseases. The Western-style diet resulted from new foods and food processing procedures that emerged during the Neolithic and Industrial periods. At the same time, the key characteristics of the diet have changed: glycemic load, composition of fatty acids and macronutrients, concentration of micronutrients and amino acids, acid/base and sodium/potassium balance and fiber content. It is assumed that pathological processes are caused by disorders in the metabolic processes of the human body, which has been adapting to Paleolithic food for thousands of years. This is caused by too rapid environmental changes during the era of industrialization.

Systematizing all the facts allows to state that the choice of whether to eat meat remains with each person, since we are what we eat. Just like thousands of years ago, today humanity is still yet to solve the problems of healthy and safe nutrition.

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DEVELOPMENT OF A PREMIX BASED ON MICELLAR CASEIN FOR FORTIFICATION OF MEAT SYSTEMS WITH VITAMIN A

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Abstract
A novel approach to the protection of unstable fat-soluble vitamins, using retinol as an example, is presented in this work. This method is based on introducing vitamin A molecules into casein micelles. Protective properties of micellar casein towards different forms of retinol (native vitamin and palmitic acid ester) in vitro and in emulsion-type meat products are investigated. A technology of the introduction using micellar casein concentrate (MCC) has been developed. Conditions similar to those in which vitamin molecules can be in meat emulsions during heat treatment are simulated in vitro. The optimal time of "encapsulation" (2 hours) and the need for additional surfactant (tween-80) are identified. The use of the casein micelles protection made it possible to increase the number of retinol molecules that did not undergo decomposition under model conditions (in vitro) from ~30% to ~80%. Using the vitamin premix the degree of degradation of vitamin molecules does not exceed 4% after heat treatment. Data received allowed us to determine the efficiency of the protection properties of casein micelles for unstable vitamin A molecules.

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Introduction

The problem of vitamin deficiency among the inhabitants of Russia is quite common and relevant to this day. A more acute deficiency is observed in people living in the north of the country. For example, in one of the settlements of the northern region in the spring, approximately 20% of the surveyed had a lack of retinol. An effective method of solving this issue is the enrichment of food with vitamins [1]. However, some of them, due to their instability, need additional protection. Recent studies suggest that milk proteins can provide such preservation. Nowadays milk proteins found a wide application in the production of various products due to their availability, low cost, physicochemical properties, and high amino acid content [2–5]. Being a milk protein casein exists as micelles and consists of α_{S1}- and α_{S2}, β-, κ-caseins and calcium phosphate [6]. The properties of micellar casein began to be widely studied after the development of microfiltration methods for its isolation from milk. Simultaneously, an active search for ways to use casein in various areas of food production started. A range of articles can be found about the application of micellar casein concentrates in the production of sports nutrition, cheeses, yogurts, and high protein/low carbohydrate drinks [3,7]. Another direction of study is the use of casein and its components for the delivery of unstable products and medicines in human organisms [8–12]. This topic has been actively studied by many scientists in recent years and is of great interest.

The introduction of various organic compounds in the casein's structure allows us to control their concentration and ensure their transport in an unchanged form. Due to its micellar structure, casein exhibits a high ability to bind ions and small molecules, stabilizing them [13]. This property of micelles is especially seen in binding with water-insoluble compounds. It happens due to hydrophobic interactions, van der Waals forces, and hydrogen bonds [14]. Among the unstable hydrophobic molecules, fat-soluble vitamins A and D have a special place. These compounds are sensitive to light, UV radiation, and elevated temperatures. Casein can absorb radiation at 200–300 nm and protect these compounds [15]. Using vitamin D as an example, the ability of casein to protect molecules from exposure to high temperatures and destructive changes that occur during storage at low temperatures was shown. It favorably distinguishes the "casein" method of storing vitamins from, for example, storing a vitamin dissolved in vegetable oil or an aqueous emulsion stabilized by surfactants (e. g., tween-80) [14]. Generally, the recombination of casein is used to introduce vitamins in casein micelle. Initially, casein is taken not in its native micellar form, but as its processing product — sodium caseinate. After the introduction of vitamin to caseinate, solutions of K_2HPO_4, calcium citrate, and $CaCl_2$ are added to the reaction mixture. Under the action of these compounds, the micellar structure of casein is restored with vitamins presumably encapsulated in micelles. However, these restored micelles still differ from native ones [16,17].

In papers [15–19] researchers concluded that recombined and native micelles can protect some molecules (including retinol) by encapsulation. Heating β-carotene (vitamin A precursor) at 80 °C for 8 hours the concentration of carotene decreased by 83.5%, while using MCC the level of degradation was only 31%. Encapsulated vitamins also showed higher stability under high-pressure treatment. It is worth noting that the maximum decrease in the amount of β-carotene was observed in the first hours of heating; subsequently, its concentration ceased to change [18]. In the study of Mohan et al. [20] it was reliably shown that in milk enriched with vitamin A, most retinol molecules are contained in the casein fraction. To prove this, researchers separated milk proteins into different protein fractions using size-exclusion chromatography, and extracted vitamin A for quantitative determination by high-performance liquid chromatography (HPLC). After extraction, the composition of each protein fraction was determined by SDS-PAGE.

For a long time, micellar casein itself has been used during the production of various types of food products, including its application as vitamin transport [13,16,21–23]. However, in meat production, other additives based on milk proteins are mainly used, such as whey proteins, caseinates, and milk powder (the main protein of which is casein) [24–29]. Using the example of sausage production technology [30], it was shown that MCC has a positive effect on the functional and technological properties of minced meat systems and cooked products. Thus, analytical studies give reason to believe that the application of micellar casein concentrate in meat product formulations is an insufficiently studied topic, especially when used as a vehicle that transports and preserves vitamins in sausage products.

Based on these data, we hypothesized that the encapsulating ability of native casein can be effectively used to fortify boiled sausages with vitamin A. During production, these sausages are subjected to prolonged exposure to relatively high temperatures. Such heating, in the absence of protection, can lead to the degradation of most retinol. Thus, this study aimed to investigate the MCC protective properties towards unstable molecules of retinol and its palmitate *in vitro* and in model minced meat systems such as boiled sausages.

To implement this idea, it was necessary to develop a method for encapsulating vitamin A molecules in casein micelles which would include such requirements as easy scalability and implementation into production. Because native micellar casein was the object of our studies, relatively simple approaches using caseinate recombination in micelles were not of interest. Also approaches based on encapsulations in native micelles accompanied by rather specific conditions, such as excessively high pressure did not suit us. The second goal was to estimate the vitamin's degree of degradation under model conditions in an unprotected form, as well as with protection in the form of casein micelles. The last task was to study the preservation rate of vitamin A in the meat system. This work is devoted to the solution of these issues.

Objects and methods

The objects of this study were retinyl palmitate (pharmaceutical drug, 50 mL, JSC "Retinoids"), retinol (derived from retinyl palmitate), concentrate of micellar casein Lactoprima Pro MicCC85 (BaltMilk, Lithuania), retinol mixed with MCC, retinol mixed with MCC and tween-80 (IGL, India), retinyl palmitate, retinyl palmitate mixed with MCC and tween-80, vitamin premix based on micellar casein concentrate, minced meat system.

The studied minced meat system was manufactured according to the formulation presented in [30]. This system was an emulsion for the production of meat products such as boiled sausages. The heat treatment included three stages (drying, frying, and cooking) until the center of a sample reached an internal temperature of 72 °C, while the temperature in the thermal chamber reached 80 °C [30].

The solvents were purified and dried by standard methods.

^{1}H NMR spectra were recorded on a DPX-250 (Bruker, Germany) (250 MHz, Scientific and Educational Laboratory of Resonance Spectroscopy, Department of Natural and High Molecular Compounds Chemistry of Southern Federal University) spectrometer using $CDCl_3$ as a solvent with the solvent residual peaks as the internal standard.

High-performance liquid chromatography was carried out using the Gilson analytical HPLC system (USA).

Products were purified by chromatography (chromatography columns were used) on Al_2O_3 (Brockmann activity III). The progress of reactions and the purity of products were monitored by thin-layer chromatography (TLC) on Al_2O_3 plates and developed with iodine vapor or UV light (UV-viewing cabinet (Spectroline, USA)).

Stirring was carried out using an overhead stirrer SH-II-6C (Huanghua Faithful Instrument Co., China).

Distillation was conducted on a Hei-VAP Core rotary evaporator (Heidolph, Germany).

For ultrasonic exposure, an ultrasonic bath Sonorex Super RK 31 (Bandelin, Germany) was used.

In the experimental part, optimal procedures are indicated (taking into account the yield of retinol).

To select model conditions various technological processes and heat treatment parameters used in the production of boiled sausages were investigated and analyzed. Studies showed that the temperature of the chamber does not usually exceed 80 °C and the time of heat treatment is less than 3 hours. Thus, we chose the harshest conditions as a model one in which molecules of the vitamin can be in meat emulsions. These conditions included maintaining a constant temperature in the range of 78–80 °C and varying the time the sample was exposed to temperature, ranging from 0 to 3 hours. It is worth noting that the processes took place in the absence of light sources.

Isolation of native retinol from a drug

A mixture of the drug (50 mL), EtOH (200 mL), and 50% aqueous KOH (80 mL) was saponified for 30 min at 80 °C with vigorous stirring. Then H_2O (300 mL) was added, and the reaction mixture was allowed to cool to rt. The crude product was extracted with *n*-hexane (4×50 mL). Most of the solvent was distilled off, and the product was purified by column chromatography (Al_2O_3, *n*-hexane). A yellow-colored fraction luminous in UV was isolated. After evaporation of the solvent yellow-orange oil (solutions have a weak green fluorescence in UV) with a total weight of 259 mg was afforded.

The resulting mixture was dissolved in 8 mL of EtOH, 0.5 mL was taken and evaporated. 3 mg of anthracene was added to the sample containing 0.5 mL of ethanol solution and the mass of retinol in the sample was calculated using the ^{1}H NMR method. There were no antioxidants in the spectrum, the mass of vitamin A was 9.1 mg, therefore, the content of retinol in the resulting mixture is 56% (145 mg).

The degree of homogenization of an MCC and retinol mixture

The study was carried out at stirring for 1, 2, and 3 h. Ultrasound (ultrasonic exposure was carried out in an ultrasonic bath) and tween-80 were used as additional factors leading to homogenization. A control sample was stirred without the addition of tween-80 or ultrasonic exposure: 1 g of casein was hydrated in a ratio of 1:20. Then 0.5 mL of retinol's solution in EtOH (9.1 mg of retinol) was added. The resulting mixture was stirred at rt for 1, 2, and 3 h in darkness. A sample with tween-80 was prepared by adding surfactant (0.5 mL) and 0.5 mL of retinol's solution in EtOH (9.1 mg of retinol) to hydrated casein. With the additional action of ultrasound, the sample was placed in an ultrasonic bath for 15 minutes before stirring. Then it was subjected to ultrasound every 30 min for 5 min.

The study of the preservation of the vitamin depending on the stirring time

1 g of casein was hydrated in a ratio of 1:20. Then tween-80 (0.5 mL) was poured in, the mixture was stirred and 0.5 mL of retinol's solution in EtOH (9.1 mg of retinol) was added. The resulting mixture was stirred at rt for 1, 2, and 3 h in darkness and heated at 80 °C in silicone oil (Merck, Germany) for oil baths for 3 h with constant stirring. After heating dibutylhydroxytoluene (BHT) (30 mg), 50% aqueous KOH (15 mL), and EtOH (30 mL) were added. Saponification was carried out at 80 °C for 30 min. Then H_2O (10 mL) was poured, and the reaction mixture was allowed to cool to rt. The product was extracted with *n*-hexane (4×10 mL). After the evaporation of the solvent yellow oil was afforded.

4 mg of anthracene was added to the sample. The amount of retinol was calculated using ^{1}H NMR spectroscopy. The yield after 1 h was 5.2 mg, 2 and 3 h — 6.9 mg.

Retinol stability study

A mixture of H_2O (10 mL) and 0.5 mL of retinol's solution in EtOH (9.1 mg of retinol) was heated at 80 °C in silicone oil (Merck, Germany) for oil baths for 3 h with constant stirring. After heating BHT (30 mg), 50% aqueous KOH (15 mL), and EtOH (30 mL) were added. Saponification was carried out at 80 °C for 30 min. Then H_2O (10 mL) was poured, and the reaction mixture was allowed to cool to rt. The product was extracted with *n*-hexane (4×10 mL). After the evaporation of the solvent yellow-orange oil was afforded.

3 mg of anthracene was added to the sample. The amount of retinol was calculated using ^{1}H NMR spectroscopy. The yield was 2.6 mg, therefore, the content of retinol decreased by 71%.

Retinol stability study using a surfactant

A mixture of H_2O (10 mL), tween-80 (0.5 mL), and 0.5 mL retinol solution in EtOH (9.1 mg of retinol) was stirred at rt for 30 min in darkness. Then the reaction mixture was heated at 80 °C in silicone oil (Merck, Germany) for oil baths for 3 h with constant stirring. After heating BHT (30 mg), 50% aqueous KOH (15 mL), and EtOH (30 mL) were added. Saponification was carried out at 80 °C for 30 min. Then H_2O (10 mL) was poured, and the reaction mixture was allowed to cool to rt. The product was extracted with *n*-hexane (4×10 mL). After the evaporation of the solvent yellow oil was afforded.

3 mg of anthracene was added to the sample. The amount of retinol was calculated using ^{1}H NMR spectroscopy. The yield was 3.3 mg, therefore, the content of retinol decreased by 64%.

Retinol stability study using MCC

1 g of casein was hydrated in a ratio of 1:20. Then tween-80 (0.5 mL) was poured in, the mixture was stirred and 0.5 mL of retinol's solution in EtOH (9.1 mg of retinol) was added. The resulting mixture was stirred at rt for 1 or 3 h in darkness and heated at 80 °C in silicone oil (Merck, Germany) for oil baths for 3 h with constant stirring. After heating BHT (30 mg), 50% aqueous KOH (15 mL), and EtOH (30 mL) were added. Saponification was carried out at 80 °C for 30 min. Then H_2O (10 mL) was poured, and the reaction mixture was allowed to cool to rt. The product was extracted with *n*-hexane (4×10 mL). After the evaporation of the solvent yellow oil was afforded.

3 mg of anthracene was added to the sample. The amount of retinol was calculated using ^{1}H NMR spectroscopy. The yield after 1 h was 8.3 mg (the content of retinol decreased by 9%), 3 h — 7.0 mg, hence the content of retinol decreased by 23%.

Preparation of a premix based on MCC for its further incorporation into sausages

Tween-80 (0.15%, this volume is within the range of recommended safe doses for its use, and also provides a good degree of homogenization [31,32]) was added to hydrated

(1:4) MCC (8 kg of MCC, 2 kg of water). Then retinyl palmitate (1.8 mL) was gradually poured into the mixture. The resulting mixture was stirred for 2 h. After stirring a premix was cooled to 4 °C and added to the cutter according to formulation [30].

Determination of vitamin A by HPLC

Sample preparation for HPLC was carried out according to GOST 32307–2013[1]. After the isolation of vitamin A, it was dissolved in acetonitrile (0.05 mL). Then 0.02 mL of solution was taken and studied by HPLC. The amount of a vitamin was calculated using the formula presented in GOST 32307–2013. For example, the mass concentration of the vitamin in the calibration solution is C_{st} mg/cm^3, peak area of an individual vitamin in a sample S_x, peak area of an individual vitamin in a calibration solution — S_{st}, the volume of solvent taken to dissolve the dry residue is V_s mL, mass of the test sample — m g.

$$X = \frac{C_{st} \times S_{st} \times 0.5 \times 1000}{S_x \times m} \text{ (mg/kg)} \quad (1)$$

Each experiment was repeated 15 times. The main variable is measured in metric scale, therefore, to select statistical processing, a comprehensive check of the normality of data distribution was carried out (graphical analysis of histograms and calculation of the Shapiro-Wilk test). Since the variables almost always showed a distribution other than normal, non-parametric Wilcoxon T test (for dependent samples, comparisons between phases of the same experiment), Kruskal-Wallis H test and Mann-Whitney U test (for independent samples, comparison of the final mass of retinol in stability studies under different conditions) were applied. In order to study the preservation of the vitamin depending on the stirring time, we also used the calculation of the Spearman's rank correlation coefficient.

Results and discussion

The most common method for the quantitative determination of fat-soluble vitamins is HPLC. However, this method, like any other, has its drawbacks. In this work, a fast and effective method for the quantitative determination of fat-soluble vitamins using nuclear magnetic resonance spectroscopy was successfully used. It is consistent with the results obtained previously [33].

Firstly, the optimal conditions were selected to introduce vitamin into casein. Vegetable oil and ethanol were chosen as solvents. The "encapsulation" process was accompanied by intensive stirring and took place at room temperature. Stirring time varied from 1 to 3 hours. Ultrasound (US), surfactants (tween-80), and their combined effect were applied as factors contributing to the transition of vitamin molecules into micelles (Table 1). The addition of surfactants is common in the enrichment of milk and milk products with fat-soluble vitamins [34]. The idea of ultrasound application is based on the obvious need to disperse fat globules of vitamin mixtures. For a preliminary visual determination of the interaction effectiveness, the degree of reaction mixture homogenization was assessed. This method of vitamin protection is easier than the ones described earlier based on the recombination of protein [11,15,17,18]. Besides, this approach allows one to preserve protein micelles in their native state.

Table 1. Homogenization of a mixture of micellar casein concentrate and retinol with stirring

	$T_{stir.} = 1$ h				$T_{stir.} = 2$ h				$T_{stir.} = 3$ h			
	—	*US*	*Surf.*	*US + Surf.*	—	*US*	*Surf.*	*US + Surf.*	—	*US*	*Surf.*	*US + Surf.*
Retinol in EtOH	—	—	*	**	**	**	***	***	**	**	***	***
Retinol in oil	—	—	*	**	*	*	***	***	*	*	***	***

The degree of homogenization: —, no homogenization; *, poor; **, good; *, excellent. $T_{stir.}$ — time of stirring**

Since micellar casein is an emulsifier, although not as good as sodium caseinate, it has been suggested that a vitamin solution with hydrated MCC be kept under vigorous stirring. However, visible results were achieved only after 2–3 hours of stirring. As can be seen from the table, homogenization and, as a result, encapsulation proceed slowly without the use of auxiliary factors. Due to the hydrophobic nature of the vitamin, oil makes it more difficult to introduce it into casein. Ethanol mixes with water and creates conditions for effective penetration of retinol into the interior and pores of casein micelle. Short-term exposure to ultrasound does not significantly affect emulsification, while long-term exposure is undesirable due to the instability of the vitamin. At the same time, the use of tween-80 significantly accelerates the mixing and encapsulation processes. After an hour of stirring polysorbate introduction leads to partial homogenization with a gradual separation of the solution without stirring. After two hours of stirring the solution looks mostly homogenized. Additional stirring for one more hour does not lead to any visible changes. There were not any noticeable differences when the combination of ultrasound and surfactant was used. Thus, based on preliminary tests the MCC/tween-80 system was chosen to produce vitamin premix (the addition of surfactant leads to more effective homogenization).

As noted earlier, quantification was carried out using NMR spectroscopy. A given amount of anthracene was introduced into the samples as a standard. In this case, an important factor in the selection of a standard substance is the absence of signals in the spectrum that would intersect with the characteristic peaks of the substance studied. Anthracene signals are at 7.46 (dd, J = 6.5, 3.3 Hz, 4H), 8.00 (dd, J = 6.5, 3.3 Hz, 4H), 8.43 (s, 2H) ppm. Retinol peaks are at 1.03 (s, 6H), 1.46 (m, 2H), 1.61 (m, 2H),

[1] GOST 32307–2013 "Meat and meat products. Determination of fat-soluble vitamins by high performance liquid chromatography". Retrieved from https://docs.cntd.ru/document/1200107182. Accessed August 05, 2023

1.71 (s, 3H), 1.89 (s, 3H), 2.00 (s, 3H), 2.02 (t, J = 6.1 Hz, 2H), 4.32 (d, J = 7.0 Hz, 2H), 5.69 (t, J = 7.0 Hz, 1H), 6.09 (d, J = 11.2 Hz, 1H), 6.10 (d, J = 14.1 Hz, 1H), 6.14 (d, J = 13.9 Hz, 1H), 6.28 (d, J = 15.1 Hz, 1H), 6.64 (dd, J = 15.1, 11.3 Hz, 1H) ppm [33]. In the ^{1}H NMR spectrum of vitamin A, upfield signals cannot be used for quantitative assessment, since other aliphatic signals are present in the region of 1–3 ppm as well as water. In the downfield region, there are signals from the methylene unit associated with the OH group and C_{sp^2}–H protons, which do not intersect with other signals and can be used to estimate the content of retinol in the sample (Figure 1).

To estimate the influence of stirring on the "encapsulation" degree of retinol molecules into protein micelles, we carried out experiments with time of preliminary stirring varied from 1 to 3 hours. After stirring for a given time samples were subjected to three hours of heating under the same conditions. Figure 2 shows selected signals in ^{1}H NMR spectra obtained in our experiments. We calculated the amount of retinol using an anthracene singlet at 8.43 ppm (marked in orange) and a triplet from one of the CH retinol units (5.69 ppm), marked in green on the structure.

Initially, we supposed that there is a dependence on retinol concentration from stirring time. To test this

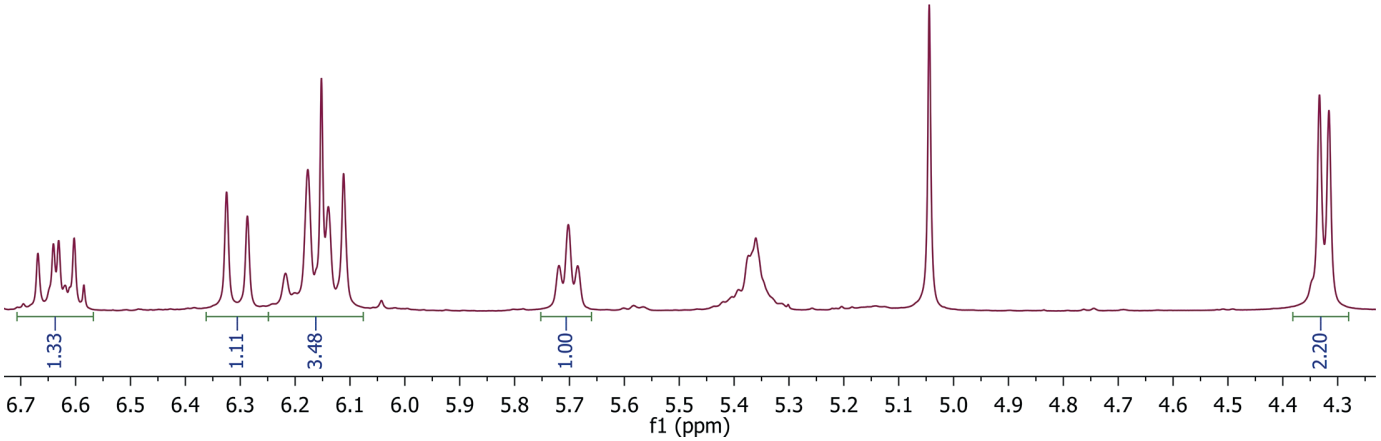

Figure 1. Fragment of the ^{1}H NMR spectrum of retinol isolated chromatographically from a drug

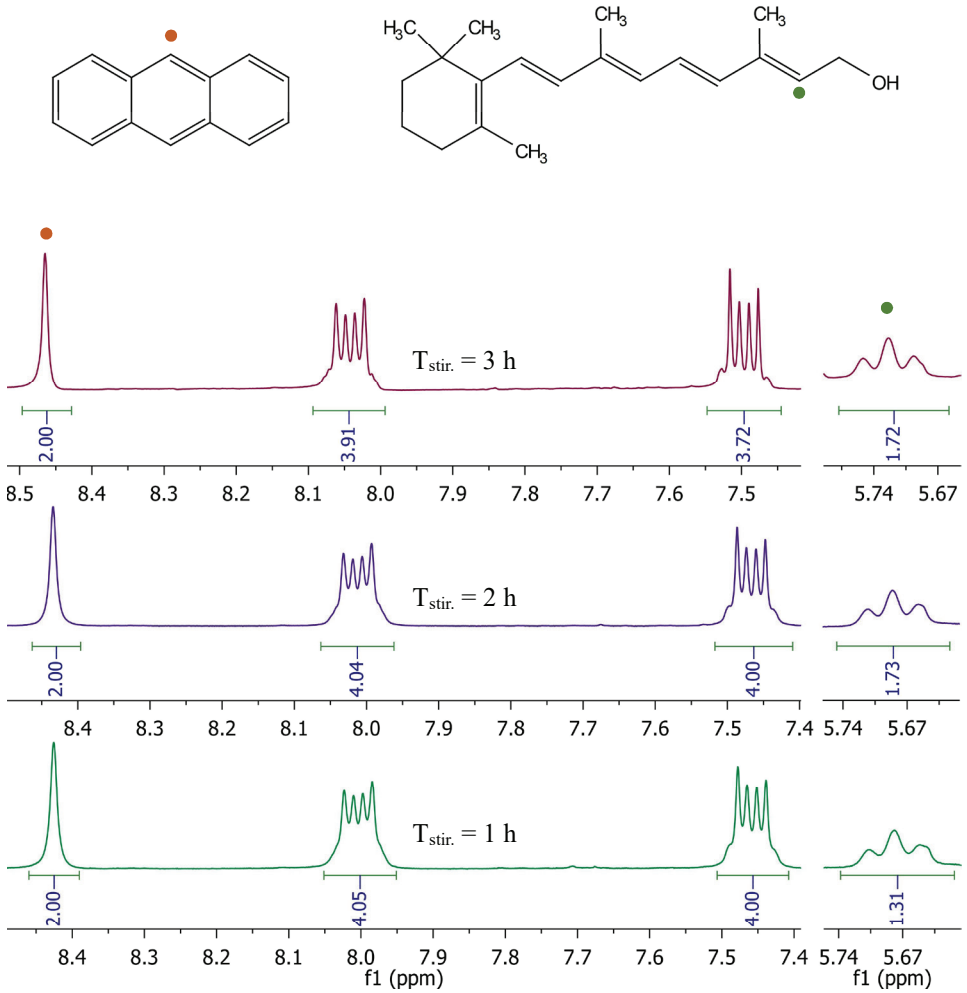

Figure 2. Fragments of ^{1}H NMR spectra of anthracene and retinol mixture with stirring for 1, 2, and 3 h

hypothesis, we calculated Spearman's correlations between two variables. Indeed, the resulting correlation coefficient confirms that the stirring time and the mass of the vitamin in the mixture are directly proportional to each other (positive correlation $r = 0.799421$, $p \leq 0.005$). To determine when the most significant increase in retinol concentration occurs, a comparative analysis was carried out.

Significant differences between the yield of retinol depending on stirring time were tested using the Wilcoxon test. Table 2 shows the results of calculating the criterion for pairwise comparisons of experimental phases, indicating the empirical value of the T-criterion and the level achieved.

Table 2. Results of comparison of the retinol mass in the samples depending on the stirring time

		Comparison between different points		
	M ± sd, mg	Before stirring	1 h	2 h
Before stirring	9.1			
1 h	5.18 ± 0.161	T = 0, p = 0.00065		
2 h	6.86 ± 0.073	T = 0, p = 0.00065	T = 0, p = 0.00065	
3 h	6.90 ± 0.065	T = 0, p = 0.00065	T = 0, p = 0.00065	T = 0, p = 0.06789

M — mean; sd — standard deviation; T — empirical value of the Wilcoxon test; p — significance level

In this work, significant differences are those in which the significance level is $p \leq 0.05$. Figure 3 shows a graph of the average retinol mass values depending on the stirring time. To visualize the spread of values curves with minimums and maximums are presented. The level of significance of differences ($p \leq 0.005$) between phases was also noted.

The result of this comparison allows us to conclude that there is a significant ($p = 0.0007$) difference in the mass of retinol before mixing and after 1 hour (the concentration decreases by 42.86%). Comparing the retinol concentration in the mixture after 1 and 2 hours the level of vitamin A increased almost by 20% which is also a statistically significant difference ($p = 0.0007$). There is no statistical difference between retinol mass after 2 and 3 h of stirring ($p = 0.067$). Thus, the optimal stirring time is 2 hours as the additional hour gives no significant difference for the increase in the vitamin concentration.

To estimate the efficiency of vitamin protection by casein it was necessary to understand at what rate retinol itself would undergo thermal degradation. Experiments were carried out using retinol solutions in sunflower oil or ethanol. The saponification stage was carried out in both cases since this stage is mandatory for the quantitative determination of vitamins in sausages. Saponification results in the removal of casein since its peptide bonds are subjected to alkaline hydrolysis. As described earlier, the alcohol solution mixes more easily with casein, so it was chosen to be used in further experiments. In Figure 4A fragments of ^{1}H NMR spectra are shown. They allow one to calculate the amount of vitamin A in samples without casein's protection after heating at 80 °C for 3 hours.

The first spectrum shows the initial concentration of the vitamin (Figure 4, A1). Since retinol was isolated from a drug and subjected to purification by column chromatography, it was decided to remove only the most mobile fraction which contained antioxidants to optimize the time. Thus, the isolated vitamin contained some impurities that did not affect the accuracy of the experiment, but the initial amount of the vitamin needed to be determined spectroscopically. NMR reliability in the determination of fat-soluble vitamins was verified by repeated quantitative evaluation of the same sample by HPLC. The discrepancy between the two methods is no more than 5%. The same results were obtained in work [33] in which pharmaceutical drugs were studied by the NMR method. Re-determination of the concentration is possible because ^{1}H NMR spectroscopy is a non-destructive method of analysis. Using the Wilcoxon test, we confirmed that the decrease in retinol concentration as a result of three-hour heating is significant, most of the retinol is decomposed, and its residual content does not exceed 30% (Figure 4, A2;

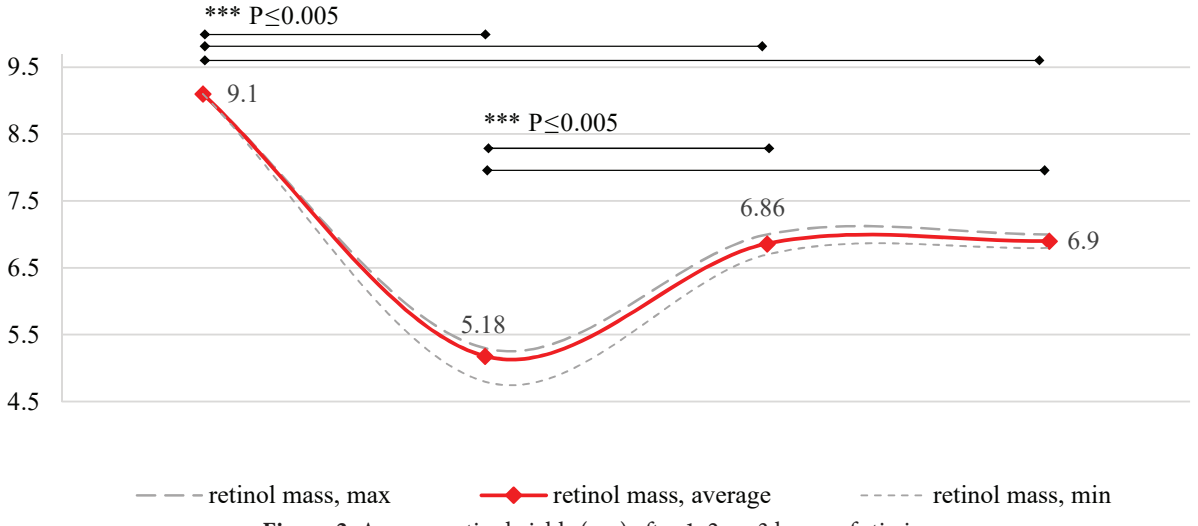

Figure 3. Average retinol yields (mg) after 1, 2, or 3 hours of stirring

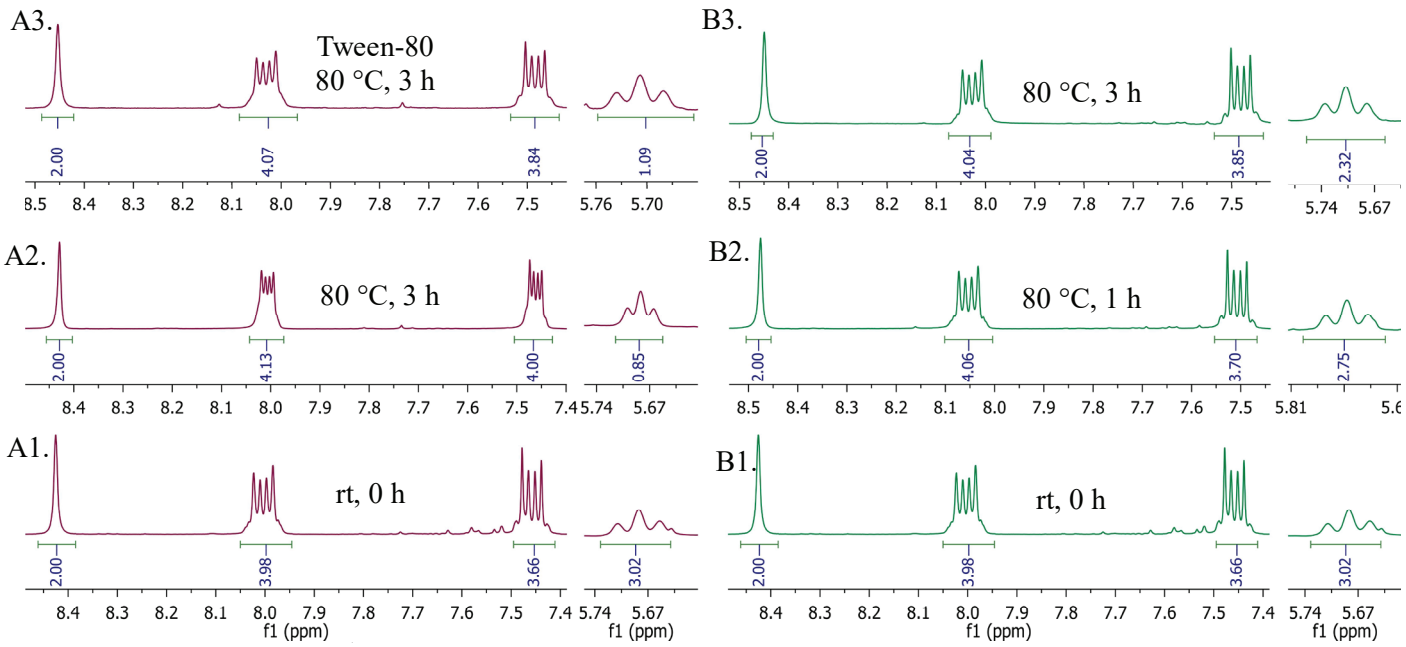

Figure 4. Fragments of ^{1}H NMR spectra of anthracene and retinol mixture without casein (A) and with the addition of MCC (B) without heating and with heat treatment for 1 and 3 h

Figure 5). The reduction of vitamin concentration in such conditions is in agreement with statistical calculations at the significance level $p \leq 0.005$.

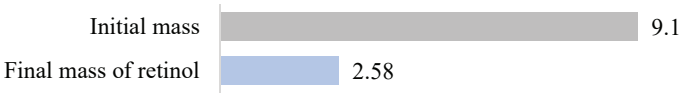

Figure 5. The average yield of retinol (mg) before and after the test was carried out (T = 0, p = 0.00065)

As expected, retinol in its pure form without any protection shows the worst results even in contrast to a vitamin sample emulsified with polysorbate (Figure 4, A3). Most likely, the micellar shell created by surfactant molecules can protect vitamin molecules to some extent. In general, it is seen that retinol at this temperature is largely subjected to destructive changes, the residual content of the vitamin after heating for 3 hours, even with the use of tween-80, is no more than 40% (Figure 4, A3). The Wilcoxon T-test allowed us to validate that the decrease in retinol amount in this experiment is significant. The difference between the mass of the vitamin in the mixture before and after heat treatment shows a statistically significant reduction ($p \leq 0.005$) (Figure 6).

These results are consistent with a study on the preservation of β-carotene by recombined casein, in which after 8 hours of heating at 80 °C the amount of vitamin A precursor was 16.5% [18].

The retinol stability using MCC was studied at 1 and 3 hours. The results of descriptive statistics are shown in Table 3.

Figure 4B shows the spectra of three retinol samples prepared using MCC and polysorbate. The first one is the sample that did not undergo any heating used for the determination of initial concentration (Figure 4, B1). The second sample was subjected to heating for 1 hour (Figure 4, B2) and the third one was heated for 3 hours (Figure 4, B3). According to the spectra, after an hour of heating, only 9% of the total number of vitamin molecules underwent destruction. After three hours, 77% of retinol was retained in the sample. All the described dependencies are presented for greater clarity in the form of a graph in Figure 7. Thus, the use of micellar protection provided more than a twofold increase in the residual content of retinol after three hours of heating at 80 °C. All experiments were repeated several times and showed high reproducibility. The results are consistent with similar data obtained by Sáiz-Abajo et al. [18].

Figure 6. The average yield of retinol (mg) before and after heat treatment (T = 0, p = 0.00065)

Table 3. Main descriptive statistics for experiments with a vitamin premix based on MCC

	Average amount, mg	Standard deviation
Initial mass	**9.1**	**0**
Mass after 1 h, t = 80 °C; Vitamin A + surf. + MCC	**8.3**	**0.151186**
Mass after 3 h, t = 80 °C; Vitamin A + surf. + MCC	**7.0**	**0.109978**

A comparison of the final retinol concentration in formulations obtained by different technologies was conducted in two stages. Firstly, in the Kruskal-Wallis test the variable H was calculated (a nonparametric analog of analysis of variance, since the data is not normally distributed) (Table 4).

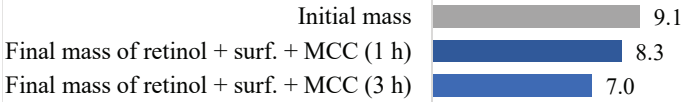

Figure 7. The average yield of retinol (mg) before and after 1 and 3 h of heating (T = 0, p = 0.00065)

Table 4. Primary results of comparison of the retinol mass in samples obtained by different techniques

	Kruskal-Wallis ANOVA by Ranks Kruskal-Wallis test: H = 55.75857 p ≤ 0.005		
	Number of measurements (N)	Sum of ranks	Average of ranks
Mass after 3 h, t = 80 °C; pure vitamin A	15	120	8
Mass after 3 h, t = 80 °C; Vitamin A + surf.	15	345	23
Mass after 1 h, t = 80 °C; Vitamin A + surf. + MCC	15	795	53
Mass after 3 h, t = 80 °C; Vitamin A + surf. + MCC	15	570	38

Pairwise comparisons were performed using the Mann-Whitney U test for independent samples. Since all the shifts were typical (i. e., between any two groups, changes in the concentration of the substance were observed only in one direction, and the sets did not intersect), the result of calculating the criterion for all pairs was similar and amounted U = 0, p = 0.000003393, which corresponds to the required significance level p ≤ 0.005. A visual comparison of the stability of vitamin A at heating at 80 °C for 3 hours in the presence of additional protection (tween-80 or its combination with MCC) is shown in Figure 8.

Even though we did not set the task of determining the localization of retinol molecules and whether they undergo encapsulation, we can indirectly assume that this probably occurs. This is supported by the rather effective protection of vitamin A provided by casein, as well as the fact that a shorter stirring (encapsulation) time leads to a decrease in the degree of protection.

The final stage of the research was to estimate the preservation of vitamin A in model minced meat systems such as boiled sausages. These systems were based on poultry meat partially replaced with micellar casein concentrate. The technology of manufacturing minced meat systems with minor modifications (the stage of obtaining hydrated casein) is presented in patent № 2801108, and the original formulation is given in Table 5.

Table 5. Formulation of model minced meat system

Component	Mass (kg/100 kg of minced meat)
Poultry meat (chicken fillet)	80
Pork rind	20
Spice mixture "Munich sausages"	0.7
Salt	2.5

A developed vitamin premix of retinyl palmitate based on MCC was added to meat systems. Casein concentrate was hydrated in a 1:4 ratio to achieve a protein percentage close to that in poultry meat. Then it was added to the recipe replacing 10% of raw meat. Firstly, we calculated the amount of vitamin added to the premix. To do this, data on the adults' physiological requirement for vitamin A (900 μg RAE/d) was taken as a basis[2]. According to the order of the Ministry of Health of the Russian Federation dated August 19, 2016, № 614[3], the standard consumption of poultry meat per year is 31 kg or about 85 g per day. The formulation of the studied systems with the partial meat replacement with MCC provides for the use of 72 kg of poultry meat per 100 kg of raw materials. It means 120 g of the finished product will contain the recommended daily intake. To eliminate the possibility of vitamin A overdose, the required dose of retinol was calculated as 10% (90 μg RAE) of the daily intake of vitamin per 100 g of the product. Thus, 90 mg of the vitamin would be used for 100 kg of the product. Taking into account the fact that the retinyl palmitate drug used contains about 55 mg of retinyl palmitate in 1 mL, 1.8 mL of the drug should be taken per 100 kg of raw materials. At the same time, the content

[2] MR2.3.1.2432–08 "Balanced diet. Norms of physiological needs for energy and nutrients for various groups of the population of the Russian Federation". Retrieved from https://fcgie.ru/download/elektronnaya_baza_metod_dokum/mr_2432–08.pdf. Accessed August 09, 2023

[3] Order of the Ministry of Health of the Russian Federation (August 19, 2016 No. 614) "On approval of recommendations on rational standards of food consumption that meet modern healthy nutrition requirements". Retrieved from https://nadn.ru/upload/iblock/58d/58df042069fa850e7d425d9f2b06244f.pdf. Accessed August 09, 2023

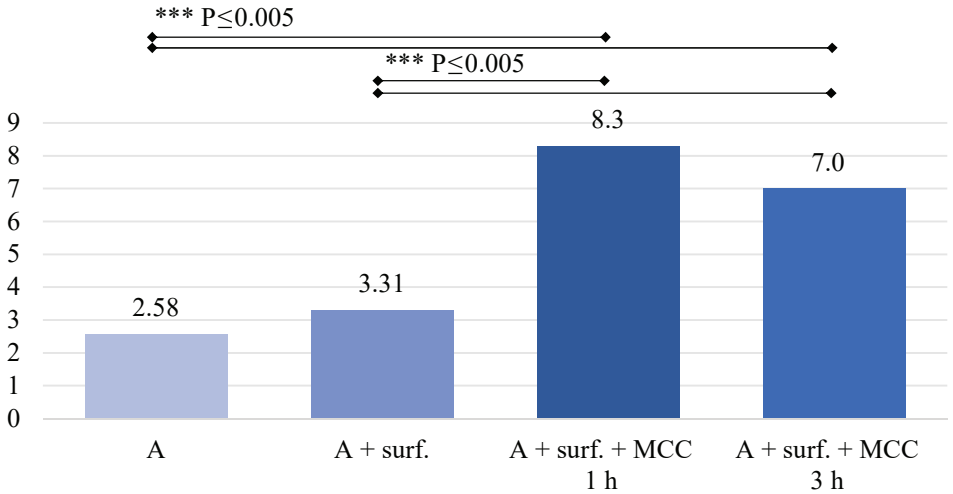

Figure 8. The average yield of retinol (mg) in samples obtained with and without using tween-80 or MCC and tween-80 (U = 0, p ≤ 0.005)

of vitamin A in the raw materials used for the production of sausages can be not taken into consideration due to its extremely low content in cooked products, which was established in different studies [35,36].

It is also worth noting that the quantitative ratio of casein/retinol in the product is much lower than in model systems. This allowed us to reduce the surfactant's amount required for more efficient homogenization to 0.15% vol.

Determination of retinol at sufficiently high concentrations was conducted by ^{1}H NMR in experimental studies. However, with the addition of vitamin A into the product according to the recommendations given, its amount would not exceed 1 mg in 1 kg of sausages. For such measurements, the nuclear magnetic resonance method is not applicable. Also due to the large mass of the sample tested, its preparation for analysis becomes difficult. Therefore, in this case, the concentration of retinol was determined by HPLC.

It was found that the model samples contained an average of 0.87 ± 0.05 mg of vitamin per 1 kg of finished product. This confirms the effectiveness of fortification of boiled sausages with a retinyl palmitate premix based on MCC.

Conclusion

It has been found that casein micelles are indeed able to protect unstable hydrophobic molecules (retinol in this case). Technology has been developed for the introduction of vitamin A into MCC, which involves the use of surfactants and intensive stirring at room temperature for two hours. With a decrease in time, the degree of protection reduces, and with an increase, it remains practically unchanged. When the vitamin is heated in the presence of casein for 3 hours at 80 °C (model conditions), the residual content of retinol reaches almost 80%, while without milk protein the maximum yield is 36%.

The study and verification of deep processes that occur during the so-called "encapsulation" is fascinating but at the same time difficult task for further research. At the same time, this goal is more fundamental than practical. In our opinion from a practical point of view, the results obtained, are enough to talk about the effectiveness and expediency of using MCC in the fortification of food products, in particular sausages, with such important substances as fat-soluble vitamins.

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STUDY OF THE EFFECT OF ONION HUSK ETHANOL EXTRACT ON THE CHEMICAL COMPOSITION AND MICROSTRUCTURE OF MEAT PATES

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Keywords: *plant-based antioxidants, total antioxidant capacity, DPPH, vitamins, fatty acid composition, amino acid composition, SDS-PAGE, proteins, histological analysis*

Abstract
The wide use of antioxidants is due to their involvement in free radical processes in foods and human body. Interest in the use of low-value raw materials providing products with functional properties and increasing their shelf life is rapidly increasing. However, any changes in the formulation and technology may affect the properties and composition of the finished product. During the work, the effect of replacing 34% (sample 1) or 17% (sample 2) beef broth with 70% water-ethanol extract of yellow onion peels in the formulation of the experimental meat pates was investigated. The control product contained only beef broth as liquid. The total antioxidant capacity by the DPPH radical method (TAC_{DPPH}), fatty acid composition and amino acid composition were determined; microelement content analysis, proteomic and microstructural studies of meat pate samples with and without the addition of extract were also carried out. For 14 days, TAC_{DPPH} values of experimental pates were higher than in control by at least 2.32 times ($P < 0.10$). Samples 1 and 2 were characterized by a decrease in the concentrations of zinc, manganese and magnesium by no more than 14% ($P < 0.10$), with a simultaneous increase in selenium, copper, potassium and calcium of 8% to 17.35% ($P < 0.10$) depending on the microelement. The mass fraction of protein in experimental pates 1 and 2 was higher by 6.76% and 2.73% ($P < 0.10$), respectively, which was due to a decrease in moisture because of ethanol evaporation. Replacing the broth in the formulation affected the decrease in the protein biological value, as evidenced by a decrease in amino acid scores (AASs). However, a decrease in the AAS difference coefficient in experimental pates 1 and 2 by 7.71% and 3.07%, respectively, led to an increase in the biological value of the pates by 7.7% and 3.06%, respectively. Based on the results of proteomic and histological analysis, it was revealed that the addition of ethanol extract did not lead to significant changes in the protein composition and microstructural characteristics of the test samples.

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Introduction

Meat and meat products play an important role in the diet, as they are a good source of energy and nutrients such as essential amino acids, proteins of high biological value, minerals (iron, zinc, selenium, manganese) and B vitamins, especially vitamin B12 [1]. On the other hand, some nutritionists associate increased consumption of meat products with an increased risk of developing a number of diseases, for example, coronary heart disease and certain types of cancer [2]. In addition, lipids and proteins contained in meat products are prone to oxidation with the formation of toxic substances, which leads to a deterioration in organoleptic characteristics and a decrease in nutritional properties [3]. To reduce these processes, synthetic antioxidants are widely used, which may negatively affect the human body [4,5].

Consumer awareness of the value of healthy foods [6] has contributed to changes in dietary habits and traditions, which has increased interest and demand for fortified foods and the replacement of synthetic preservatives with natural ingredients [7,8]. This trend stimulates the search for and study of new sources of plant-based antioxidants to develop food products with improved nutritional value and beneficial properties. Numerous studies show the effectiveness of using plant-based antioxidants instead of artificial antioxidants in extending the shelf life of meat products [9,10,11,12,13]. Thus, Kim et al. [9] demonstrated that the addition of 70% ethanol extracts of *Pimpinella brachycarpa* and *Aralia elata* to raw beef burgers helped to reduce the concentration of lipid peroxidation products and microorganisms, as well as to improve the color stability of meat when stored at 4 °C for 12 days. In another

work, Das et al. [11] showed that the use of mature *Moringa oleiferia* leaf extract in cooked goat meat burgers at the level of 100 mg/100 g reduced oxidative spoilage over a longer period of time compared to the common synthetic antioxidant, butylated hydroxytoluene. In addition, the positive physiological effects of compounds with antioxidant properties have been proven [14,15]. The widely known properties of plant-based antioxidants are due to their ability to neutralize free radicals (FR), as well as interrupt oxidative chain reactions and stimulate the activity of the body's antioxidant system.

At the moment, there are numerous technologies for creating products of animal origin in combination with plant raw materials. The authors of the publication [16] characterize such products as foods with an increased biological value and a balanced amino acid composition. In articles [17,18], the authors consider methods for producing meat products with a high content of polyunsaturated fatty acids, probiotics, biologically active plant compounds (antioxidants, dietary fiber) while maintaining their sensory characteristics.

In the meat products market, pates are in great demand, which is due to the unique original taste because of the plant components they contain (pistachios, prunes, etc.) [19].

Furthermore, the technology for the production of meat pates is one of the most convenient for adding additional components, such as plant extracts, emulsions, powders, dietary fiber, etc., since a high degree of homogenization contributes to their uniform distribution in the product. However, due to high homogenization, humidity, the use of by-products, and the presence of a significant amount of fatty raw materials, pates in coating are perishable food products, so the use of antioxidants in their production is an important component. Scientific research has also demonstrated the effectiveness of using extracts of spices, medicinal and food plants as natural antioxidants, and some scientific works have shown their antimicrobial potential [20,21]. A particularly interesting area is the use of low-value plant raw materials to provide products with functional properties and increase their shelf life, which makes it possible to convert by-products into goods with high commercial value. There is an increase in research of antioxidants obtained from processing wastes from the fruit and vegetable industry, which reduces the cost of functional food products and also complies with the principles of a circular economy [22,23]. Previously, we demonstrated that the addition of yellow onion peel ethanol extract to the formulation of meat pate contributed to an increase in the total antioxidant capacity in relation to transition metal ions and the content of phenolic compounds simultaneously with a decrease in oxidative spoilage [24]. However, any changes in the formulation and technology of meat products may affect the properties and composition of the finished product. Therefore, the purpose of this work was to study the effect of 70% ethanol extract of yellow onion peels, rich in plant-based antioxidants, namely quercetin and its glycosides, on chemical composition and microstructure of meat pates.

Objects and methods

The objects of the study were yellow onion peels (*Allium cepa*) purchased at the VkusVill store, manufactured by Agroleto LLC, Krasnodar, Russia, and developed meat pates with and without the addition of 70% water-ethanol extract of yellow onion peels.

To prepare onion husk extract (OHE), crushed onion husk (particle size 5 mm or less) were mixed with 70% ethanol in a ratio of 1:15 (g: ml), infused for 24 hours at 22 ± 2 °C, and then filtered through a folded paper filter. The extract was stored in a sealed dark vial at 4 °C. The total antioxidant capacity according to the DPPH method (TAC_{DPPH}) was 2.919 ± 0.058 mmol equiv. quercetin / l.

Three samples of the experimental meat pates were produced in the Department of Applied Scientific and Technological Development of the V. M. Gorbatov Federal Research Center for Food Systems in accordance with the formulation presented in [24] and in Table 1.

Table 1. Formulations of the experimental meat pates

Ingredients	Pate samples		
	Control	Sample 1 (max)	Sample 2 (1/2 max)
Main raw materials, kg per 100 kg of blanched products			
Beef flank, blanched	35	35	35
Beef liver, trimmed, blanched	23	23	23
Lean pork, blanched	20	20	20
Pork heart, blanched	10	10	10
Wheat flour	5	5	5
Whole cow's milk powder	2	2	2
Fried onions	5	5	5
Spices and other materials, g per 100 kg of unsalted raw materials			
Table salt	1400	1400	1400
Granulated sugar	300	300	300
Ground black pepper	100	100	100
Ground allspice	50	50	50
Ground mustard	500	500	500
Ground nutmeg	50	50	50
Liquid, liters per 100 kg of main raw materials			
Beef broth	20	13.2	16.6
OPE	0	6.8	3.4

Control product and two experimental products with the addition of the maximum tolerable concentration (100 mg rutin/day) of antioxidants (Sample 1) and 50% of the maximum concentration (Sample 2) in equivalent quercetin based on the adequate intake (AI) of flavanols and their glycosides in accordance with the Appendix 5 "Values of daily consumption of food and biologically active substances for adults as part of specialized food products and dietary supplements" of the Uniform sanitary-epidemiological and hygienic requirements for products (goods) subject to sanitary-epidemiological supervision

(control) (as amended as of November 14, 2023)[1]. OHE was added instead of broth at the levels of 6.8 l/100 kg of the main raw materials for test sample 1 and 3.4 l/100 kg of the main raw materials for test sample 2, which corresponded to TAC_{DPPH} values of 19.85 and 9.94 mmol equiv. quercetin/100 kg of the main raw materials, respectively (or 6.0 and 3.0 g of quercetin/100 kg of the main raw materials).

The technology for preparing experimental meat pates corresponded to the technology presented in [24]. Briefly, meat raw materials were prepared, cut into pieces of 200 to 300 g and blanched separately in water at 95 ± 5 °C for 15 to 20 minutes (liver), 120 minutes (pork heart) or 40 minutes (beef flank). At the same time, the onions were peeled, chopped in Bosch MCM3501M food processor (Bosch, Škofja Loka, Slovenia) and blanched with sunflower oil in a frying pan until golden brown. Heat-treated meat raw materials and onions were separately ground in Hurakan HKN-12SC meat grinder (Hurakan, Guangzhou, China) to a particle size of 2 to 3 mm. The pate was mince using a cutter blender (Robot-Coupe, Montceau-Les Mines, France). Fried onions, dry ingredients, broth and OHE were added to the finished emulsion and processed until a homogeneous minced mass was obtained. Chopping was carried out at 3000 rpm for 5 minutes. This product was used as a control sample. The technological difference that changed the composition of the final product was the replacement of 34% (Sample 1) and 17% (Sample 2) of beef broth with OHE. The final product temperature at the end of homogenization was higher than 40 °C. The product was packaged in 100 ± 1 g vacuum packaging (VakumPak-M, Webomatic, Bochum, Germany), PA/PE, 150 × 200 mm, 70 μm thickness; cooked in a water bath (EKROS4310, St. Petersburg, Russia) for 20 to 30 minutes at a temperature of 72 °C in the geometric center of the bar; then cooled to 4 °C and stored at 4 °C. Temperature was monitored using WT-1 digital thermometer (Xuzhou Sanhe Automatic Control Equipment Co., Ltd., Xuzhou, China).

To study the effect of OHE on the chemical composition and microstructure of meat pate, water-soluble and fat-soluble vitamins, minerals, microelements, amino acids, fatty acids (FA) were determined in freshly prepared samples; atherogenic index was calculated; proteomic analysis was carried out using electrophoresis; and histological analysis was carried out. TAC was determined by the DPPH method on the day of preparation (day 0) and on days 3, 5, 7 and 14 of storage at 4 °C.

To determine the TAC of pates, ethanol extracts were prepared, for which an average sample of the experimental product was mixed with 96% ethanol in a ratio of 1:5 (g: ml), homogenized using hand-held S10 homogenizer (Stegler, China) for 2 minutes at 9000 rpm. Ethanol extracts were infused for 60 minutes at 22 ± 2 °C, followed by filtering through a folded paper filter, and then stored at minus 40 °C. TAC values of onion peel and meat pate extracts were determined by the DPPH radical method on SF-2000 spectrophotometer (OKB Spektr, Russia) according to the published method [25]. A stock 1 mM ethanol solution of the DPPH radical (Santa Cruz Biotechnology, USA) was prepared in a dark glass container, which was infused in the dark at a temperature of 22 ± 2 °C for 12 hours. Before measurements, DPPH working solution with a concentration of 100 μM was prepared, the absorbance of which was at least 1.00 ± 0.05 optical units. To determine TAC values of extracts, 1.52 ml of DPPH working solution and 80 μl of a sample or 96% ethyl alcohol as a control sample or quercetin at concentrations of 100 to 275 μM were added to glass tubes to plot a calibration curve. The reaction mixture was shaken vigorously and incubated in the dark at 22 ± 2 °C for 30 min. Measurement of the absorbance of solutions was carried out in cuvettes with a distance between the working faces of 1 cm at a wavelength of 517 nm. Measurements for each sample were carried out in quadruplicate. TAC values were calculated from the calibration plot ($R^2 > 0.99$) using equation (1) and expressed in mmol equiv. quercetin/l of OHE or in μmol equiv. quercetin/100 g of product.

$$TAC = \frac{\left(\frac{D_k - D_o}{D_k}\right) \times 100\% - 4.6904}{0.3081} \quad (1)$$

where D_k is the absorbance of the control sample; D_o is the absorbance of the sample.

To study the physicochemical composition of meat pates, the mass fractions of protein were determined according to GOST 25011–2017[2], the mass fractions of carbohydrates according to MU 1–40–3805[3] and the mass fractions of fat according to GOST 23042–2015[4].

Concentrations of water-soluble and fat-soluble vitamins in experimental meat pates were measured according to GOST R 55482[5] and GOST 32307[6], respectively.

To study the variability of the microelement composition of the control and experimental meat pates, the content of iron, zinc, copper, lead, cadmium was determined according to GOST 30178–96[7], the content of

[1] "Uniform sanitary-epidemiological and hygienic requirements for products (goods) subject to sanitary-epidemiological supervision (control) (as amended as of February 22, 2022)" Retrieved from https://docs.cntd.ru/document/902249109 Accessed January 20, 2024

[2] GOST 25011–2017. "Meat and meat products. Protein determination methods: Retrieved from https://docs.cntd.ru/document/1200146783 Accessed February 7, 2024 (In Russian)

[3] MU1–40–3805. "Methodological instructions on laboratory quality control of public catering products" Retrieved from https://docs.cntd.ru/document/1200049293 Accessed February 7, 2024 (In Russian)

[4] GOST 23042–2015. "Meat and meat products. Fat determination methods" Retrieved from https://docs.cntd.ru/document/1200133107 Accessed February 7, 2024 (In Russian)

[5] GOST R55482. "Meat and meat products. Method for determination of water-soluble vitamins" Retrieved from https://docs.cntd.ru/document/1200104685 Accessed February 7, 2024 (In Russian)

[6] GOST 32307. "Meat and meat products. Determination of fat-soluble vitamins by high performance liquid chromatography" Retrieved from https://docs.cntd.ru/document/1200107182 Accessed February 7, 2024 (In Russian)

[7] GOST 30178–96. "Raw material and food-stuffs. Atomic absorption method for determination of toxic elements" Retrieved from https://docs.cntd.ru/document/1200021152 Accessed February 7, 2024 (In Russian)

magnesium, sodium, potassium, manganese according to GOST R 55484–13[8], the content of calcium according to GOST R 55573–13[9], the content of selenium according to GOST 31707–12[10], the content of arsenic according to GOST R 51766–01[11], the content of mercury according to GOST R 53183–08[12].

The amino acid composition of pates was determined according to GOST 34132–2017[13], the content of oxyproline according to GOST 23041–2015[14], the content of tryptophane according to GOST R 70149–2022[15].

The biological value of pates was assessed by calculating amino acid scores (AASs), AAS difference coefficient (AASDC,%), rationality coefficient of amino acid composition (R_C, fractions of units), coefficient of utility and indicator of comparable excess content of essential amino acids (EAAs) *(σ,* mg/g of reference protein) according to [26].

The biological value of pate protein was determined by calculating amino acid scores for essential amino acids according to the formula (2).

$$C_i = \frac{A_i}{A_{i,\,ref}} \cdot 100 \qquad (2)$$

where C_i is the amino acid score of the i-th EAA, %; A_i is the content of i-th EAA in 1 g of the protein under study, mg/g; $A_{i,\,ref}$ is the content of the i-th EAA in 1 g of "reference" protein according to FAO/WHO, mg/g.

The excess amount of EAAs not used for plastic needs was determined by calculating the AAS difference coefficient (AASDC, %) according to the formula 3.

$$AASDC = \frac{\sum_{i=1}^{n} |(C_i - 100)|}{n} \qquad (3)$$

where C_i is the AAS of the i-th EAA, %; n is the number of EAAs.

The balance of EAAs in relation to the physiologically necessary norm (reference) was characterized by the rationality coefficient of amino acid composition (R_C, fractions of units), which was calculated according to the formula 4.

$$R_C = \frac{\sum_{i=1}^{k} (A_i \cdot k_i)}{\sum_{i=1}^{n} A_i} \qquad (4)$$

where A_i is the content of the i-th EAA in 1 g of the protein under study, mg/g; k_i is the coefficient of utility of the *i*-th EAA to the limiting amino acid, fractions of units.

The coefficient of utility (utilization) reflects the balance of EAAs in relation to the reference and was calculated according to the formula 5.

$$k_i = \frac{c_{min}}{c_i} \qquad (5)$$

where C_{min} is the minimum EAA score of the protein under study in relation to the reference protein, fractions of units; C_i is the score for the i-th EAA in relation to the reference protein, fractions of units.

Calculation of the indicator for comparable excess content of EAAs (σ, mg/g of reference protein) showing the total mass of EAAs not used for anabolic needs, was calculated according to the formula 6.

$$\sigma = \frac{\sum_{i=1}^{n} (A_i - C_{min} \cdot A_{i,э})}{C_{min}} \qquad (6)$$

Fatty acid composition (36 acids) of the test samples was determined according to GOST R 55483–2013[16]. The calculation of the atherogenic index (AIP) of pates was carried out according to the formula (7) given by Ulbricht T. L.V. and Southgate D. A.T., 1991 [27].

$$AIP = \frac{(C_{12} + C_{14} + C_{16} + Trans\ FA)}{(PUFA + C_{18:1} + other\ MUFA)} \qquad (7)$$

The determination of the tolerable level of toxic elements in meat pates was carried out according to TR TS 021/2011 "On food safety"[17], SanPiN2.3.2.560–96 "Hygienic requirements for the quality and safety of food raw materials and food products"[18].

One-dimensional electrophoresis was carried out in a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE) in VE-10 chamber (Helicon, USA) at a constant current and voltage of 60 V and 120 V, for 2 hours, until the front stain (bromophenol blue) reached the bottom edge of the gel sheets. A marker consisting of molecular weight standards (Thermo, Latvia) was used as a standard solution. Staining was carried out with Coomassie brilliant blue G-250 (PanReac, Spain). To remove the

[8] GOST R55484–13. "Meat and meat products. Determination of sodium, potassium, magnesium and manganese by flame atomic absorption" Retrieved from https://docs.cntd.ru/document/1200103312 Accessed February 7, 2024 (In Russian)

[9] GOST R55573–13. "Meat and meat products. Determination of calcium by atomic absorption and titrimetric methods" Retrieved from https://docs.cntd.ru/document/1200105941 Accessed February 7, 2024 (In Russian)

[10] GOST 31707–12. "Foodstuffs. Determination of trace elements. Determination of total arsenic and selenium by hydride generation atomic absorption spectrometry (HGAAS) after pressure digestion" Retrieved from https://docs.cntd.ru/document/1200098581Accessed February 7, 2024 (In Russian)

[11] GOST R51766–01. "Raw material and food-stuffs. Atomic absorption method for determination of arsenic" Retrieved from https://docs.cntd.ru/document/1200025461 Accessed February 7, 2024 (In Russian)

[12] GOST R53183–08. "Foodstuffs. Determination of trace elements. Determination of mercury by cold-vapour atomic absorption spectrometry (CVAAS) method after pressure digestion" Retrieved from https://docs.cntd.ru/document/1200076584 Accessed February 7, 2024 (In Russian)

[13] GOST 34132–2017. "Meat and meat products. Determination of amino acids composition of animal protein" Retrieved from https://docs.cntd.ru/document/1200146930 Accessed February 7, 2024 (In Russian)

[14] GOST 23041–2015. "Meat and meat products. Method for determination of oxyproline" Retrieved from https://docs.cntd.ru/document/1200123926 Accessed February 7, 2024 (In Russian)

[15] GOST R70149–2022. "Meat and meat products. Determination of the mass fraction of tryptophan by spectrophotometric method" Retrieved from https://docs.cntd.ru/document/1200184801 Accessed February 7, 2024 (In Russian)

[16] GOST R55483–2013. "Meat and meat products. Determination of fatty acids composition by gas chromatography" Retrieved from https://docs.cntd.ru/document/1200103852 Accessed February 7, 2024 (In Russian)

[17] TR TS 021/2011. "Technical Regulations of the Customs Union On food safety (as amended as of July 14, 2021)" Retrieved from https://docs.cntd.ru/document/902320560#8Q20M0. Accessed April 4, 2022

[18] SanPiN2.3.2.560–96. "Hygienic requirements for the quality and safety of food raw materials and food products". Retrieved from https://docs.cntd.ru/document/9052436. Accessed April 03, 2022

unbound stain, 10% acetic acid (Reagent Component, Russia) was used. To increase resolution, staining with silver nitrate (PanReac, Germany) was additionally performed according to Blum's method [28]. Computer densitometry of one-dimensional electropherograms in a wet state was carried out using Bio-5000 Plus scanner (Serva, Germany) in 600 ppi 2D-RGB mode in triplicate. Visualized protein fractions were interpreted using UniProt database [29].

To study the microstructure, three pieces of 1.5×1.5×0.5 cm were selected from each sample, which were placed in the chamber of MIKROM-HM525 cryostat (Thermo Scientific, USA). Slices 14 μm thick were made (three sections from each piece), mounted on Menzel-Glaser glasses (Thermo Scientific, USA) and stained with Ehrlich hematoxylin and 1% aqueous-alcoholic eosin solution (BioVitrum, Russia) according to the generally accepted method [30]. The study of histological preparations and their photography were carried out on AxioImaiger A1 light microscope (Carl Zeiss, Germany) using AxioVision 4.7.1.0 image analysis software (Carl Zeiss, Germany).

Statistical analysis was carried out using Microsoft Excel and Statistica 10.0 software packages. The results were presented as means and standard deviations (M ± SD). Statistical significance was calculated using nonparametric Mann-Whitney U-tests (for two independent groups). A probability of $P < 0.1$ was selected as the level of significance.

Results and discussion

The results of determining TAC_{DPPH} in ethanol extracts of meat pates stored at 4 °C and the difference between the control and test samples are presented in Table 2.

Table 2. TAC_{DPPH} values of meat pates

Days	TAC_{DPPH}, μmol equiv. quercetin/100 g of product				
	Control	Sample 1 (max)	Sample 2 (1/2 max)	Δ (S1-C)	Δ (S2-C)
0	15.45 ± 1.05	49.59 ± 2.66*	35.84 ± 1.91*,#	34.14	20.39
3	4.36 ± 0.63	44.33 ± 3.20*	25.45 ± 5.75*,#	39.96	21.08
5	7.22 ± 0.67	41.18 ± 3.22*	21.13 ± 1.89*,#	33.97	13.92
7	6.51 ± 0.59	38.09 ± 2.09*	25.54 ± 0.84*,#	31.58	19.03
14	8.24 ± 0.51	41.79 ± 1.31*	24.34 ± 0.25*,#	33.55	16.10

*, significant difference between the control and test samples, Mann-Whitney U-test, P-value < 0.10; #, significant difference between test samples, Mann-Whitney U-test, P-value < 0.10

During 14 days of storage, a decrease in total antioxidant capacity was observed in all samples. TAC values in the test samples during all days of storage were significantly higher than that in the control pate. Thus, TAC_{DPPH} values of the experimental product with the maximum concentration of OHE (Sample 1) exceeded those of the control sample by 34.64 ± 3.15 μmol equiv. quercetin/100 g of product, and the corresponding values of the experimental pate with 50% OHE concentration (Sample 2) exceeded those of the control sample by 18.10 ± 3.02 μmol equiv. quercetin/100 g of product. It was determined that reducing the concentration of OHE introduced into the meat product by 50% significantly reduced the contribution of the plant extract to TAC_{DPPH}, as evidenced by the excess of the difference between Sample 1 and the control pate (Δ(S1-C)) and the differences between Sample 2 and the control at day 0 of approximately 1.7 times. Based on the results of the experiment, it was determined that within 14 days of storage, the addition of OHE to the formulation of meat pate provides a significantly increased TAC in relation to nitrogen-containing free radicals in comparison with the control sample, with previously proven reduction in oxidative spoilage presented in [24]. Other researchers have shown the effectiveness of using peanut skin extract to inhibit oxidative spoilage in meat burgers, i. e. the reduction of lipid and protein oxidation products [31]. Based on the results of work [32], it was determined that the introduction of an antioxidant complex into the formulation of meat burgers contributed to an increase in the antioxidant capacity of the test sample by 9 times in comparison with the control, while the increased antioxidant capacity values were maintained when the products were stored for 28 days.

The results of determining the concentrations of water-soluble and fat-soluble vitamins in meat pates and the calculated percentages of AI of these vitamins according to the values of daily consumption of nutrients and biologically active substances for adults in the composition of specialized food products (SFP) and biologically active supplements[19] are presented in Table 3.

In all pate samples, the content of vitamins D3, B7 and C was below the detection limit. The concentrations of vitamins A, E, B1 and B12 significantly decreased in the order of Sample 2 > Control > Sample 1, and vitamins B2, B3, B5, and B9 significantly decreased in the order of Sample 1 > Sample 2 > Control. According to the results of determining vitamins in all experimental meat pates, a high content of B vitamins was noted, while not exceeding the tolerable upper intake level of all vitamins except B12. It is known that products of animal origin, in particular liver, which was the main component of the experimental meat pate, are characterized by a high content of B vitamins, especially B12, which is an important nutrient for the cognitive and neurological functions of the body [33]. In addition, beef liver is rich in components such as protein, heme iron, and zinc [33]. Liver contains more than 10 μg of vitamin B12 per 100 g, but its absorption is markedly reduced when its concentration in the product exceeds 2 μg, with only about 20% of the vitamin being effectively absorbed [34]. Thus, a significant excess of vitamin B12 over AI in all the resulting pates will contribute to the absorption of about 2.6 μg/100 of product, which is about 85% of the daily requirement. During the work, it was shown that the addition of an ethanol extract of plant-based antioxidants

[19] "Uniform sanitary-epidemiological and hygienic requirements for products (goods) subject to sanitary-epidemiological supervision (control) (as amended as of February 22, 2022)" Retrieved from https://docs.cntd.ru/document/902249109 Accessed January 20, 2024

Table 3. Vitamin content and their% of AI in meat pates

Vitamin	Intake, per day		Control		Sample 1 (max)		Sample 2 (1/2 max)	
	AI	UL	C, in 100 g	% of AI	C, in 100 g	% of AI	C, in 100 g	% of AI
A, μg	900	3000	156.90 ± 2.23	17.43	147.95 ± 2.79*	16.44	188.35 ± 2.97*,#	20.93
D3, μg	10	15	< 1.0	0	< 1.0	0	< 1.0	0
E, mg	15	150	1.34 ± 0.06	8.93	1.21 ± 0.03*	8.07	1.67 ± 0.03*,#	11.13
B1, mg	1.5	5.0	1.59 ± 0.08	106.0	2.25 ± 0.06*	150.0	2.52 ± 0.05*,#	168.0
B2, mg	1.8	6.0	2.10 ± 0.05	116.67	2.54 ± 0.06*	141.11	2.37 ± 0.06*,#	131.67
B3, mg	20	60	17.25 ± 0.39	86.25	19.36 ± 0.19*	96.80	18.27 ± 0.24*,#	91.35
B5, mg	5	15	5.46 ± 0.07	109.20	6.27 ± 0.11*	125.40	5.64 ± 0.46 #	112.80
B6, mg	2.0	6.0	0.36 ± 0.04	18.0	0.41 ± 0.01	20.50	0.48 ± 0.04*,#	24.0
B7, μg	50	150	< 1.0	0	< 1.0	0	< 1.0	0
B9, μg	400	600	77.08 ± 0.21	19.27	79.09 ± 2.27*	19.77	78.71 ± 0.39*	19.68
B12, μg	3.0	9.0	13.23 ± 0.99	441.0	10.85 ± 0.25*	361.67	14.86 ± 0.19*,#	495.33
C, mg	90	900	< 1.0	0	< 1.0	0	< 1.0	0

*, significant difference between the control and test samples, Mann-Whitney U-test, P-value < 0.10; #, significant difference between test samples, Mann-Whitney U-test, P-value < 0.10; C, concentration of vitamins, mg or μg depending on the vitamin; UL, tolerable upper intake level.

Table 4. Microelement composition of meat pates

Microelement	Intake, per day		Control		Sample 1 (max)		Sample 2 (1/2 max)	
	AI	UL	C, in 100 g	% of AI	C, in 100 g	% of AI	C, in 100 g	% of AI
Iron, mg	18 / 10^	40 / 20^	3.50 ± 0.01	19.44 / 35.0	3.33 ± 0.03 *	18.5 / 33.3	3.56 ± 0.04 *,#	19.78 / 35.6
Magnesium, mg	400	800	23.58 ± 0.20	5.90	23.37 ± 0.25	5.84	20.49 ± 0.11 *,#	5.12
Sodium, mg	1300**	—	598.96 ± 12.05	—	661.45 ± 6.11 *	—	687.27 ± 6.49 *,#	—
Potassium, mg	2500	3500	419.54 ± 2.57	16.78	462.60 ± 2.74 *	18.50	479.18 ± 5.47 *,#	19.17
Zinc, mg	12	25	3.73 ± 0.03	31.08	3.45 ± 0.04 *	28.75	3.07 ± 0.04 *,#	25.58
Copper, mg	1	3	3.44 ± 0.02	344	3.91 ± 0.03 *	391	3.76 ± 0.01 *,#	376
Calcium, mg	1000	2500	15.61 ± 0.02	1.56	17.19 ± 0.07 *	1.72	18.31 ± 0.01 *,#	1.83
Selenium, μg	55 / 75^	150	35.0 ± 1.0	50.0	38.0 ± 1.0 *	54.29	38.0 ± 1.0 *	54.29
Manganese, mg	2	5	0.08 ± 0.0	4.0	0.08 ± 0.0	4.0	0.09 ± 0.01 *,#	4.0
Lead, mg	—	—	< 0.01	—	< 0.01	—	< 0.01	—
Cadmium, mg	—	—	< 0.01	—	< 0.01	—	< 0.01	—
Arsenic, mg	—	—	< 0.01	—	< 0.01	—	< 0.01	—
Mercury, mg	—	—	< 0.002	—	< 0.002	—	< 0.002	—

*, significant difference between the control and test samples, Mann-Whitney U-test, P-value < 0.10; #, significant difference between test samples, Mann-Whitney U-test, P-value < 0.10; ^, norm for women/men; C, concentration of microelements, mg or μg; UL, tolerable upper intake level; **, only in SFP for athletes' nutrition.

obtained from onion peels to the formulation of meat pate did not have a negative effect on the composition of vitamins, and in the case of such compounds as B1, B2, B3, B5, B6, B9, the increase in concentrations was proportional to the addition of OHE.

The results of determining the concentrations of microelements and toxic elements in pates, as well as the calculated percentage of AI according to the values of daily intake of nutrients and biologically active substances for adults in the composition of specialized food products (SFP) and biologically active supplements[20] are presented in Table 4.

According to TR TS 021/2011 "On food safety"[21], the concentrations of toxic elements such as lead, cadmium, arsenic and mercury in all samples were significantly below the tolerable level, which indicates the safety of meat pates. It was determined that the concentrations of microelements were significantly different between samples. The concentrations of iron and manganese decreased in the order of Sample 2 > Control > Sample 1, and the content of sodium, potassium and calcium decreased in the order of Sample 2 > Sample 1 > Control. It was noted that the highest amount of zinc and magnesium was contained in the control sample and exceeded the similar values of Sample 1 and Sample 2 by 0.28 and 0.66 mg of zinc/100 g of product (P < 0.10), respectively, and by 0.21 and 3.09 mg magnesium/100 g of product (P < 0.10), respectively. Copper concentration was highest in the pate with the maximum amount of OHE (Sample 1) and amounted to 3.91 ± 0.03 mg/100 g, which significantly exceeded the amount of the microelement in the Control and Sample 2 by 0.47 and 0.15 mg/100 g (P < 0.10), respectively. An increase in selenium content in the test samples was noted, which amounted to 38.0 ± 1.0 μg/100 g of product. Analysis of the microelement composition demonstrated that the addition of OHE to meat pate led to a decrease in the con-

[20] "Uniform sanitary-epidemiological and hygienic requirements for products (goods) subject to sanitary-epidemiological supervision (control) (as amended as of February 22, 2022)" Retrieved from https://docs.cntd.ru/document/902249109 Accessed January 20, 2024

[21] TR TS021/2011. "Technical Regulations of the Customs Union On food safety (as amended as of July 14, 2021)" Retrieved from https://docs.cntd.ru/document/902320560#8Q20M0. Accessed April 4, 2022

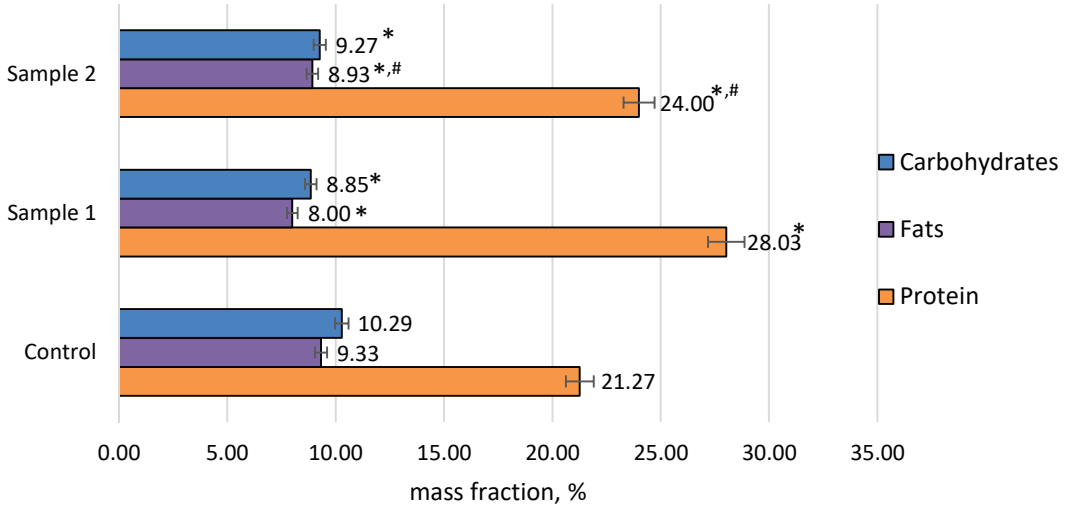
centrations of zinc, manganese and magnesium by no more than 14% ($P < 0.10$), simultaneously with an increase in the amount of selenium, copper, potassium and calcium from 8% to 17.35% ($P < 0.10$) depending on the microelement. This corresponds to the data of [35], in which the authors demonstrated that onion peels are a rich source of calcium (1.8 to 16.5 mg/g), potassium (11.1 to 15.9 mg/g), selenium (0.00003 to 0.00093 μg/g) and other microelements. It is known that mineral substances perform a plastic function in human body participating in the metabolism of almost any human tissue. But often not only their content, but also their ratio is important. Thus, the optimal ratio of calcium and magnesium is 4:1, and an increase in the concentration of magnesium may impair the digestibility of calcium [36]. However, we found that meat pates are characterized by a high magnesium content. In this connection, a decrease in the concentration of magnesium and an increase in the amount of calcium in the experimental pates led to a more optimal ratio of these elements. Thus, Ca: Mg ratio in the control sample was 0.72:1, and for samples 1 and 2 it was 0.74:1 and 0.89:1, respectively. Selenium is an essential mineral for humans, as its deficiency is associated with an increased risk of mortality, poor immunity, and decreased cognitive function. At the same time, it is known that selenium has antiviral and antioxidant effects and is necessary for the normal functioning of the reproductive system of men and women, and also reduces the risk of developing autoimmune thyroid diseases [37,38]. At the moment, the problem of selenium deficiency is observed all over the world [39], and therefore, increasing the amount of this microelement by 8.6% ($P < 0.10$) in experimental pates due to the addition of onion peels extract is especially important. Copper is considered a redox metal and is an essential nutrient for all species studied to date. It is known that deficiency of this microelement may contribute to the development and progression of a number of diseases, including cardiovascular diseases and diabetes [40]. In addition, the biological role of copper is to cross-link collagen via Cu-dependent lysyl oxidase and maintain the activity of superoxide dismutase (CuZn SOD) [41,42]. The high copper content in experimental pates is due to the use of beef liver in the formulation in the amount of 23% of all main raw materials, which is characterized by a high content of not only protein and iron, but also copper and vitamins [43].

Figure 1 shows the mass fractions of the main nutrients in the experimental meat pates with and without the addition of OHE to their formulations.

The diagram clearly shows that the addition of onion peel extract to meat pates contributed to a significant increase in the amount of protein and a decrease in fats and carbohydrates, with more addition of OHE leading to greater changes. Thus, the mass fraction of protein in the control product was inferior to samples 1 and 2 by 6.76% and 2.73% ($P < 0.10$), respectively. The concentration of carbohydrates in the experimental pates decreased by no less than 1.04% ($P < 0.10$), and concentration of fat decreased by no less than 0.4% ($P < 0.10$). The noted changes in the main indicators of the nutritional value of pates were associated with the replacement of beef broth with ethanol extract of onion peels by 34% in Sample 1 and 17% in Sample 2. It is known that ethyl alcohol is a volatile compound, the vaporization of which increases with increasing temperature. Since the pate preparation technology included a hot chopping step, during which the minced pate was homogenized for 5 minutes at a temperature above 40 °C, the ethanol contained in the extract evaporated. In addition, in [24], we demonstrated that the addition of OHE to the formulation of meat pates led to a decrease in the mass fraction of moisture by 4.17% (Sample 1) and by 1.47% (Sample 2).

Experimental data characterizing the fatty acid and amino acid compositions of the developed meat pates are presented in Table 5 and Table 6, respectively.

Analysis of the fatty acid composition of the experimental meat pates presented in Table 5 showed that the addition of OHE contributed to a slight change in the



*, significant difference between the control and test samples, Mann-Whitney U-test, P-value < 0.10; #, significant difference between test samples, Mann-Whitney U-test, P-value < 0.10

Figure 1. Main indicators of the nutritional value of pates

Table 5. Fatty acid composition of meat pates

No.	Fatty acid	Control	Sample 1	Sample 2
8	Myristic acid C14:0	2.67 ± 0.09	2.73 ± 0.04	2.70 ± 0.08
9	Myristoleic acid C14:1	0.37 ± 0.02	0.44 ± 0.02*	0.42 ± 0.04
10	Pentadecanoic acid C15:0	0.40 ± 0.01	0.38 ± 0.01	0.37 ± 0.01*
12	Palmitic acid C16:0	22.01 ± 0.21	22.56 ± 0.21*	22.12 ± 0.16 #
13	Palmitoleic acid C16:1	3.20 ± 0.15	3.50 ± 0.03*	3.24 ± 0.08 #
14	Margaric acid C17:0	2.52 ± 0.03	2.4 ± 0.03*	2.30 ± 0.01*,#
16	Stearic acid C18:0	20.84 ± 0.42	19.14 ± 0.42*	18.36 ± 0.61*
17	Oleic acid C18:1	33.37 ± 0.47	36.17 ± 0.70*	34.50 ± 0.85 #
18	Elaidic acid C18:1	2.33 ± 0.07	2.10 ± 0.17	1.98 ± 0.11*
19	Linoleic acid C18:2ω6	9.72 ± 0.14	8.54 ± 0.36*	11.69 ± 0.25*,#
20	Linolelaidic acid C18:2ω6	0	0	0.05 ± 0.09
21	Linolenic acid C18:3ω3	0.30 ± 0.04	0.27 ± 0.04	0.28 ± 0.06
22	Arachidic acid C20:0	0.07 ± 0.12	0	0.19 ± 0.02 #
23	Arachidonic acid C20:4ω6	1.28 ± 0.24	1.0 ± 0.01	0.96 ± 0.01*,#
25	Dihomo-γ-linolenic acid C20:3ω6	0.68 ± 0.14	0.59 ± 0.02	0.54 ± 0.03 #
28	Gondoic acid C20:1ω9	0.25 ± 0.01	0.18 ± 0.16	0.30 ± 0.01*,#

*, significant difference between the control and test samples, Mann-Whitney U-test, P-value < 0.10; #, significant difference between test samples, Mann-Whitney U-test, P-value < 0.10.

concentration of some fatty acids, which is also explained by the presence of myristic, palmitic, palmitoleic and other acids in onion waste [35]. Based on the results of the studies, atherogenic index (AIP) of all pates was calculated, which was 0.52 for the Control and Sample 1 and 0.50 for Sample 2. It was determined that the AIP of the experimental pates was 29.7% below the average AIP of the human diet for the Control and Sample 1 and 32.4% below the average AIP of the human diet for Sample 2, which is 0.74 according to A. B. Lisitsyn et al. [44].

Table 6. Amino acid composition of meat pates

No.	Amino acid	Content of bound amino acids (g/100 g of product)		
		Control	Sample 1	Sample 2
1	Aspartic acid	2.75 ± 0.28	2.86 ± 0.09	2.61 ± 0.32
2	Glutamic acid	4.75 ± 0.75	4.97 ± 0.40	4.47 ± 0.85
3	Serine	1.07 ± 0.23	1.14 ± 0.12	0.98 ± 0.26
4	Histidine	1.0 ± 0.07	0.96 ± 0.07	1.02 ± 0.02
5	Glycine	1.20 ± 0.15	1.24 ± 0.08	1.14 ± 0.17
6	Threonine	1.24 ± 0.17	1.3 ± 0.08	1.18 ± 0.18
7	Arginine	1.72 ± 0.49	1.77 ± 0.41	1.65 ± 0.53
8	Alanine	1.49 ± 0.25	1.55 ± 0.15	1.42 ± 0.28
9	Tyrosine	0.87 ± 0.15	0.92 ± 0.08	0.83 ± 0.16
10	Cystine	0.19 ± 0.04	0.20 ± 0.02	0.18 ± 0.04
11	Valine	1.1 ± 0.17	1.16 ± 0.07	1.02 ± 0.19
12	Methionine	0.79 ± 0.10	0.79 ± 0.08	0.78 ± 0.09
13	Phenylalanine	0.91 ± 0.14	0.94 ± 0.10	0.89 ± 0.15
14	Isoleucine	0.70 ± 0.09	0.71 ± 0.07	0.69 ± 0.10
15	Leucine	1.66 ± 0.22	1.71 ± 0.15	1.60 ± 0.23
16	Lysine	1.12 ± 0.21	1.13 ± 0.19	1.10 ± 0.22
17	Proline	2.45 ± 1.80	1.44 ± 0.06	1.46 ± 0.06
18	Oxyproline	0.14 ± 0.01	0.17 ± 0.01*	0.09 ± 0.01*,#
19	Tryptophane	0.32 ± 0.01	0.34 ± 0.01*	0.38 ± 0.01*,#
	Total	25.48 ± 5.08	25.33 ± 2.24	23.48 ± 3.85

*, significant difference between the control and test samples, Mann-Whitney U-test, P-value < 0.10; #, significant difference between test samples, Mann-Whitney U-test, P-value < 0.10

When studying the amino acid composition of experimental pates, a slight increase in tryptophane and a decrease in proline and hydroxyproline were noted. However, no significant differences were found between the control and test samples, as evidenced by the same amount of total amino acid content. The results of the calculated biological value of the protein in the experimental products relative to the standard FAO/WHO scale are presented in Table 7 and Table 8.

Table 7. Amino acid score for essential amino acids in pates

Amino acid	Amino acid concentration, g/100 g of protein				Amino acid score, %		
	Reference protein, FAO/WHO	Control	Sample 1	Sample 2	Control	Sample 1	Sample 2
Isoleucine	4	3.29	2.53	2.88	82.25	63.25	72
Leucine	7	7.8	6.1	6.67	111.43	87.14	95.29
Lysine	5.5	5.27	4.03	4.58	95.822	73.27	83.27
Methionine + cysteine	3.5	4.61	3.53	4	131.71	100.86	114.29
Phenylalanine + tyrosine	6	8.37	6.64	7.17	139.5	110.67	119.5
Threonine	4	5.83	4.64	4.92	145.75	116	123
Tryptophane	1	1.5	1.21	1.58	150	121	158
Valine	5	5.17	4.14	4.25	103.4	82.8	85

Table 8. Indicators of meat pate biological value

Indicators	Control	Sample 1	Sample 2
Minimum amino acid score, C_{min}	0.83	0.63	0.72
Amino acid score difference coefficient, AASDC,%	25.47	17.76	22.40
Biological value of pate,%	74.54	82.24	77.60
Rationality coefficient of amino acid composition, Rc, fractions of units	0.71	0.69	0.72
Indicator of comparable excess content of EAAs, σ, mg/g of reference protein	148.69	158.89	140.69

According to the results of the AAS calculation, it was revealed that the control product had 2 limiting amino acids, i. e. isoleucine and lysine, while the experimental products had 4 limiting amino acids, which also include leucine and valine. Moreover, for all samples, isoleucine was the first limiting amino acid. It was noted that the AASs of all essential amino acids in the experimental products decreased, which indicated a decrease in the biological value of the protein, presumably associated with replacing part of the beef broth with OHE. With the maximum addition of the extract, a greater decrease in AAS was observed. According to Uniprot database [29], beef flank contains water-soluble proteins such as myoglobin (17.0 kDa), myosin (16.9 kDa), triosephosphate isomerase 1 (23.0 kDa) and hemoglobin (15.4 kDa). These proteins are characterized by a high content of essential amino acids, in particular leucine, lysine, valine, threonine and isoleucine. Presumably, when blanching beef flank, these proteins pass into the broth, which, according to the formulation of the pates, was used as a liquid, and in the case of Samples 1 and 2 was replaced by OHE in the amount of 34% and 17%, respectively. This fact led to a decrease in AAS of EAAs in studied samples. Amino acid scores of essential amino acids were used to calculate indicators of the biological value of pates.

It was found that the addition of OHE to the meat pate reduced the minimum AAS along with an increase in the amount of limiting amino acids. Greater addition of the extract contributes to more significant changes. However, it was noted that the experimental pates were characterized by a smaller excess amount of essential amino acids not used for plastic needs, as evidenced by a decrease in the AAS difference coefficient by 7.71% in the case of Sample 1 and by 3.07% in the case of Sample 2. In addition, biological value of the experimental pates increased by 7.7% (Sample 1) and by 3.06% (Sample 2), which was also comparable to the change in the added volume of OHE. The slight variability in rationality coefficient of amino acid composition of all samples indicated an equal balance of essential amino acids in relation to the physiologically necessary norm (reference) in both the Control and Samples 1 and 2. Despite the fact that C_{min}, biological value and AASDC of Sample 2 were better than that of Sample 1, the control product and pate with a lower content of OHE (Sample 2) were characterized by lower σ by 10.11% and 18.2%, respectively, which indicated a smaller mass of essential amino acids not used for anabolic purposes.

As a result of studying samples of meat pate using one-dimensional electrophoresis with equal application (all samples were added in volume of 15 μl) and various staining options, electropherograms were obtained, presented in Figure 2.

The difference in protein visualization between the control and test samples was insignificant. There were no significant differences, such as the absence or, conversely, the appearance of *de novo* protein fractions between the samples. All the main muscle proteins were preserved. The formulation of the pate used pork heart, lean pork, beef liver and beef flank. The highest intensity of proteins was detected in the control sample, less pronounced intensity was in Sample 2 and the lowest concentration of proteins was in Sample 1. Such a visualization of proteins in the electropherogram is due to the fact that Sample 1 has the highest content of alcohol extract (6.8 ml per 100 g of product) instead of beef broth (in the control sample), so the concentration of proteins present in the broth decreases accordingly.

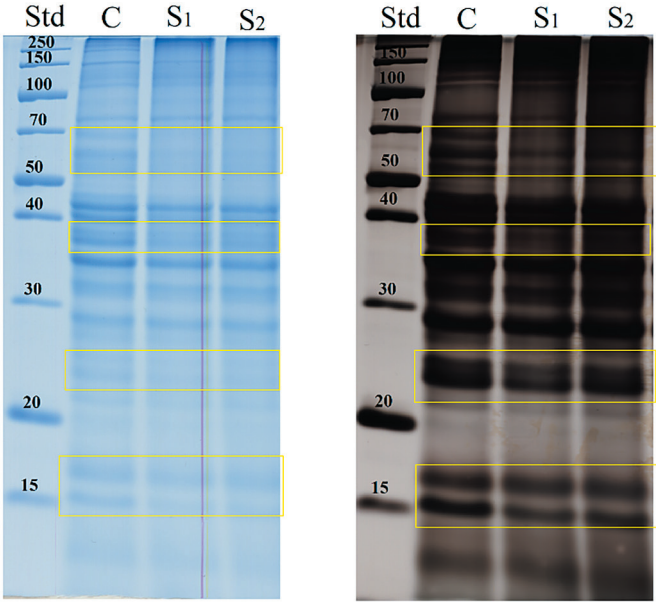


Figure 2. Electropherogram of 10% PAGE of meat pates on day 0: Std is molecular weight standard: 250, 150, 100, 70, 50, 40, 30, 20, 15 (from top to bottom); C is Control; S1 is Sample 1; S2 is Sample 2

When conducting a bioinformatic analysis of electropherograms, differences were identified in the intensity of staining of proteins present in skeletal muscles, with a molecular weight in the range of 15 to 17 kDa and 23 to 25 kDa, probably being fractions of hemoglobin (15.4 kDa) [45], myosin light chains (16.9 kDa) [46], myoglobin (17.0 kDa) [47], triosephosphate isomerase 1 (23.0 kDa) [48], troponin T (23.0 kDa) [49] and troponin I (25.0 kDa) [50]. Differences were also found in protein fractions in the range of 37 to 38 kDa, presumably being fractions of cathepsin B (36.9 kDa) [51] and beta-2-glycoprotein 1 (38.2 kDa) [52], which are present in pork heart and beef liver, respectively. The intensity of protein fractions with molecular weights of 55 kDa, 61 kDa, 68 kDa differed in staining intensity between the Control and Samples 1 and 2, and presumably may correspond to aldehyde dehydrogenase 1A1 (54.8 kDa) [53], leucine-rich protein 1 (61.1 kDa) [54] and 2-hydroxyacyl-CoA lyase 2 (68.1 kDa) [55], which are expressed in beef flank and beef liver.

Histological analysis revealed that all samples were characterized by the same structure and a high degree of constituent component grinding. The main part was represented by individual fragments of cross-striated muscle fibers and hepatocytes located singly or in small groups of

2 to 20 pieces, between which there was a small amount of fine-grained protein mass (Figure 3).

The samples also contained fragments of liver and loose fibrous connective tissue that retained their structural organization, individual fat drops evenly distributed throughout the minced meat, single fragments of heart muscle tissue and plant components related to wheat flour, natural spices (mustard, black pepper and allspice, nutmeg) and onions. The dispersion of the main part of the samples was 10 to 170 μm. All samples were characterized by a dense arrangement of structural elements; no differences in microstructural organization were identified. Thus, the histological analysis of the test samples showed that the introduction of OHE into the pate did not lead to a change in its main structural characteristics and did not affect the microstructure of the components included in the product. Studying the effect of OHE on the microstructure of test samples was of practical interest, due to the lack of similar studies in the scientific literature, where the focus is on the antioxidant and antimicrobial aspects, as well as the sensory characteristics of the finished

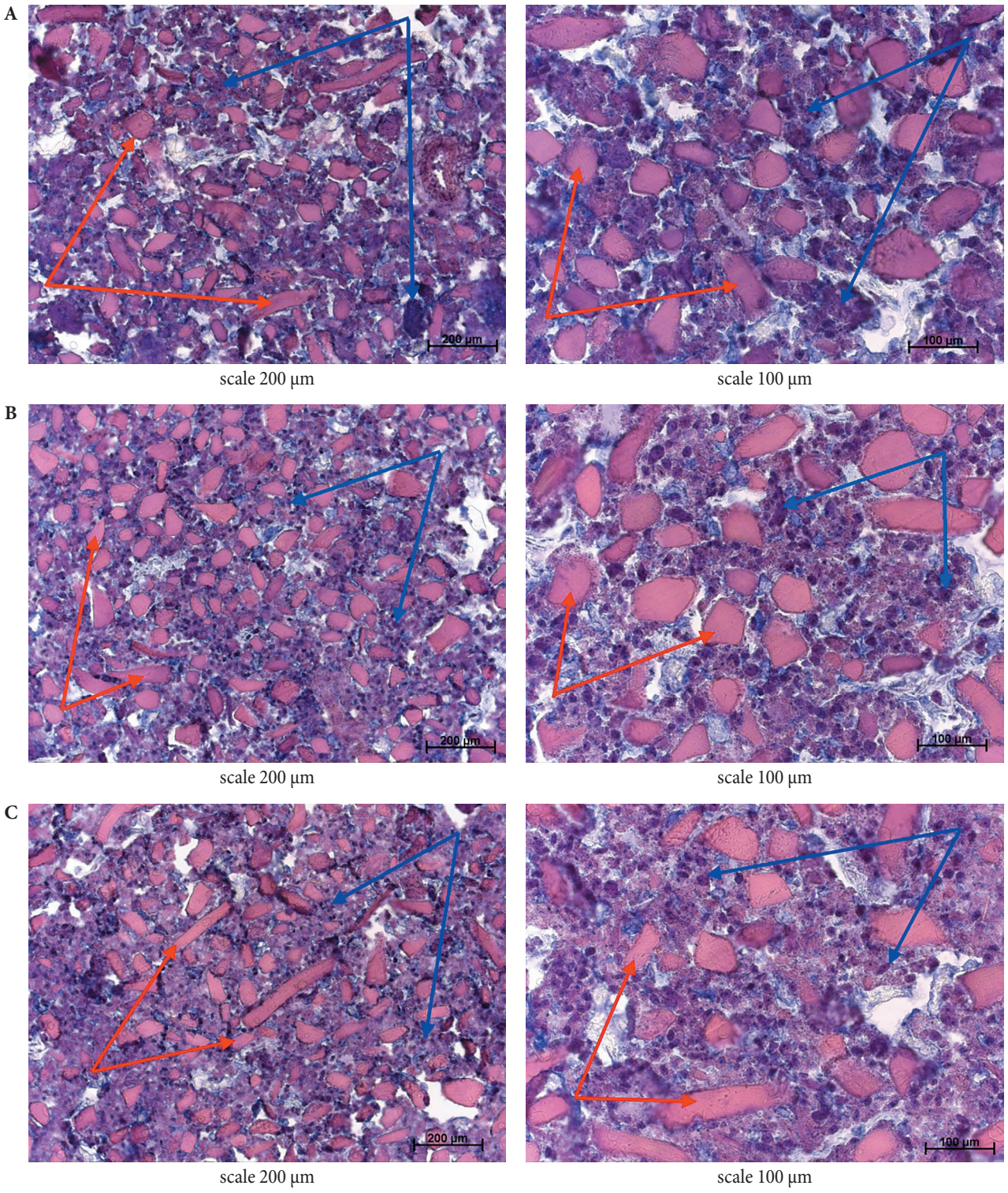


Figure 3. Microstructure of the studied samples (A is Control, B is Sample 2, C is Sample 2). Hematoxylin and eosin staining. Red arrows denote fragments of muscle fibers; blue arrows denote liver fragments and individual hepatocytes

products [56,57]. At the same time, the revealed absence of potential differences in the structure of animal tissue fragments, as the main source of protein, confirms the obtained results of proteomic analysis.

Conclusion

The introduction of 70% ethanol extract of yellow onion peels into the standard formulation of meat pate contributed to the enrichment of the product with plant-based antioxidants, which within 14 days, significantly increased the total antioxidant capacity against nitrogen-containing free radicals by no less than 2.32 times ($P < 0.10$) compared to the control sample. The addition of the extract to the meat product led to an increase in the percentage of adequate daily intake of B vitamins, a decrease in the concentrations of zinc, manganese and magnesium by no more than 14% ($P < 0.10$), simultaneously with an increase in the amount of selenium, copper, potassium and calcium from 8% to 17.35% ($P < 0.10$) depending on the microelement. Fatty acid and amino acid compositions of the experimental products showed minor changes. All pates were characterized by a reduced atherogenic index in comparison with the average AIP of the human diet by 29.7% for the control sample and by 32.4% for the experimental products. The noted increase in the mass fraction of protein in Samples 1 and 2 by 6.76% and 2.73% ($P < 0.10$), respectively, together with a slight change in amino acid composition, led to a decrease in the minimum amino acid score simultaneously with a decrease in the excess amount of essential amino acids not used for plastic needs. This was evidenced by a decrease in the amino acid score difference coefficient by 7.71% in the case of Sample 1 and by 3.07% in the case of Sample 2, which led to an increase in the biological value of the experimental pates by 7.7% when added the maximum amount of extract and by 3.06% for Sample 2. According to the results of proteomic analysis, it was revealed that the addition of the extract did not lead to significant changes in the protein composition: such fundamental differences as the absence or, conversely, the appearance of *de novo* protein fractions between the control and experimental pates was not detected, while all the main muscle proteins were preserved. Histological analysis revealed that all samples were characterized by the same structure and a high degree of constituent component grinding. The addition of onion peel extract to meat pate, both in the maximum and in half of the maximum volumes, did not affect the density of the structural element arrangement, and there were no differences in the microstructural organization for all experimental products.

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ANALYSIS OF SUBSTANCES MIGRATION WITHIN THE TROPHIC CHAIN "SOIL — PLANT — RAW MEAT MATERIALS"

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Abstract
The trophic chain, which manifests the correlation at nutritional level between various macro- and microorganisms, is an important factor of the ecosystem; it can show the migration of various substances within the chain "soil — water — plant — animals". The trophic chain in Borgoy depression area was studied due to the profound correlation between the compositional characteristics of the soil and pronounced organoleptic features of meat of the sheep that feed on grass in this area. For the experiments, control and experimental samples of soil, water, plants and mutton meat were examined. The samples taken near the saline lake within the Borgoy depression on the west of Beloozersk village served as an experimental sample. The samples taken 30 km from the salt lake near Petropavlovsk village in the Republic of Buryatia served as control sample. Experiments have shown that the soil of the Borgoy depression is a saline soil, with a depth of the salt horizon of 0–30 cm, and the salt belongs to chloride-sulfate-soda type of salinity. It is noted that the saline soil is characterized by a much higher content of carbonates, chlorides and cations of sodium, potassium and magnesium. Correlation was found between the isotopic composition in soil, vegetation and the raw meat materials. More profound certain organoleptic features of Borgoy mutton were noted. It's highly probable that this fact is associated with the peculiarities of the mineral, chemical, and amino acid compositions of meat of the livestock that lives in the pastures of the Borgoy depression, characterized by saline soils. Despite the increased content of heavy metals such as lead and copper in the soil, data on sheep muscle tissue showed that all values of toxic elements content are within the permissible concentration range. The transfer of heavy metals from the soil to the aboveground part of plants is hindered by the underground root part, which serves as barrier.

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Introduction

The trophic chain, as the nutritional relation between various macro- and microorganisms, contributes to the transformation of energy and matter. All plants, animals and microscopic organisms are closely related according to the principle food-to-food consumer [1]. A number of scientists study the trophic chain as an important factor in the ecosystem to confirm the fact of transfer of various substances within the chain: soil-water-plant-animals. Works [2–3] show that the trophic chain characterizes the process of transit of the substances in a certain ecosystem, while accumulation is observed of the substances valuable for the heterotrophs and the contaminants too.

The issues of the substances transition in the process of trophic communication and its significance have been the subject of research for many domestic and foreign scientists. Thus, the influence of trophic transfer and transformation of CeO_2 nanoparticles was studied by spraying the soil where lettuce was growing for 30 days with radioactive CeO_2 nanoparticles, followed by giving the lettuce leaves to the land snails to eat. The second half of the lettuce was treated by foliar application of the radioactive nanoparticles directly onto the leaves. Experimental data have proven that with direct trophic influence, the rate of transition of the test substance into the tissues and feces of snails is higher [4].

The work [5] presents the results of a study of the accumulation of heavy metals in the trophic chain "soil — vegetable feed — cattle — human". The content of heavy metals in all objects of study was studied, and the coefficient of biological absorption of metals was calculated. The research results showed: at all levels of the trophic chain, the greatest accumulation was observed for zinc and copper; along with an increase in the trophic chain level, the transition coefficients of such toxic metals as lead and cadmium increased too.

The authors of [6] studied the transit of heavy metals and phyto-hormones in the trophic chain "soil — plant — bee — honey". It was revealed that honey builds up heavy metals that accumulate in the soil in accordance with the laws of the trophic chain.

The processes of transit of heavy metals in the soil — potato — Colorado beetle ecosystem were studied. The influence of the content of heavy metals in the soil on their content in potato leaves and the body of the Colorado potato beetle has been confirmed [7].

Bioaccumulation of organochlorine pesticides in marine organisms (oysters, crabs, etc.) due to trophic connections with surface sediments has been proven. The authors proposed to use the obtained results to create the criteria for monitoring water quality [8].

The authors [9] studied the specific features of the Caspian sea trophic chain: plankton — mollusks — fish. Research allows identifying the reason for the quality deterioration of fish food base.

The issues of the influence of microelements in the trophic chain "soil — plant — animal" under certain environmental conditions are considered. The critical levels of microelements and the ways of their detoxification are shown [10].

An analysis of the level of toxicants contamination of certain links within the trophic chain "soil — feed — animal's body — livestock products" was implemented. A method has been proposed for calculating the coefficients of substances transfer along the food chain to the animal's organs to control the quality of livestock products [11].

Analysis of the composition of natural stable isotopes of carbon, nitrogen and sulfur is one of the potential tools for verifying geographic origin [12–14]. Plants and non-migratory animals that feed on them potentially have region-specific isotopic compositions determined by climatic and environmental conditions.

Researchers have found that the consumer characteristics of raw meat in the form of tissues and organs of the farm livestock depend not only on the type of the animal, its gender, breed, variety, but also on the growing and feeding conditions [15–18].

It has been shown that mutton that lives in the area of the Borgoy depression of Dzhida district of the Republic of Buryatia has more profound organoleptic characteristics: richer taste of meat and richer broth, sweetish taste, and so on [19], however, there are studies on the trophic chain "soil — plant — Borgoy mutton"

Thus, an analysis of the literature showed that the quality of raw meat can be affected by the composition of soil, plant materials, and feed for the free-range livestock. In connection with the above, the purpose of the study was to analyze the migration of substances, isotopes of carbon, nitrogen and some trace elements within the trophic chain "soil — water — plant — raw meat" to assess the quality of mutton from the sheep that live in the Borgoy depression.

Material and methods

To conduct the experiments, the control and experimental samples of soil, water, plants and mutton were analysed. As control samples the samples of soil, water and plants were taken 30 km from a saline lake, near Petropavlovka village in the Republic of Buryatia. The experimental samples were the samples of soil, water, and plants taken near a saline lake within the Borgoy depression on the west of Beloozersk village (Figure 1). Characteristics of control and experimental samples of soil, water, plants and mutton meat are given in the Table 1.

The sampling area is a hilly terrain of the Borgoy steppe spanning along the river Dzhida, here there are three fairly large lakes: Nizhneye Beloe, Verkhneye Beloe and Kamenny Klyuch, plus several small lakes, including those of a temporary, seasonal nature — they are presented in the form of flooded meadows.

Table 1. List and characteristics of the researched objects

Objects of research	Characteristics	
	Control sample	Tested sample
Sampling location	Republic of Buryatia, Dzhida district, 30 km from the saline lake — near Petropavlovka village	Republic of Buryatia, Dzhida district, near saline lakes within the Borgoy depression on the west of Belozersk village
Sampling date	September-October 2022	September-October 2022
Soil samples within the depth of 0–10 cm	Within the sampling location, the soils are chestnut powdery-carbonate	Within the sampling location, the soils are saline
Samples of plants that sheep living in the sampling areas feed on	Vegetation of the sedge and common sedge pastures	Vegetation of the sedge and common sedge pastures
Water samples	From a stream near the soil sampling location	From a well within the Borgoy depression
Mutton samples	Mutton of the sheep living near the soil and vegetation sampling site	Mutton of the sheep living within the Borgoy depression

Figure 1. Sampling location

The landscape of the area is characterized by medium-hilly, gentle relief, has the general outline of a flat plain, slightly inclined (3–5°) towards the river Dhzida. The terrain is a bit undulating, with poorly developed microrelief. The terraces of the river Dzhida, Verkhnee lake and Nizhneye Beloe lake are expressed.

The specific feature of the Borgoy steppe and, to a greater extent, whole area around Beloozersk village, which received its name from the Beloe (White) Lake located next to it, is a white coating on the soil surface along the shores of lakes — this is the evaporation of saline mineral salts from the lake and sedimentation of salty fogs in windy weather.

Table 1 presents the characteristics of the researched objects.

In the soil and water samples, water extract analysis was run to determine the type of soil salinity and water quality; isotopic and mineral composition was studied in the samples of soil, plants and raw meat. Soil and water parameters were determined and analyzed in five repetitions in the laboratory of the Institute of General and Experimental Biology of the Siberian Branch of the Russian Academy of Sciences.

To study the meat properties, the samples of muscle tissue of the semitendinosus muscle were taken from Buryat type sheep of Transbaikal breed (n = 3) at the age of 5–6 months of spring-summer free-range pasture feeding. The animals were slaughtered in the slaughterhouse of the consumer cooperative "Khamtaa" (Dzhida district, the Republic of Buryatia). To conduct research, the hip cuts were selected from the pairs of the control carcasses and experimental carcasses, cooled at a temperature of 2–4 °C, packaged in polymer film and delivered to the research site in Ulan-Ude within 24 hours.

To study the isotopic composition, semitendinosus muscles were selected from hip cuts, packed in a polymer film, frozen at a temperature of minus 14–16 °C, packed in a thermal bag to slow down the thawing process, and delivered by aircraft to the Center for shared use of isotope mass spectrometry based in the Institute's laboratory of ecology and evolution problems named after. A. M. Severtsov RAS (Moscow). The studies were done within three days after the delivery of the samples.

In the laboratory of VSGUTU, the organoleptic characteristics of meat and mutton broth were studied, in the laboratory of the Center for Hygiene and Epidemiology of the Republic of Buryatia, the content of some microelements, including toxic ones, was determined.

The organoleptic characteristics of mutton and mutton broth were evaluated after the thermal processing. Mutton was cooked as follows: meat lump of 1 kg was placed in a container with cold water in a ratio of 1:3 and boiled until the temperature in the center of the lump reached 75 °C for about 60 minutes, 30 minutes before the end of cooking table salt was added (at rate of 1% by weight of mutton lump). After cooking, the meat was cooled down, sliced and sent for tasting. The meat was rated on a nine-point scale according to the following parameters: appearance, smell, taste, texture, juiciness. To evaluate the mutton broth, it was poured into transparent glasses and its appearance, color, smell, taste, flavor richness was assessed.

The content of toxic microelements was defined with the help of atomic-adsorption method of Spectr AA240 (Agilent, USA) spectrophotometer in the vegetative feeds according to the state standard GOST 30692–2000[1], in the soil — according to the FR.1.31.2013.14150 "M–MVI–80–2008[2], in the water — according to the GOST R57165–2016[3], in the ram meat materials — according to the GOST 30178–96[4]. Content of mercury and arsenic by atomic adsorption spectrometry using the universal spectrometric complex USK "Gamma-plus" (ZAO "NTC Expertcenter", Moscow, Russia). In the raw meat the arsenic content was determined according to the GOST R51766-2001[5], of the mercury — by GOST 26927–86[6], in the vegetation — by GOST 34427–2018[7], in water — by GOST 31950–2012[8], in soil — by PND F 16.1.2.23–2000[9].

The water extract of the soil was analyzed in accordance with the Guidelines for Chemical Analysis of Soils. The dry residue was tested after evaporating an aliquot and its drying in an oven at a temperature of 105 °C. The calcined residue was found by calcining the dry residue in a SNOL muffle furnace (SnolTerm, Russia) at a temperature of 550 °C.

Alkalinity was determined by titration with 0.02H H_2SO_4 solution with phenolphthalein added. Chlorine was titrated with $AgNO_3$ (0.02H solution) in the presence of 10% K_2CrO_4. Ca+ and Mg+ were determined by the trilonometric method, SO_4^{2-} — with the addition of glue, ending on a PE-5300VI spectrophotometer (EKROSHIM, Russia). The K+ and Na+ contents were determined using a PFA-378 flame photometer (Yunico-Sys, Russia).

The content of stable isotopes was determined using Finnigan Delta V Plus mass spectrometer (Thermo Electron Corporation, USA). Isotopic ratios were calculated in ppm using the folowing formula (1):

$$\delta n X_{sample} = [(R_{sample}/R_{standard}) - 1] \times 1000, ‰ \quad (1)$$

where X is the element (nitrogen or carbon), n is the number of the heavy isotope, R is the molar ratio of the heavy and light isotopes of the element.

The results of the measurement were brought to international standards. For nitrogen — N2 of atmospheric air, for carbon the "Viennese" equivalent of PeeDee belemnite (PDB); the analytical error in determining the isotopic composition did not exceed 0.3‰.

The obtained experimental data were statistically processed by finding the arithmetic mean, standard error and confidence interval. The significance of the differences was considered as significant at $p < 0.05$.

Results and discussion

In result of the experimental studies presented in the Table 2, it was found that the studied soils were of saline type, with a salt horizon depth of 0–30 cm, of chloride-sulfate-soda type of salinity, and a chestnut powdery-carbonate soil. It was noted that the saline soil is characterized by a much higher content of carbonates and bicarbonates (28.8 and 612.44 mg/l) than the chestnut soil (4.8 and 219.6 mg/l), as well as the higher amount of anions and cations (447.42 and 155.2 versus 127.5 and 64.4 mg/l accordingly). The composition of saline soil anions is dominated by chlorides (104.9 mg/l), the composition of cations is sodium (69.4 mg/l), calcium (54.0 mg/l) and magnesium (30.0 mg/l). In chestnut soil their content is almost 2 times less (25.2, 22.0 and 15.6 mg/l, respectively). The data obtained from the water analysis (Table 2) showed that the water from the well features high alkalinity (carbonates and bicarbonates — 28.8 and 612.44 mg/l) and a high content of sodium, calcium and magnesium chlorides, which is apparently related to the soil salinity. The water from the stream is much softer and has much lower content of anions and cations.

The work [20] also indicates that in Borgoy depression the salinity of the soil is soda-based, and the chemistry of the salinity is predominantly sulfate-sodium. The authors note that the soils have a slightly-alkaline and alkaline reaction of the environment; the composition of the CEC (cation exchange capacity) is dominated by exchangeable Na. There is a high content of exchangeable magnesium throughout the profile of chemical elements, which can be attributed to the specific regional features. A highly saline type with a high predominance of sodium and magnesium is demonstrated by the ratio of Ca: Mg: Na cations equal to 1:4:5. In the saline soil the maximum of salts is clearly reached in the upper horizons; the total salt content along the profile reaches 0.89–2.75%. The salinity of the soils of the Borgoy depression was also pointed out by the authors

[1] GOST 30692–2000 "Fodders, mixed fodders and animal raw foodstuff. Atomic absorption method for determination of copper, lead, zinc and cadmium" Retrieved from https://docs.cntd.ru/document/1200013014 Accessed September 05, 2023

[2] FR.1.31.2013.14150 "M–MVI-80–2008 Methods for measuring the mass fraction of elements in soil samples, soils and bottom sediments by atomic emission and atomic absorption spectrometry (Replaced with FR.1.31.2004.01278 according to the letter, OOO Monitoring, Ref. No. 267 of". Retrieved from https://www.russiangost.com/p-275510-fr131201314150.aspx Accessed September 05, 2023

[3] GOST R57165–2016 "Water. Determination of elements by inductively coupled plasma atomic emission spectrometry" Retrieved from https://docs.cntd.ru/document/1200140392 Accessed September 05, 2023

[4] ГОСТ 30178–96 "Raw material and food-stuffs. Atomic absorption method for determination of toxic elements" Retrieved from https://docs.cntd.ru/document/1200021152 Accessed September 05, 2023

[5] GOST R51766–2001 "Raw material and food-stuffs. Atomic absorption method for determination of arsenic" Retrieved from https://docs.cntd.ru/document/1200025461 Accessed September 05, 2023

[6] GOST 26927–86 "Raw material and food-stuffs. Methods for determination of mercury" Retrieved from https://docs.cntd.ru/document/1200021114 Accessed September 05, 2023

[7] GOST 34427–2018 "Foodstuff and animal fodder. Determination of mercury by Zeeman atomic absorption spectrometry" Retrieved from https://docs.cntd.ru/document/1200159810 Accessed September 05, 2023

[8] GOST 31950–2012 "Water. Method for determination of total mercury by flameless atomic absorption spectrometry" Retrieved from https://docs.cntd.ru/document/1200096958 Accessed September 05, 2023

[9] PND F 16.1.2.23–2000 "Measurement method for measuring the mass fraction of total mercury in soil, soil and bottom sediment samples on a RA-915 mercury analyzer with RP-91C attachment" Retrieved from https://www.russiangost.com/p-162459-pnd-f-161223–2000.aspx Accessed September 05, 2023

of the scientific work [21], who studied the genesis, geography and classification of steppe and forest-steppe soils in the basin of the Baikal Lake.

The content of toxic microelements is very important for characterizing the quality of raw meat, which microelements can also migrate through the trophic chain. In this regard, the content of heavy metals in the studied objects of Borgoysky Nature Reserve region was tested (Table 3).

The data presented in the Table 3 showed that the soil has high lead content of 40 mg/kg, which exceeds the maximum permissible standards by 1.25 times. High content of copper was noted (30 mg/kg). The increased content of iron was noted in the water (0.76 mg/dm^3), which is 1.5 times higher than the maximum permissible concentration according to the standard SanPin 1.2.3685–21[10].

Analysis of the content of heavy metals in pasture vegetation, presented in Table 3, showed that in the above-ground parts of plants their content is significantly lower than the level of maximum permissible concentrations.

The presented data on vegetation are consistent with the results of scientists represented in the works [22–25]. The increased content of microelements, including lead (57.3–92.8 mg/kg), was noted in the upper humus horizon of the saline soils of the West Trans-Baykal area in the work [23], which is associated with a relatively large amount of carbonates thus serving as a reservoir (barrier) for all elements. Chestnut soils of the steppe ecosystems of West Trans-Baykal area in the work are characterized by the increased content of lead (2.9 times), which is determined by the soil-forming rocks [24].

The heavy metals are prevented from their transportation from the soil to the above-ground part of plants by the humus substances of the soil and the underground root part, which plays a barrier role. In this regard, the vegetation that serves as food for grazing sheep is safe from an environmental point of view. No connection was found between the increased lead content in the soil and vegetation [22]. The lead content in herbaceous vegetation on the chestnut soils of West Trans-Baykal area in the work does not exceed the permissible standards.

Data on the muscle tissue of the sheep showed that all parameters of the toxic elements content are within the permissible concentrations, which confirms the food safety and quality of Borgoy mutton.

Next, the isotopic composition of the trophic chain "soil — plants — raw meat materials" was analyzed; the isotopic signature of the animals reflects the integral information about its trophic relations, starting with the plants that form the soil environment of the pastures [26].

Figure 2 presents the results of a study of the isotopic composition in the trophic chain "soil-plant-raw meat materials".

[10] Sanitary and epidemiological rules and regulations SanERR1.2.3685–21 "Hygienic standards and requirements for ensuring the safety and (or) harmlessness of environmental factors for humans" Retrieved from https://docs.cntd.ru/document/573500115 Accessed September 05, 2023

Analysis of the isotope composition of soils showed that in the saline soil there is a highly profound fractionation of the heavy carbon isotope, C13, and the chestnut soil is richer with the light isotope. Discrimination δ13C (Δ‰), that is, the difference in the content of the heavy isotope 13C in them is large and equal to 11.68‰ ($p < 0.05$). This is apparently caused by high content of carbonates, sodium chlorides, calcium and magnesium in the saline soil and the lower content of carbon and nitrogen (1.4 and 0.2%). The same phenomena can be seen with the fractionation of the heavy isotope 15N. The difference in the δ15N content is lesser and amounts to 1.51‰.

The obtained data are consistent with the scientific literature data. Thus, in articles [26,27] it is noted that the isotopic composition of soils depends on its composition: saline soils, as a rule, are characterized by high content of carbon isotope in contrast to the other types of soils. Data on the isotopic composition of carbon are consistent with the data of articles [28,29], which show the "isotopic signature" of grass vegetation from the pastures in the muscle tissue of raw livestock meat with a δ13C value not exceeding the level of –24.0‰.

The research results presented in the Figure 2 showed that the isotopic composition of carbon in soils differs from the isotopic composition of carbon in the plant material by 1.19‰ in the chestnut soils and by 13.47‰ in the saline soil; the weighting takes place in the process of destruction of vegetation residues by microorganisms.

The analysis of the literature data revealed that the obtained data are consistent with the literature data, so in the article [22] the following fact is presented: usually the value of soil carbonates δ13C is 14–16‰ more than that value of organic matter.

The vegetation on the saline soil is represented by hard sedge; in the vegetation mass on the chestnut soils this herb occupies a dominant position; the codominant is the false wheatgrass. This is probably why the vegetation on the studied soils has the similar carbon isotope indices, but the plant residues on the saline soil are lighter ($\Delta = 0.6$‰). The degree of fractionation of the 15N isotope during their life activity is higher — δ15N is 6.4‰. Low discrimination of δ13C (1.19‰) and δ15N (2.01) proves the high rates of vegetation mineralization on the chestnut soils.

In the food chain "plant-to-animal", as a result of physiological processes, muscle tissue of the animal is enriched with the heavy carbon isotope, but depleted in the 15N isotope. In the analyzed samples, the weighting of carbon in the Borgoy mutton is 2.2, in the control sample — 2.03‰, and the δ13C discrimination between them is low and amounts to 0.43‰. Enrichment of δ15N occurs in control sample of meat ($\Delta = 0.34$‰), and lighting occurs in Borgoy mutton meat ($\Delta = 0.55$‰). The control sample of mutton, compared to Borgoy mutton, is more enriched in the heavy carbon isotope 13C ($\Delta = 0.43$‰) and the heavy nitrogen isotope ($\Delta = 0.09$‰). Thus, there is a correlation between the isotopic composition of soil, of vegetation and raw meat.

Table 2. Results of the soil and water samples analysis (n = 5)

No.	Water samples	Calcined residue, mg/l	Dry residue, mg/l	Alkalinity, mg/l		Cl^- mg/l	SO_4^{2-} mg/l	Σ of anions,%	Ca^+ mg/l	Mg^+ mg/l	Na^+ mg/l	K^+ mg/l	Σ of cations,%
				CO_2^{2-}	HCO_3^-								
	Water												
1	Experimental sample	598.1 ± 35	674.0 ± 45	28.8 ± 5.2	612.4 ± 65.4	104.9 ± 21.3	7.5 ± 1.2	447.4 ± 20.4	54.0 ± 9.5	30.0 ± 8.2	69.4 ± 11.3	1.8 ± 0.2	155.2 ± 56.2
2	Control sample	194.2 ± 28*	312.1 ± 32.2*	4.8 ± 1.6*	219.6 ± 58.2*	5.7 ± 2.2	7.5 ± 3.4	127.5 ± 36.9*	22.0 ± 6.5*	15.6 ± 6.3*	25.2 ± 12.0*	1.6 ± 0.09	64.4 ± 12.3*
	Soil												
	Soil samples	Calcined residue, mg/l	Dry residue, mg/l	Alkalinity, mg/l		Cl^- %	SO_4^{2-} %	Σ of anoins, %	Ca^+ %	Mg^+ %	Na^+ %	K^+ %	Σ of cations,%
				CO_2^{2-}	HCO_3^-								
3	Experimental sample	2.92 ± 0.2	3.32 ± 0.82	0.81 ± 0.02	1.46 ± 0.01	0.13 ± 0.03	0.019 ± 0.002	1.69 ± 0.04	0.01 ± 0.001	0.0048 ± 0.0003	0.12 ± 0.06	0.031 ± 0.004	0.162 ± 0.006
4	Control sample	0.32 ± 0.05*	0.48 ± 0.09*	0.01 ± 0.002*	0.09 ± 0.002*	0.05 ± 0.002*	0.003 ± 0.001*	0.11 ± 0.001*	0.012 ± 0.06	0.0036 ± 0.0012	0.031 ± 0.008*	0.003 ± 0.001*	0.05 ± 0.01*

Note: * The difference between the values of groups 1 and 2, 3 and 4 is significant, since $p < 0.05$.

Table 3. Content of the microelements in the analyzed samples obtained from the location of Borgoy depression

No.	Name of toxic elements	Soil		Water		Pasture vegetation (fodders)		Muscle tissue of sheep	
		Content, mg/kg	MAC/ SanPin 1.2.3685–21	Content, mg/dm³	MAC/ SanPin 1.2.3685–21	Content, mg/kg	MAC/ SanPin 1.2.3685–21	< 0,01	< 0,5
1	Lead	40.0 ± 1.2	32	< 0.003	0.01	< 0.1	5.0	< 0.01	< 0.05
2	Cadmium	0.54 ± 0.12	—	< 0.0001	0.001	< 0.1	0.3	< 0.01	< 0.1
3	Arsenic	2.7 ± 0.21	—	< 0.005	0.01	—	—	—	—
4	Copper	30.0 ± 1.5	—	< 0.001	1.0	—	—	—	—
5	Iron	—	—	0.46 ± 0.05	0.3	—	—	< 0.001	< 0.5
6	Mercury	< 0.005	2.1	< 0.0001	0.0005	0.0085	0.05		

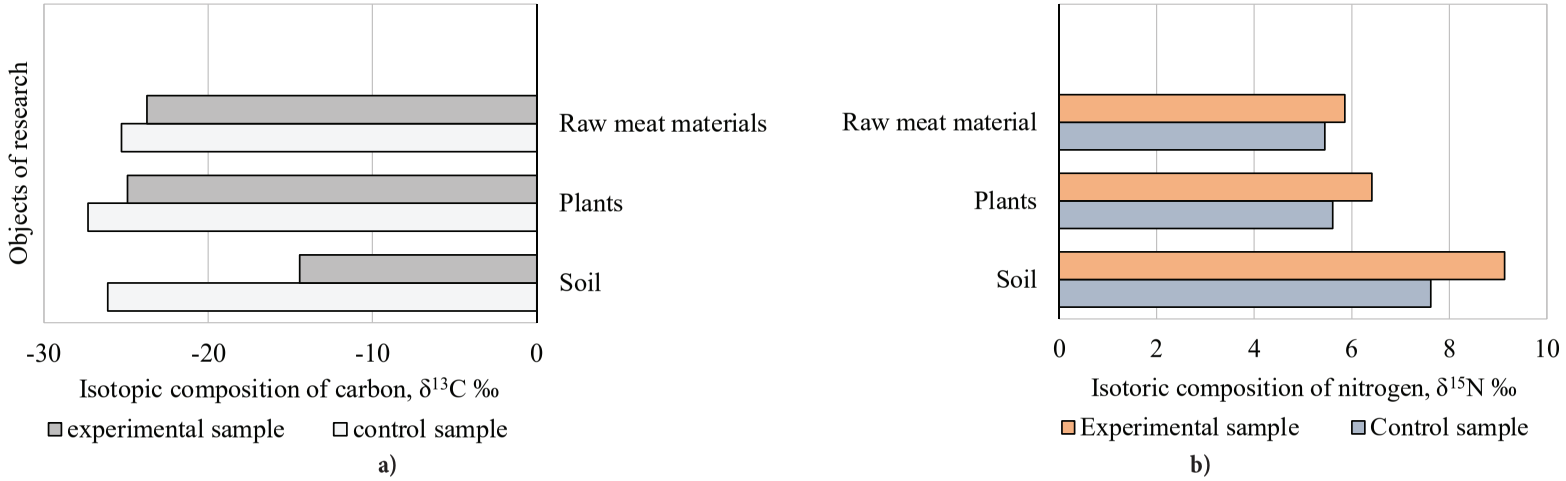

Figure 2. Results of isotopic analysis

The similar correlation between the trophic chain of "soil — plants — livestock meat" was established in regards to the carbon and nitrogen isotopes in [28,29]. The data obtained are consistent with the conclusions that the isotopic composition of soil organic matter is a cumulative characteristic that reflects the ratio of photosynthetic types of vegetation and depends on the type of vegetation, the level of its fractionation during soil processes, and climatic conditions [27].

To study the quality parameters of Borgoy mutton, the organoleptic values of the meat and broth of the control sample and experimental sample of mutton were studied, the data are presented below in the profile diagrams (Figure 3 and Figure 4).

The data of the profile diagram (Figure 3) presented for assessing the quality of the boiled mutton showed that the experimental sample and control sample did not differ in their appearance ($p > 0.05$). More pronounced rich aromatic taste, a sweetish taste and a more delicate texture were noted in Borgoy mutton, which is confirmed by plenty of points for taste — by 8.4%, for smell (aroma) — by 6.6% compared to the control ($p < 0.05$).

Figure 4 presents the profile diagram data for assessing the quality of the broth, which showed that the broth cooked from the meat of experimental sample is richer, more saturated, highly fragrant with an expressed meat taste in comparison with the control sample. Analysis of the obtained data showed that the broth cooked from the control sample and experimental sample did not have any special differences in appearance and color ($p > 0.05$). But when tasting the broth the higher average points were conferred upon the experimental sample: for taste it got 7.4% higher assessment, for smell — more by 7.2%, for richness — by 5.7% compared to the control sample ($p < 0.05$).

The obtained data confirm that Borgoy mutton has more pronounced organoleptic characteristics [19]. Probably, the migration of micro- and macroelements, which the soil of the Borgoy depression is rich in, through the chain "soil — water — plant — animal", contributes to the accumulation of micronutrients that form the peculiar organoleptic characteristics of Borgoy mutton.

Conclusion

The trophic chain, which characterizes the correlation at the nutritional level between various macro- and microorganisms, is an important factor in the ecosystem and can show the migration of various substances within the chain "soil — water — plant — animals". The trophic chain in Borgoy depression area was studied due to the profound correlation between the compositional characteristics of the soil and pronounced organoleptic features of meat of the sheep that feed on grass in this area. To conduct the research the control and experimental samples of soil, water, plants and mutton were examined. The experiment was implemented on the samples taken near a salt marsh lake within Borgoy depression to the West of Beloozersk village. The control samples were the samples taken 30 km from a salt lake — near Petropavlovka village of the Republic of Buryatia. Experiments showed that the soil of Borgoy depression is a saline soil with a salt horizon depth of 0–30 cm, and of chloride-sulfate-soda type of salinity. It is noted that the saline soil features much higher content of carbonates, chlorides and cations of sodium, potassium and magnesium. Correlation was found between the isotopic composition in the soil, vegetation and the raw meat. Borgoy mutton has more profound organoleptic characteristics; this fact is probably associated with the peculiarities of the mineral, chemical, amino acid composition of the meat of the livestock that lives in the free-range pasture conditions of the Borgoy depression on the saline soils. Despite the increased content of heavy metals like lead and copper in the soil, analytic data on the muscle tissue of sheep showed that all values of the toxic elements content are within the acceptable concentrations. The transfer of heavy metals from the soil to the above-ground part of plants is prevented by the underground root part, which serves as barrier. The research showed that Borgoy mutton has high quality characteristics.

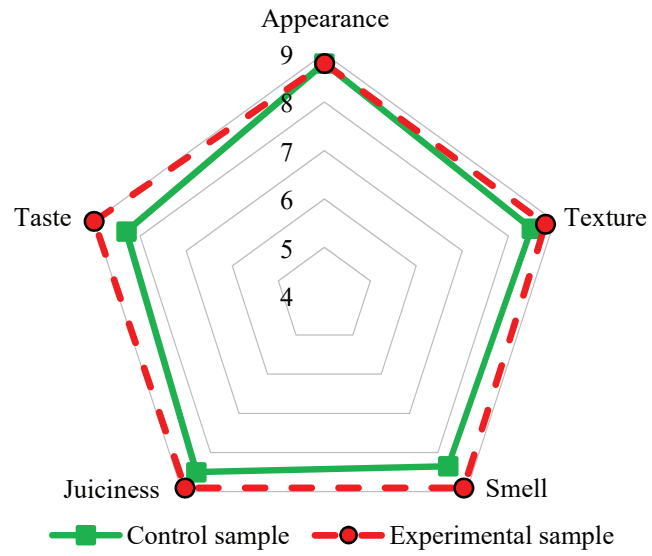

Figure 3. Profile diagram of the control and experimental samples of boiled mutton

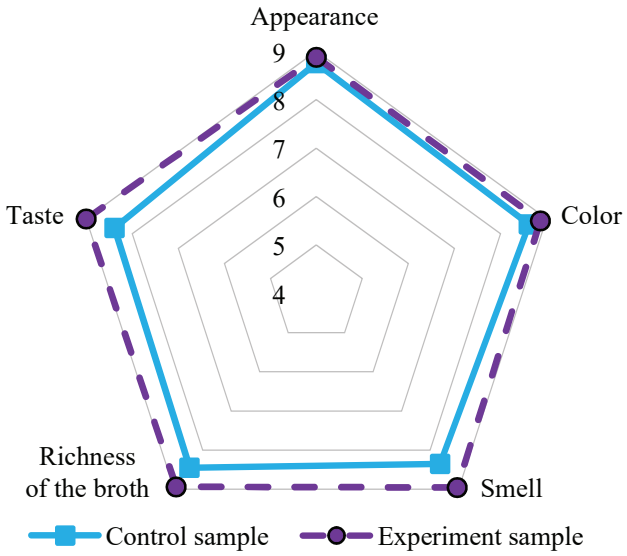

Figure 4. Profile diagram of broth cooked from the control and experimental samples of mutton

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The authors were equally involved in writing the manuscript and bear equal responsibility for plagiarism.

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