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ABOUT THE JOURNAL

The journal "Theory and practice of meat processing" is a peer-reviewed scientific journal covering a wide range of issues: formation of the composition and properties of meat raw materials including various methods for raising animals and poultry; the main questions of meat raw material processing, improvement of technologies for meat product manufacture including functional foods, effects of meat and meat product consumption on human health.

The primary objectives of the journal "Theory and practice of meat processing" are to create content for distribution of international knowledge in the world scientific community, promote research performed by scientists from Russia and foreign countries, enhance presentation of their scientific achievements in the international arena and highlight promising research directions in the meat industry.

The main tasks of the journal consist in publishing results of theoretical and experimental studies carried out in Russian and foreign organizations, as well as on the authors' personal initiative; bringing together different categories of researchers, university and scientific professionals; creating and maintaining a common space of scientific communication, bridging the gap between publications at the regional, federal and international levels.

The editors strive to expand the pool of writers and welcome new authors.

The journal content areas reflect the modern state of different scientific and technical problems, the industrial implementation of scientific results and advanced national and foreign experience, such as regulation of feeding rations and keeping conditions, targeted modification (selection, hybridization, operative manipulations with animals and poultry), the study of effects of the main meat nutrients (proteins, lipids), micro- and macroelements, vitamins, their compatibility and preservation during meat and poultry product manufacture on meat quality and properties.

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DO INSECTS AS FEED INGREDIENT AFFECT MEAT QUALITY?

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Keywords: *animals' diet, insects, regulation, poultry, rabbit, pig*

Abstract

The development of sustainable feed ingredients for monogastric livestock is nowadays considering insect meals and oils to replace or supplement conventional feedstuffs. Although the regulation on the use of insect products differs among countries resulting in restrictions on use in the diets of monogastric meat producers, global research is exploring all the strengths and weaknesses of their inclusion. Therefore, whereas the scientific literature has extensively studied both the relationship between insect farming systems and safety (potential health risks), and between the dietary use of insects and the nutritional value of diets and production performance of farm animals (fish, poultry, swine, rabbit), the relationship between insect-containing diet and meat quality has only recently been considered. The present review therefore aims to collect the results of the studies that have related the dietary use of some insect species, such as the black soldier fly (Hermetia illucens), the yellow mealworm (Tenebrio molitor) and the silkworm (Bombyx mori), on the physicochemical and sensory traits of the poultry, swine and rabbit meat. The variable that has been most affected by the inclusion of insects as feed on livestock meat quality is the fatty acid (FA) profile, which, as is well known, in monogastrics tends to reflect that of the diet. Therefore, the black soldier fly inclusion has always originated meats with a more saturated FA profile, the yellow mealworm a more monounsaturated fatty acid profile, whereas the silkworm a more unsaturated fatty acid profile and rich of valuable omega-3 FA, but rarely changed the related physicochemical variables, or the sensory profile of the meat.

Introduction

Statistics about demographic trends depict that by 2050 the World's population should reach 9.1 billion people. This scenario is putting pressure on the search for alternative and sustainable feed resources for the livestock sector. This was officially emphasised by the Food and Agriculture Organisation (FAO) of the United Nation in Report "The future of food and agriculture — Alternative pathways to 2050" [1]. Among the possible alternative feed (and food) sources, insects are seen as one of the most effective alternatives to improve global food and feed security, with remarkable potential sustainability [2]. In fact, insects generally reproduce quickly, have fast growth and high feed conversion efficiency, and can be reared on a wide range of bio-waste streams, thus becoming an effective natural tool to recycle waste into nutritionally rich feed/food ingredients. Compared with traditional protein production, that of insect has a really low hydric and ecological footprint, and its production requires small lands to be exploited for very high yield [3].

Why insects in animals' diet?

In addition to the above reasons, the use of insects in animal feeding is supported by the fact that numerous animal species among birds and mammals consume insects as part of their natural diet. About 80% of birds are reported to include insects in their diets [4], among which there are also the chicken (*Gallus gallus*), the turkey (*Meleagris gallopavo*), the guinea fowl (*Numida meleagris*), the quail (*Coturnix coturnix*), and the ostrich (*Struthio camelus*), which are species of interest for food production in different Regions of the World [5–8].

Fish also eagerly consume insects in nature: both terrestrial and aquatic insects are considered part of the natural diet of both freshwater (black carp (*Mylopharyngodon piceus*), African catfish (*Clarias gariepinus*), common catfish (*Ameiurus melas*) [9,10]) and marine fish (chum salmon (*Oncorhynchus keta*), Atlantic salmon (*Salmo salar*) [11,12]).

There are also some mammal species (such as the wild boar) that have a varied diets, in which insects are a part of them. Among the monogastric mammals used as a food source in many Countries, there is the farmed rabbit, which naturally does not include insects in its diet, but the lipid source generally consisting of sunflower oil and soybean oil could be partially replaced with insect oil, as alternative and sustainable feed resource.

Insects as feed ingredient

In 2014 the first international conference on "Insects to feed the world" was organized by the FAO [13] which proposed, from the first time, the use of insects as promising alternative food and feed sources as a possible solution related to the expected demographic growth. Since that time, a new research field has emerged and an impressive number of articles have been published (see reviews of [14–29]), with an exponential increase in scientific knowledge on insects as feed.

Most studies have focused on the potential industrial application of certain insect species and have shown that

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black soldier fly (*Hermetia illucens*) and yellow mealworm (*Tenebrio molitor*) have great potential in providing large-scale and high-quality nutrients for aquaculture, poultry and pig diets, exploiting bio-waste and organic side-streams [30].

In parallel, an increasing number of research is testing the feed use of insects by focusing on the circular economy of small and medium-sized livestock farm, with the aim of making them independent from the global market, thus differently sustainable, or focusing on niche products such as meat from native breeds to preserve the biodiversity, or raised according to the organic system [29].

Regulation

Geographically, there are three main legislative scenarios: 1) the Anglo-Saxon countries (Australia, Canada, New Zealand, UK, USA), for whom insects are not treated as unique/novel food and feed, and therefore the food and feed agencies have generally approved import and sales upon fulfilment of certain quality and safety requirements; 2) the Asian, centre- and south-American, and African countries, for which insects are usually considered a conventional food and feed; 3) the European Union, for which it is necessary to establish rules and supply approval before permitting any trade of a specific food/feed product.

The result from these extremely diversified scenarios, is that also the legislation structure of different areas around the World regarding insects' use as food and feed is extremely heterogeneous. To respond to the new food and feed trends worldwide, and thus market exigencies and perspectives, the legislative frameworks of countries are rapidly changing.

In the European Union (EU), there is no history related to possible use of insects as food. In 2015, the European Parliament established that insects could fall into the "novel foods" category, and consequently they are subject to the consequent approval processes. Recently, EFSA [31] approved the yellow mealworm as a novel food, and the European Commission is discussing a regulation authorising this insect as a food.

Likewise, there is no history in the EU regarding the use of insects as feed application. Only in 2014 the FAO conference triggered a gradual change in the EU legislative framework, which is still evolving. Currently, processed animal proteins (PAPs) extracted from insects are allowed to be used in aquaculture, laboratory, companion and fur animals (regulation (EU) no. 2017/893), whereas for poultry species and pigs discussion is still going on, thus legislation still needs to be updated. Conversely, the fat fraction extracted from insects is allowed as feed ingredient for any animal species. The regulation allows the use of seven insect species: the black soldier fly (*Hermetia illucens*), the yellow mealworm (*Tenebrio molitor*), the lesser mealworm (*Alphitobius diaperinus*), the house cricket (*Acheta domesticus*), the field cricket (*Gryllus assimilis*), the banded cricket (*Gryllodes sigillatus*), and the common house fly (*Musca domestica*), specifying also the substrates allowed to feed insects. Regulation (EU) 2017/1017 permits the use of live or dead terrestrial invertebrates with or without treatment as feed material, but not as processed as described in Regulation (EC) no. 106/2009. Thus, invertebrates are considered as a suitable material for a feed at all the stages of their lives, except for species that adversely affect plants, animal, or human health.

Within the EU, however, starting from the above-mentioned common legislative framework every country has adopted this legislative framework differently. As an example, Belgium, The Netherlands, Great Britain, Denmark and Finland, refer to national laws which allow the production, marketing and trade of insect-based products. Instead, Germany show a limited degree of tolerance, whereas other states (i. e., Italy), have close to zero tolerance.

Recently (on May 25 2021), delegates of the EU-27 in the EU Standing Committee on Plants, Animals, Food and Feed (PAFF Committee) backed draft regulation aimed at setting EU harmonised standards for insect frass and the addition of silkworm to the list of seven approved insect species for use as protein in aquaculture feed [32]. The silkworm species was already approved for use in the feed of non-food producing animals, and was evaluated by the EFSA in its insect protein risk profile opinion in 2015. On 22 June 2021 the ENVI (Committee on the Environment, Public Health and Food Safety of the European Parliament) backed the draft Commission Regulation amending Annex IV to Regulation (EC) No 999/2001 which would remove the ban on the use of PAPs (including insect-derived protein) in poultry and pig feed, which was approved by the European Commission on 17 August 2021, and it will enter into force on the twentieth day following that of its publication in the Official Journal of the European Union [32].

Insect nutritional sources and formulation

To exploit the maximum potential as feed ingredient, insects are processed to obtain whole insect meal (full-fat), protein meal (PAPs; only for aquaculture, pet and fur animals) which can be defatted or contain some proportion of lipids according to the extraction method, and fat/oil (for all animal species). In addition, there are also bioactive compounds, such as the chitin [33], the 1-deoxynojirimycin (1-DNJ, [34]), and the lauric acid [35] that can be extracted from insects and used for different industrial applications.

The high variability of the nutritional composition of insect species can be an issue when focusing on its use as feed ingredient. In particular, the quantity and quality of insect lipids play a leading role, since their nutritional contribution varies primarily according to the insect species, their living substrate and growth stage, and then, according to the degree of lipid extraction of the insect meal [2]. Therefore, the use of defatted insect meal ensures a more constant feed formulation.

The extracted insect fat/oil can serve as feed ingredient alternative to fish and other vegetable oils, but also as food ingredient, in the cosmetic industry and as biofuel. The fatty acid (FA) profile of insects lipids can be very extreme: two examples are the *Hermetia illucens* and the *Bombyx mori*. The fat extracted from *Hermetia illucens* larvae contains 60–79% SFA. Conversely, the *Bombyx mori* chrysalis is very rich in omega-3 PUFA [36] and its oil presents a favourable n-6/n-3 ratio of 0.17 (personal communication). Thus, combining full-fat or partially defatted insect meal from different species could help ensure the best FA profile for animal feed [3]. The amino acid profile also differs between the different species of insects; in the case of the *Tenebrio molitor* larva the content of all individual amino acids was found to be higher than that of barley, fish, brewer's yeast, beef/veal, and crustaceans, except for lysine, which was slightly higher in brewer's yeast [31].

Research studies conducted so far have tested a wide range of levels of substitution (5–100%, mainly with fishmeal or soybean meal) or inclusion (0.75–60%) of insect meal, to find the best level, to cover nutrient requirements and to maximise growth and health performance, and product quality from farmed aquatic and terrestrial animals (see review of [17]). Pioneering research is also testing the effect of feeding live larva on poultry, but has so far only focused on improving animal welfare [37] or laying hen egg production and quality [38].

Insects in feeds and meat quality

The effects of dietary insect products (larva meal, prepupa meal, oil) inclusion on the quality of food-producing terrestrial animals have been studied mainly in poultry, with sporadic and recent interest in porcine and rabbit species. The purpose of this review is to provide updated literature on the use of insect-based products as feed for meat-producing animals, detailing the effects on the physico-chemical-sensory quality of the meat obtained. The review will consider the black soldier fly (*Hermetia illucens*), the yellow mealworm (*Tenebrio molitor*), and the silkworm (*Bombyx mori*), the first two for the greatest commercial interest in the EU, the third because it is potentially interesting in improving the dietary-nutritional value of meat. To be used as feed compounds, insect products can be used partially processed (dried larva) or processed (partially or totally defatted meals, dechitinised meals, fats/oils). The use of live larva in livestock feed is in its infancy and currently poses some technical limitations. Regarding the inclusion/substitution level of insects and insect-derived products in feed, a wide range has been tested, and for each insect species the best inclusion/substitution range is being identified.

Dietary inclusion of black soldier fly (Hermetia illucens) and meat quality

The black soldier fly larva is one of the most used organism for aquaculture and one of the best studied for both aquaculture and poultry feeding. The black soldier fly larva averagely contains 43.1±5.05 g protein /100 g DM, and the amino acid profile is rich in leucine (6.72 g/100 g protein), lysine (6.22±1.08 g/100 g protein), and valine (5.38±0.82 g/100 g protein). Nutritionally important is also its contribution in calcium (24.1±12.8 g/kg DM) and phosphorus (6.01±1.77 g/kg DM) (see review of [29]). As aforementioned, the amount of larva fat and its FA profile are extremely variable and depend on the type of substrate. A description of the results obtained on the meat quality of poultry, pigs and rabbits is provided below.

Poultry

Based on numerous studies (Table 1), the black soldier fly as meal or fat in poultry diets has no [39–48] or limited influence [41,42,47–50] on physical meat quality (pH, colour, water holding capacity (WHC), shear force) of broiler's chicken, quail, barbary partridge, and muscovy duck. Similarly, the poultry meat proximate composition also showed alternate results, and they do not seem related to the insect meal inclusion level. Differences in meat nutrients composition were mainly observed for more protein content [42,50], for lowered [45] or increased [43,51] essential amino acids, and for enrichment in minerals, like calcium [51], sulfur [49], and cupper [40]. In general, the sensory evaluation of poultry meat derived from animals fed diets supplemented with black soldier flies did not differ from that obtained from control diets [41,44,51]. Instead, the black soldier fly inclusion as meal or fat had a major contribution in modifying the FA profile of the lipids in the poultry meat [40–43,45,47,50–54].

Considering that the FA profile of the meat of monogastric animals is in line with the pattern of that of their diet, and that the black soldier fly (whatever its form) is rich in saturated fatty acids (SFA; approximately 70% of the total FAME, of which 43% is represented by the C12:0 [48] it follows that the proportion of SFA in meat increases as a function of the dietary inclusion level of the black soldier fly. In majority of the cases, this implies a worsening of the n-6/n-3 ratio [43], but either insect defatting or their food substrate, may not change [49] or improve [45] the omega-6/omega-3 ratio.

If the FA profile of the meat is changed by the inclusion of the black soldier fly in the poultry diet, changes in the lipid oxidation of the meat is also expected. However, most of the studies did not observe changes in the oxidation of meat lipids in animals fed black soldier fly [40,51,53]. However it is interesting to note that Choi et al. [39] observed a significantly low TBARS value on fresh meat after 7 days of refrigerated storage, and authors attributed it to the improved antioxidant activity (measured through the DPPH radical scavenging activity) of the meat due to the inclusion of the black soldier fly in the diet.

Table 1. Effect of dietary inclusion of black soldier fly on broiler meat quality.

Item	Avian species	Insect form	Substitution level range (%)	Inclusion level range (%)	Impact	References
pH, cooking loss[1], shear force[1]	chicken	meal	50–75	0.5–1.0 5–15	NS NS NS	[39] [1][41] [42]
Colour[1; 2], TBARS[a], DPPH radical scavenging	chicken	meal	25–50	0.5–1.0 5–15	P < 0.05 P < 0.05 P < 0.05	[39] [1][49] [2][42]
pH[1; 2], colour[1; 2], lipid oxidation, cooking loss, shear force	chicken	meal	 50–75	5–20 5	NS P < 0.05 P < 0.05	[43] [1][50] [2][41]
Amino acid, FA[b] profiles	chicken	meal		5–20	P < 0.05	[43]
FA profile	chicken	meal	25–50 50–75	 5 5–15	NS P < 0.05 P < 0.05 P < 0.05	[49] [50] [41] [42]
Mineral profile	chicken	meal	25–50		P < 0.05	[49]
Bioactive peptides, volatile profile	chicken	meal	100		P < 0.05	[55] [56]
Proximate composition	chicken	meal	50–75	5 5–15	P < 0.05 NS P < 0.05	[50] [41] [42]
Sensory evaluation	chicken	meal	50–75		NS	[41]
pH, colour, WHC[c], proximate composition, amino acid, FA and mineral profiles, sensory evaluation	chicken	meal		5–15	NS	[44]
FA profile	chicken	fat	100 50–100 50–100		P < 0.05 P < 0.05 P < 0.05	[52] [53] [54]
pH[1], thaw loss, proximate composition[1], TBARS	chicken	fat	50–100		NS NS	[53] [1][54]
Cholesterol	chicken	fat	50–100		P < 0.05/NS	[53]
Sensory evaluation	chicken	fat	50–100		NS	[53]
pH, colour, total moisture loss, shear force, heme iron, shelf life	quail	meal		10	NS	[45]
Proximate composition, cholesterol, amino acid, FA profiles, sensory evaluation	quail	meal		10	P < 0.05	[45]
pH, colour, WHC, shear force	quail	meal	25–100		NS	[46]
Proximate composition, sensory evaluation, cholesterol, TBARS	quail	meal		10–15	NS	[51]
Mineral, FA, amino acid profiles	quail	meal		10–15	P < 0.05	[51]
pH, cooking loss	quail	meal		10–15	P < 0.05	[48]
Colour, shear force, amino acid profile	quail	meal		10–15	NS	[48]
pH, shear force, cook loss, proximate composition	barbary partridge	meal	25–50		NS	[47]
Colour, FA profile	barbary partridge	meal	25–50		P < 0.05	[47]
pH, colour, proximate composition, TBARS	muscovy duck	meal		3–9	NS	[40]
FA profile, mineral profile	muscovy duck	meal		3–9	P < 0.05	[40]

[a]TBARS = Thiobarbituric acid reactive substances; [b]FA = fatty acids; [c]WHC = water holding capacity

Pig

The results until now obtained on feeding pigs with inclusion of black soldier fly meal highlighted any influence on the tested physical meat quality (pH, colour WHC, shear force; Table 2) [57,58]. As regards the meat proximate composition, Altmann et al. [57] did not observe differences, whereas a more recent study of Chia et al. [59] found higher protein content in groups fed with black soldier fly meal, but not in that with the highest substitution level.

The meat from pigs fed black soldier fly meal had higher concentrations of K, Fe and Zn [59], thus providing additional functional and nutritional minerals for humans. The dietary inclusion of 4% black soldier fly meal increased the marbling score of the *Longissimus thoracis* muscle, and upregulated the expression of genes related to lipid metabolism and to myosin heavy chain (MyHC–IIa) isoform, likely inducing the muscle fibre transition towards more oxidative fibres [58]; however the effect was not observed at the 8% inclusion level, and therefore further research should be provided to support this finding.

As expected, the lipids FA profile of the pork meat was affected by the dietary inclusion of black soldier fly meal, but to a lesser extent than in poultry. In fact, even if some single FA significantly differed (C12:0, C14:0, C16:1, C18:3 n-3, C20:4 n-6, C20:5 n-3, C22:6 n-3), no difference in FA classes or in n-6/n-3 ratio was observed [58]. The only sensory evaluation conducted so far, which considered 26 attributes, revealed statistically significant differences only in odour intensity and juiciness with the inclusion of insect meal in the diet, both considered as sensory improvements [57].

The exiguous number of research conducted until now on the use of black soldier fly meal in pig diets does not allow a detailed assessment of their effect on meat quality, but the response appears to be slightly more subdued than that observed in poultry, with positive implications especially in terms of FA profile.

Rabbit

For rabbit diets, the studies so far conducted only considered the fat obtained from the defatting of the black soldier fly meal (Table 3). Dalle Zotte et al. [60] evaluated the total replacement of the dietary fat source: linseed oil vs black soldier fly fat at 2 inclusion levels (3% and 6%), whereas [61] the partial (50%) or total replacement of soybean oil with black soldier fly fat.

The first study highlighted differences in rabbit meat colour (increased redness) and in oxidative stability (lower TBARS), whereas the second one did not find difference in meat physical traits, proximate composition and sensory evaluation. Both studies obtained the same response in terms of TBARS values, and similar response for the FA profile, that is oriented towards higher saturated FA proportion (C12:0 and C14:0).

These two studies provided new insights into the use of black soldier fly fat in rabbit diets, considering it an excellent substitute for commonly used oils and fats.

*Dietary inclusion of yellow mealworm (**Tenebrio molitor**) and meat quality*

The yellow mealworm larva is gaining attention as a source of protein for food purposes worldwide, and, recently, the EU included it in the Union list of authorised novel foods [62]. Yellow mealworm is also considered

Table 2. Effect of dietary inclusion of black soldier fly on pork meat quality

Item	Feeding length	Insect form	Substitution level range (%)	Inclusion level range (%)	Impact	References
Proximate composition, mineral profile	98 days	meal	25–100	6–14	$P < 0.05$	[59]
pH, colour, drip loss, shear force	46 days	meal		4–8	NS	[58]
Marbling score, IMF, IMP, mRNA expression to lipid metabolism and MyHC, FA profile	46 days	meal		4–8	$P < 0.05$	[58]
pH, colour, cooking loss, shear force, proximate composition, TBARS[a]	25–110 kg LW	meal	50–100		NS	[57]
Sensory evaluation	25–110 kg LW	meal	50–100		$P < 0.05$	[57]

[a]TBARS=Thiobarbituric acid reactive substances

Table 3. Effect of dietary inclusion of black soldier fly on rabbit meat quality

Item	Insect form	Substitution level range (%)	Inclusion level range (%)	Impact	References
Colour, TBARS[a], FA[b] profile	fat	100 (linseed *vs* BSF)	3–6	$P < 0.05$	[60]
pH, colour, WHC[c], shear force, proximate composition, sensory evaluation	fat	50–100		NS	[61]
FA profile, TBARS	fat	50–100		$P < 0.05$	[61]

[a]TBARS=Thiobarbituric acid reactive substances; [b]FA= fatty acids; [c]WHC=water holding capacity

a nutritionally suitable substitute for fishmeal and soybean for aquaculture and poultry diets, although its cost is currently too high and cannot financially compete with standard feed sources. It should be emphasised that the strength of this feedstuff therefore lies in the high protein content (Nx6.25: 56–61%), characterised by a high biological value, as it includes all the essential amino acids, in favourable proportions. Furthermore, it is a rich source of phosphorus [29] and potassium [31]. Fat (25–30%) contains approximately 24% saturated FA and polyunsaturated FA, and 50% monounsaturated FA, resulting in an omega-6/omega-3 ratio of 24 [31].

Poultry

Contrasting results were obtained for physicochemical traits of meat from chicken broilers, apparently not depending on the inclusion level of yellow mealworm meal (Table 4). However, the majority of the studies did not observe change in the meat pH, colour, moisture loss, shear force, and fatty acid profile [63–65]. Instead, [66] observed that the variables considered (WHC, lipids, ash, TVB-N, and sensory attributes) worsened as the level of yellow mealworm in the diet increased, and the authors partly attributed this trend to the possible presence of oxidised fat in dried insect meal. On the other hand, no other studies, testing higher yellow mealworm inclusion levels, found adverse effects on sensory traits; on the contrary, [67] observed an improvement in meat juiciness and tenderness. When other poultry species were considered, such as Barbary partridge [47] and quail [68], no substantial differences in the meat physicochemical traits were observed due to the use of moderate to high levels of yellow mealworm in the diet. Only meat colour changed in both bird species, however with an unclear pattern, whereas the FA profile of the barbary partridge meat was significantly affected by the dietary yellow mealworm, particularly at the higher substitution level (50% of the soybean meal) [47]. FA changes resulted in the reduction of the C18:0 and omega-6/omega-3 ratio ($P<0.05$) and the increase of C14:0, C15:0, C16:1, and C18:1.

Pig

Only one research has considered the use of yellow mealworm meal as dietary protein source in growing pigs [69] (Table 5). Authors tested the hypothesis that partial or complete replacement of a conventional protein source by yellow mealworm larva meal can influence the intermediary metabolism of the pig.

The omics-techniques on key metabolic tissues (liver, muscle and plasma) demonstrated that the insect meal from yellow mealworm can be used as a dietary source of protein in pigs without strongly impairing their metabolism, and consequently their growth performance. In addition, the unaltered plasma and liver triglycerides and cholesterol concentrations rather indicated that the insect meal had no effect on lipid metabolism in pigs. Studies in this regard are in their infancy, and further research is desirable to evaluate pros and cons, in order to provide precise information on the possibility of using yellow mealworm meal in pig feeding.

Table 4. Effect of dietary inclusion of mealworm on broiler meat quality

Item	Avian species	Insect form	Substitution level range (%)	Inclusion level range (%)	Impact	References
Proximate composition, sensory evaluation	chicken	meal		17	NS	[63]
pH, colour, WHC[a], proximate composition, TVBN[b], sensory evaluation	chicken	meal		1–3	$P<0.05$	[66]
pH, colour, drip loss, proximate composition, FA[c] profile	chicken	meal		7.5	NS	[64]
pH, colour, WHC, shear force	chicken	meal		2–8	NS	[65]
Sensory evaluation	chicken	meal		8.1	$P<0.05$	[67]
pH, shear force, cook loss, proximate composition, cholesterol	barbary partridge	meal	25–50		NS	[47]
Colour, FA profile	barbary partridge	meal	25–50		$P<0.05$	[47]
WHC	quail	meal		7.5–30	NS	[68]
Colour	quail	meal		7.5–30	$P<0.05$	[68]

[a]WHC=water holding capacity; [b]TVBN=Total volatile basic nitrogen; [c]FA= fatty acids

Table 5. Effect of dietary inclusion of mealworm on pork meat quality

Item	Feeding length	Insect form	Substitution level range (%)	Inclusion level range (%)	Impact	References
transcriptome, lipidome and metabolome of liver and muscle	4 week	meal		5–10	NS	[69]

Rabbit

The dietary inclusion of 4% yellow mealworm meal did not modify the proximate composition of the rabbit meat (Table 6). The amino acids content was modified by the dietary inclusion of insect meal, but the pattern observed in the 2 meat portions (hind leg and saddle) was not unidirectional, suggesting going deeper into the study of this effect. Notably, phenylalanine and lysine significantly decreased in hindleg meat, whereas threonine, isoleucine and methionine significantly increased, and tryptophan decreased in saddle meat [70]. In the same study, the authors observed that the effect of the dietary inclusion of 4% yellow mealworm on the FA profile of rabbit meat was negligible, leading only to an increase in total MUFA (from 22.0 to 22.9% total FAME, for control and treated groups, respectively; $P<0.05$) due to the increase in C12:0, and a decrease in EPA and DHA ($P<0.05$); The other FA classes and the omega-6/omega-3 ratio remained unchanged.

Based on these first results, despite being derived from a single study, it appears that a low inclusion level of dried mealworm larva in the rabbit diet does not appear to have adverse effects on meat quality. However, future studies are needed to consolidate the results obtained so far.

*Dietary inclusion of silkworm (**Bombyx mori**) and meat quality*

The silkworm pupa is characterised by high protein content (53.9% in the full-fat meal, 66.7% in the defatted meal), by a variable amount of lipids (29% in the full-fat meal, 9.5% in the defatted meal), the latter able to provide an extremely healthy FA profile (omega-3 FA: 29.5% in the full-fat, and 31.5% total FAME in the defatted meal), suggesting that it is a valuable nutritional ingredient for feed of different monogastric livestock species [36].

Poultry

Silkworm pupa meal has been successfully included in chicken broilers diet (Table 7), as it produced no effect neither on colour values and lipids content [71] nor on sensory analysis [63] [67] of the meat. A slight effect has been observed for lipids (3.56 vs 4.48% for leg meat of control and treated, respectively) in the study of [63] and for protein and ash contents (however not coherent with the inclusion level; [71]). Meat pH increased with the silkworm meal inclusion level but it did not impair meat colour [71].

The best result of the dietary inclusion of silkworm pupa meal in chickens concerns the FA profile of the meat lipids: the PUFA n-3 increased, and the omega-6/omega-3 ratio decreased with the increase of the dietary silkworm meal substitution level ($P<0.01$). The C18:3 n-3 content in breast meat ranged from 6.75 to 15.0 to 28.4 mg/100 g meat, for control, 25% and 50% silkworm meal inclusion level, respectively ($P<0.05$; [71]).

Rabbit

The silkworm pupa has been tested on rabbits diet in two forms: meal [70,72] and oil [73,74] (Table 8). Meat proximate composition was not modified by the silkworm pupa products [70,72,74] as well as the meat physicochem-

Table 6. Effect of dietary inclusion of mealworm on rabbit meat quality

Item	Insect form	Inclusion level (%)	Impact	References
Proximate composition	meal	4	NS	[70]
FA[a] profile, amino acid profile	meal	4	$P<0.05$	[70]

Table 7. Effect of dietary inclusion of silkworm on broiler meat quality

Item	Avian species	Insect form	Substitution level range (%)	Inclusion level range (%)	Impact	References
Proximate composition, sensory evaluation	chicken	meal		17	NS	[63]
Colour	chicken	meal	25–50	7–14	NS	[71]
pH, proximate composition, FA[a] profile	chicken	meal	25–50	7–14	$P<0.05$	[71]
Sensory evaluation	chicken	meal		7.8	NS	[67]

[a]FA= fatty acids

Table 8. Effect of dietary inclusion of silkworm on rabbit meat quality

Item	Insect form	Substitution level range (%)	Inclusion level range (%)	Impact	References
Proximate composition	meal		4	NS	[70]
FA[a] profile, amino acid profile	meal		4	$P<0.05$	[70]
Proximate composition	meal	50–100	5–10	NS	[72]
FA profile	meal	50–100	5–10	$P<0.05$	[72]
pH, WHC[b], proximate composition, sensory evaluation, TBARS[c]	oil	100	1.30	NS	[74]
FA profile	oil	100	1.30	$P<0.05$	[73]

[a]FA = fatty acids; [b]WHC = water holding capacity; [c]TBARS = Thiobarbituric acid reactive substances

ical traits (pH, WHC), oxidative status (TBARS), and sensory traits [74].

The amino acid profile slightly changed with the silkworm pupa meal inclusion in the rabbit diet: tryptophan increased and lysine decreased ($P<0.05$) in hind leg meat, whereas isoleucine increased ($P<0.05$) in saddle meat [70]. Also the cholesterol content significantly decreased in both meat cuts of animals fed the silkworm pupa meal, thus reducing the already low level of cholesterol in the rabbit meat [70].

Similarly to what observed for the poultry meat, the greatest effect of the use of silkworm pupa products is on the FA acid profile of the rabbit meat obtained.

The 5 and 10% inclusion level of silkworm meal considerably increased the levels of C18:3 n-3, C22:5 n-3 and C22–6 n-3 in hind leg meat [72], but a lower inclusion level (4%) obtained similar results for omega-3 FA, and favourably reduced the omega-6/omega-3 ratio of the same meat portion (ratio of 4.5, compared to that of 7.8 of the control group; $P<0.01$ [70]. The 100% substitution of sunflower oil with silkworm pupa oil significantly ($P<0.01$) lowered n-6 FA and increased n-3 FA, resulting in a omega-6/omega-3 ratio of 7.7 (compared to that of 18.9 of the control group) in hind leg [73].

Conclusion

The great economic impulse towards the use of insects as food and feed for the ecological-environmental sustainability purpose has generated new companies producing insect meal and derivatives. A flywheel of interest has therefore been generated on several fronts, and the use of these products increasingly requires confirmation of safety and efficacy. In the last 5 years, numerous research has been conducted relating to the use of insects for alimentary use. Many of these aimed at the feed sector, which however has mainly considered the effect of their use on animal *ante-mortem* variables, whereas the study of the effects on nutritional, rheological and sensory quality of the meat has only intensified significantly in the last 2 years. This review focused on collecting and describing the results of research conducted so far on the effect of insects as feed on the meat quality of terrestrial monogastric animals. The results showed different effects, more of these depending on the insect species than on the animal species that benefited from them. Overall, no adverse effects were observed on meat quality. Only the meat's FA profile was affected by the insect species included in the diet, suggesting its improvement through manipulation of the insect substrate, or the use of mixtures of insect meal or oil from different insect species.

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THE INFLUENCE OF BROOD CHICKENS BY-PRODUCTS PROCESSING WITH PROBIOTIC CULTURE STARTER ON CHANGE OF THEIR FUNCTIONAL AND TECHNOLOGICAL PARAMETERS

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Keywords: *enzymatic hydrolysis, poultry wastes, bioresource, chicken by-products, gizzard, comb*

Abstract
By-products are the potential source of animal protein obtained from brood chickens and egg-laying hens. Certain by-products like gizzards and combs are quite tough and possess low nutritional and biological value due to their high content of connective tissue. Biotechnological processing improves the quality parameters of collagen-containing by-products. In this article a probiotic starter culture of propionic acid bacteria, which have high proteolytic activity, was used to treat the gizzards and combs of brood chickens. Before processing of by-products with starter culture, physical and chemical parameters and the yield of by-products in relation to poultry live weight were analyzed and recorded. 5%, 10% and 15% starter culture were added to the tested samples of chopped by-products, the samples were kept at a temperature of 30 °C, and every 4 hours the following functional and technological parameters were monitored: moisture binding capacity, water holding capacity (MBC and WHC) and yield of the product after heat treatment. The results proved that increase of starter culture amount and longer exposure of by-products to hydrolysis led to decrease of functional and technological parameters values, but for the combs those parameters remained at a sufficiently high level compared to the gizzards, as the gizzards were exposed to more intense hydrolysis than combs. The decrease in the pH value correlated with the dynamics of MBC and WHC changes; and dynamics of the product yield after the heat treatment. Also the stained histological preparations were studied in order to assess the influence of biotechnological processing on by-products microstructure, where significant differences were found in the morphological structure of muscle and collagen fibers of hydrolysates of combs and gizzards exposed to action of bacterial concentrate. The results of rheological studies showed that hydrolyzed chicken combs differed from gizzards; the combs were denser and featured more elastic structure due to a lower degree of hydrolysis by bacterial enzymes. In general, the properties of collagen-containing by-products (muscular gizzards and combs) change significantly after being exposed to enzymes of propionic acid bacteria.

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Introduction

Poultry farming is the most dynamically developing branch of the agro-industrial complex all over the world. The poultry processing products are important resource for the country's food security. The active development of this industry is facilitated not only by the high consumer properties of the poultry processing products, but also by their market availability in comparison with other types of raw meat food products [1].

Because of raising intensity of poultry industrial farming and its processing, it becomes necessary to search for new ways of rational use of secondary products and by-products still rich in proteins, but not only in proteins but also in other biologically important components.

All over the world the poultry processing plants generate a large volume of by-products and offals: heads, legs, bones, viscera and feathers [2,3]. This waste is often recycled into feed for farm animals and house pets, used for soil fertilization, or just disposed of. It is necessary to solve the problem of utilization of secondary poultry processing products taking into account the intensity and dynamism of the development of poultry farming and poultry processing in Russia. Irrational utilization of these wastes leads to environmental pollution; spread of contagious diseases, as well as it is just a loss of useful biological production resources like protein, enzymes and lipids [4].

Transformation methods, involving the use of these components for production of bioproducts with added value, can be a promising direction in reducing the concentration of unprocessed waste in the environment. The problems of studying secondary resources as sources of protein hydrolysates, enzymes, polyunsaturated fatty acids, are being solved now by both Russian and foreign researchers [4,5].

Poultry by-products mainly consist of collagen proteins, for which extraction various types of hydrolysis are widely used: hydrothermal, acidic, alkaline and enzymatic.

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The characteristics of each type of hydrolysis are shown further in Figure 1 [6].

Comparing different types of hydrolysis of secondary raw materials, it can be concluded that the enzymatic treatment is more physiological, because it runs both with the help of proteolytic enzymes and with the use of live bacterial producer cultures [7]. The processing of by-products with enzymes and microorganisms allows almost complete preservation of all essential amino acids [6, 8]. The enzymatic method is predominantly chosen to obtain functional and bioactive hydrolysates [9,10]. However, the use of enzyme preparations in industrial volumes is currently not widely used due to their high costs. An alternative is to introduce into emulsified collagen-containing by-products substrates the living culture of microorganisms, which allows reducing the cost of processing of the collagen-containing raw materials.

Application of biotechnological methods to the by-products to increase the stability of agricultural production, to obtain high-quality and environmentally friendly food, as well as to process the wastes and solve food security issues, can be referred now to the priorities of development of the agricultural and industrial complex [11].

Processing of secondary collagen-containing raw materials with bacterial concentrates allows reducing the toughness of raw materials, increasing their nutritional and biological value due to the production of lactic acid and other metabolites by bacteria, as well as due to their proteolytic activity [8].

Under the influence of microorganisms the functional, technological, physical & chemical and morphological characteristics of raw materials change, as well as the nutritional and biological value of the finished product which changes too. Positive changes in food substrates during microbial fermentation are shown in Figure 2. Partially hydrolyzed protein products feature improved functional properties like solubility, fat absorption, foam stability and emulsifying properties [12]. Propionic acid bacteria have the same characteristics in reference to food substrates, which properties are promising for processing of secondary poultry products. Scientists [13] proved the high biochemical activity of propionic acid bacteria in meat mass, which contributes to formation of optimal functional and technological properties of raw meat materials. Also, a high proteolytic activity has been found, due to which the processes of protein chains breakdown are accelerated. In the process of metabolism of propionic acid bacteria, there is an intense accumulation of volatile fatty acids and amine nitrogen, which are involved in formation of the peculiar taste and smell of meat products. The positive changes in structural and mechanical, microbiological parameters and biological value of food substrates based on meat raw materials, indicated in Figure 2, were also found during treatment of meat by-products with propionic acid bacteria [13].

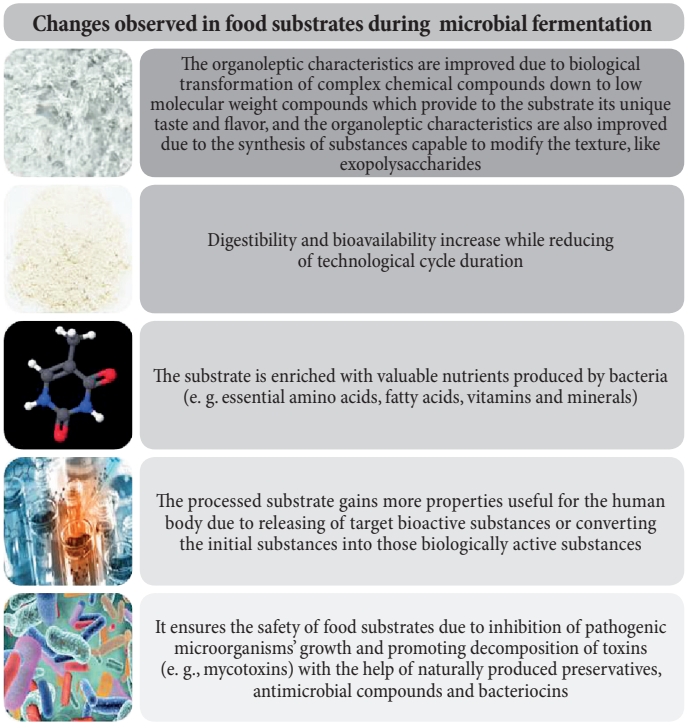


Figure 2. The influence of microbial fermentation on changes of food substrates properties

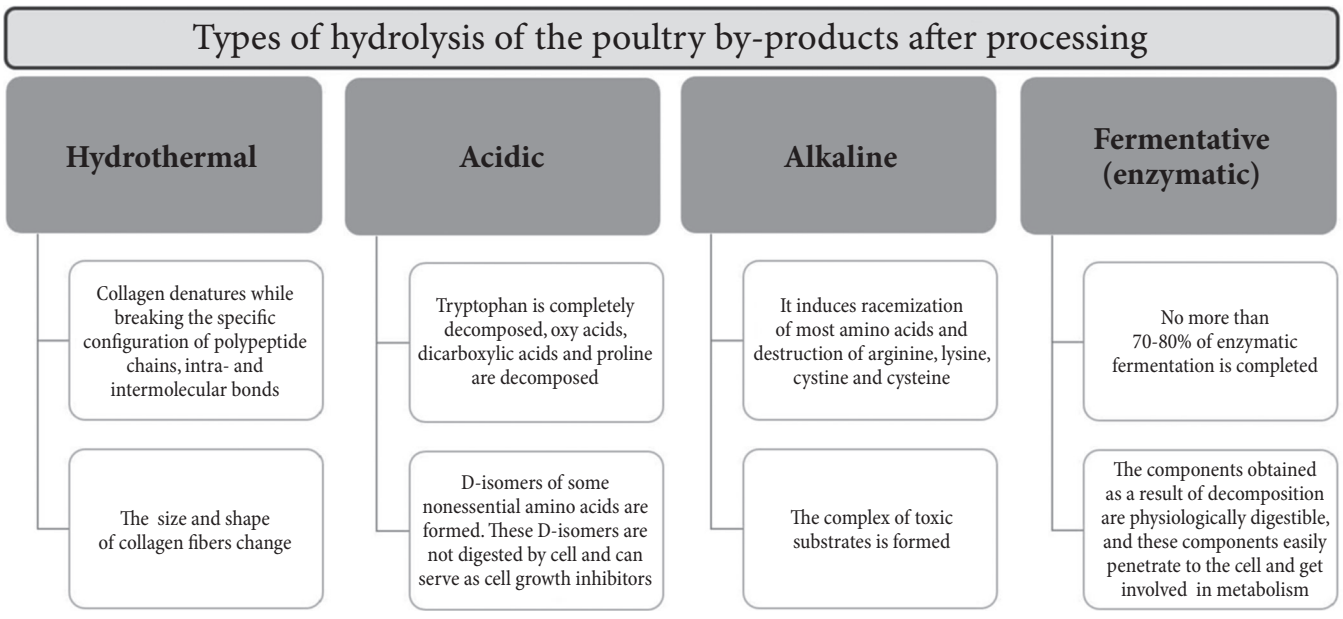


Figure 1. Characteristics of different types of hydrolysis of secondary poultry processing products.

The goal of this research is to study the influence of biotechnological processing on functional and technological parameters and microstructure of secondary products of brood chickens by-products — gizzards and combs.

Objects and methods

The object of the study is the gizzards and combs of brood chickens, which were later used in the preparation of collagen emulsions. The brood chickens were slaughtered at age of 11 months, the average live weight was 4 kg, the total number of slaughtered brood chickens was 300 pcs.

The yield of gizzards, heads and combs separately was defined as a percentage of the processed raw materials. Also, in the raw material the following physical and chemical parameters were determined according to generally accepted methods: the mass fraction of protein[1], of moisture[2], fat[3] and ash[4].

To improve the functional and technological parameters of the gizzards and combs of brood chickens, they were treated with the probiotic starter culture "Propionix" (LLC "Propionix", Moscow), which is a concentrated microbial mass of the *Propionibacterium freudenreichii subsp. shermanii* — KM 186 with an activity of 10^{10}–10^{11} CFU / cm^3.

For biotechnological treatment gizzards and combs were chopped in the chopper MFP 076 at second speed (Binatone, China) until an emulsion was obtained. 5%, 10% and 15% probiotic starter culture was added to the chopped by-products, mixed thoroughly and kept in a thermostat at a temperature of 30 °C for 16 hours with monitoring of parameters every 4 hours. As a control sample we used the chopped by-products without adding a probiotic starter culture. The following designations were assigned to the samples: C–C, G-C — control samples of chicken combs and chicken gizzards, respectively; C-Pro-5, C-Pro-10, C-Pro-15 — experimental samples of chicken combs with 5%, 10% and 15% probiotic starter culture, respectively; G-Pro-5, G-Pro-10, G-Pro-15 — experimental samples of chicken gizzards with 5%, 10% and 15% probiotic starter culture, respectively. The following parameters were monitored: pH level, moisture-binding capacity, water-holding capacity and yield after heat treatment.

The moisture-binding capacity (MBC) was determined by pressing, and the water-holding capacity (WHC) was determined thermogravimetrically, assessing the amount of moisture released from the sample during heat treatment. The yield of the product after heat treatment was determined by the difference in the mass of the samples before and after cooking at a temperature of 75 °C.

The pH values were measured in water extracts prepared by mixing 10 g of by-products samples with 40 ml of distilled water for 2 min. pH was measured with the digital pH meter model 710 A + with a measurement range from 0 to 14 pH units, measurement error of 0.02 pH units (Orion Research, Inc., USA).

To assess the influence of propionic acid bacteria on the structural components of raw materials, microstructural studies were conducted on the sliced sections of samples after their staining with hemotoxylin-eosin and picrofuchsin by the Van Gieson method. Histological preparations were studied with LEICA DMRXA microscope (Germany). The images of slices were taken with a digital video camera LEICA DFC290 (Germany) in the format of TIFF graphic files in RGB color space, which images served as objects of morphometric studies. For morphometric studies, the ImageScope M image analysis program (Germany) was used. The following parameters were analyzed: specific area of collagen fibers (%), which was defined as the ratio of the absolute area of all tissues of the histological preparation to the area of collagen (connective) tissue; average thickness of collagen fiber bundles (μm); average thickness of myocytes (μm).

The experiment on animals, including their housing, welfare, care and all manipulations, were run in compliance with the Decree No. 267 of the Ministry of Health of the Russian Federation of June 19, 2003 "On Approval of laboratory practice regulations", as well as the Council Directive 86/609/EEC of November 24, 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes.

All measurements were taken in triplicate. The results were processed statistically in Excel software (Office Microsoft: 42936865). The results were considered reliable at a significance level of $P \leq 0.05$.

Results and discussion

Physical and chemical parameters and the yield of raw materials to the live weight of poultry are presented in Table 1.

Table 1. Parameters of brood chickens by-products

Parameters	Gizzard of brood chickens	Combs of brood chickens
Yield, % of live weight	0.59 ± 0.03	0.11 ± 0.01
Mass fraction of protein, %	17.88 ± 0.02	14.81 ± 0.03
Mass fraction of fat, %	4.38 ± 0.01	10.47 ± 0.01
Mass fraction of moisture, %	75.08 ± 0.03	71.82 ± 0.03
Mass fraction of ash, %	1.76 ± 0.01	1.26 ± 0.01

The data on the chemical composition of poultry gizzards found in the scientific literature showed their significant variation depending on conditions of poultry method of farming and feeding and other factors also. For example, Zhumanova et al. found the protein content in chicken gizzards at the level of 20 g per 100 g of the product, which is

[1] GOST 25011–2017 "Meat and meat products. Protein determination methods". Moscow: Standartinform, 2018. — 14 p.

[2] GOST R51479–1999 "Meat and meat products. Method for determination of moisture content". Moscow: Standartinform, 2006. — 4 p.

[3] GOST 23042–2015 "Meat and meat products. Methods of fat determination". Moscow: Standartinform, 2019. — 9 p.

[4] GOST 31727–2012 "Meat and meat products. Determination of total ash". Moscow: Standartinform, 2019. — 8 p.

slightly higher than the values we obtained [14]. In terms of protein and moisture content, the results obtained by Abdullah and Buchtova (2016) are closer to the values we obtained: protein content — 17.34%, fat content — 0.76%, moisture content — 78.60%, ash content — 0.97% [15].

The results of pH change, presented below in Figure 3 and Figure 4, show that the acidity of the mass decreases due to the vigorous activity of propionic acid bacteria on the gizzards of brood chickens, and the combs apparently are less susceptible to hydrolysis due to the weak activity of bacteria on this substrate. This fact can be explained by the specificity of the combs structure and chemical composition, where collagen predominates. Changes in titratable acidity showed the similar dynamics; lactic acid accumulates faster in the gizzard-based substrate. That fact confirms the activity of propionic acid bacteria. At the same time the combs-based substrate based happened to be stable in terms of the lactic acid content; its amount increased only insignificantly by the end of the experiment. Mejri et al. (2017) explain the decrease in pH by the accumulation of acidic metabolic products of bacteria when processing raw materials with starter cultures [16].

The results of determining the moisture-binding capacity (MBC) of brood chickens gizzards samples are shown below in Figure 5, and MBC of combs are shown in Figure 6. A decrease in the MBC values was noted along with hydrolysis of by-products and with increase in the amount of starter culture. This process correlates with pH decrease, which was also noted by group of Gorlov et al. [17]. The MBC values varied from 76.2% in the experimental sample of broiler gizzards with 15% probiotic culture, up to 99.4% in the control sample during the initial period of hydrolysis (Figure 5). Gorlov et al. defined the level of MBC for chicken gizzards as 89.07%, which is slightly lower than the values we obtained [17]. The decrease of MBC along with increase in amount of bacterial starter culture can be explained by the fact that the starter culture is used in liquid form — the whey contains the active propionic acid bacteria. When the amount of starter culture is increased, the content of additional moisture introduced into

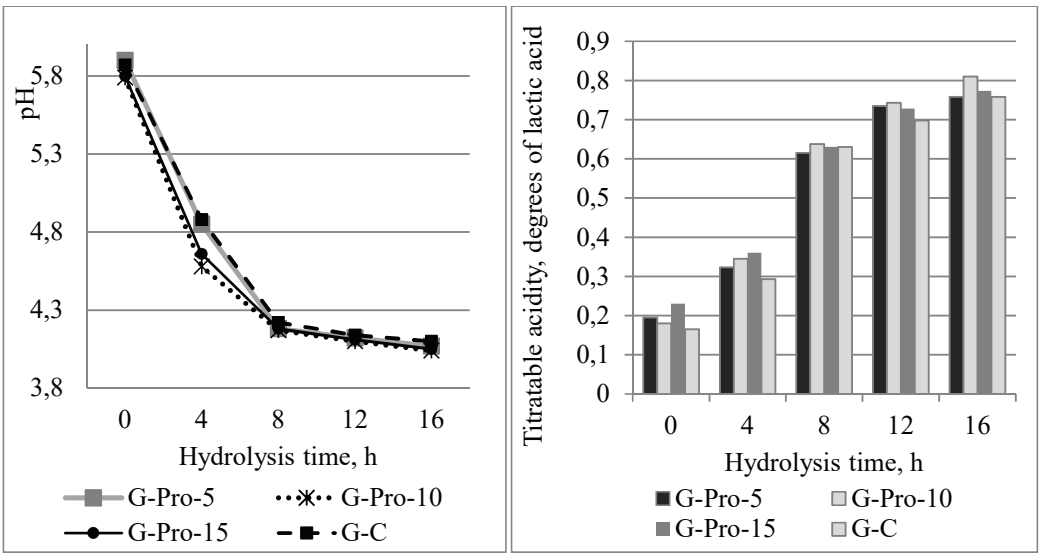


Figure 3. Changes in pH values and titratable acidity during hydrolysis of brood chickens gizzards

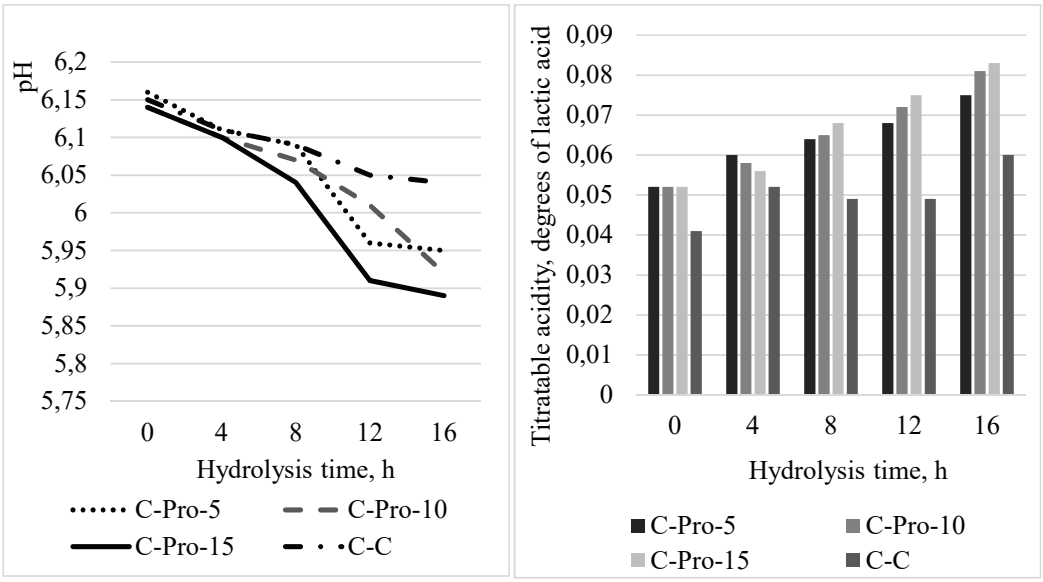


Figure 4. Changes in pH and titratable acidity values during hydrolysis of chicken combs

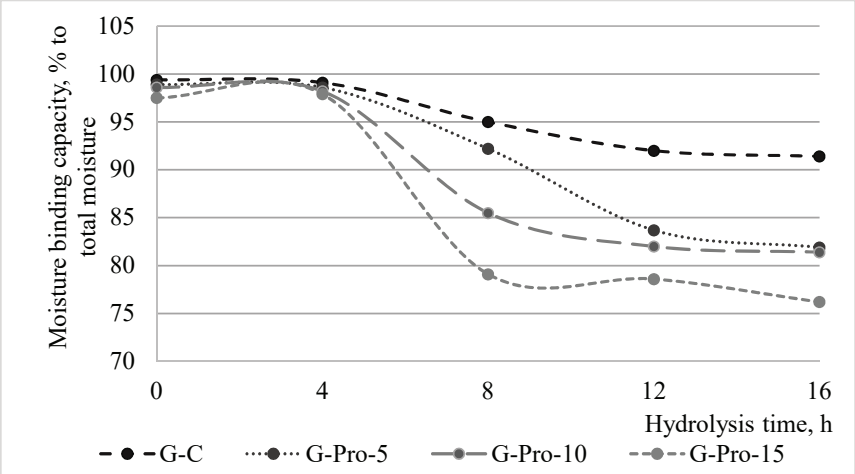


Figure 5. Changes in MBC values during hydrolysis of brood chickens gizzards

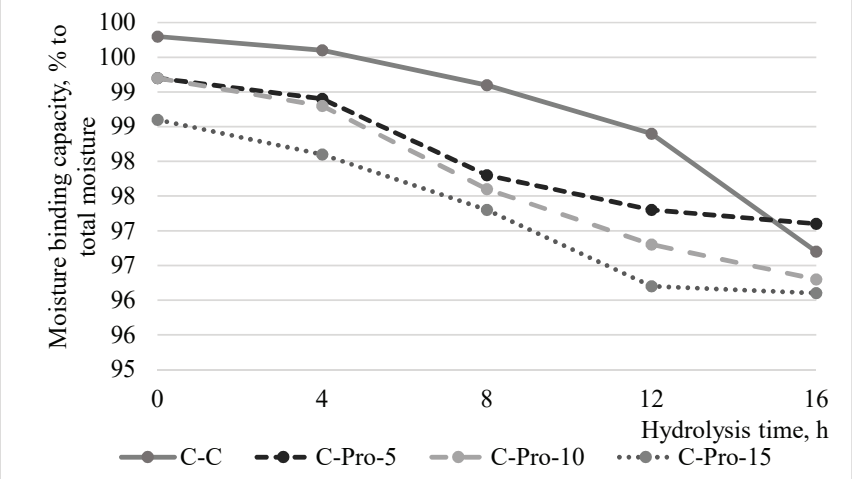


Figure 6. Changes in MBC values during hydrolysis of the gizzards of brood chickens

the by-product mass also increases. In samples of by-products, the intensity of hydrolysis increases with addition of starter culture, which is clearly seen in gizzard samples. The peptides formed during hydrolysis are not able to bind significant amount of excessive moisture. This is also confirmed by the results obtained for determination of WHC and losses during heat treatment of samples of by-products (Figure 7 and Figure 8).

Gombozhapova et al. [18] explain the decrease in MBC and WHC of raw meat during its prolonged mechanical processing by partial denaturation of proteins [18]. The capacity of meat to bind water is a complex characteristic influenced by structural and biochemical changes that occur to proteins [19,20]. During the fermentation of raw meat material its pH decreases, and similar changes occur in proteins structure. Tavdidishvili et al. [21] underline the importance of the WHC parameter, because the moisture loss during heat treatment and the sensory parameters of the product depend on its level [21].

When analyzing the dynamics of the structural and mechanical properties of hydrolysates (refer to the Figure 9 and Figure 10 below), a trend was found for increase in the flexibility properties of both control and experimental samples of chicken combs and gizzards during the fermentation period of 16 hours long; the plasticity increases due to destruction of native protein macromolecules and, as a consequence, it causes their functional properties decrease. It has been proven that hydrolyzed gizzards of brood chickens feature softer, more flexible structure in samples fermented with a concentrate of propionic acid bacteria — while during 16 hours of hydrolysis, the total deformation increased by 18.9–38.5%, and elastic deformation decreased by 22.5–38.4% in comparison with the control samples. Hydrolyzed chicken combs differed from hydrolyzed chicken gizzards by their denser and more elastic structure, which is explained by lower degree of hydrolysis under the action of bacterial enzymes.

Histological examination of microstructure in the hydrolyzed muscular gizzards of brood chickens of the control group found the fragments of the muscle wall with small areas of the mucous membrane (Figure 11a). The bundles of smooth muscle fibers are located in mutually perpendicular directions. Collagen fibers of the connective tissue matrix are grouped into compact bundles and stained with picrofuchsin to get crimson shades (Figure 11c). In the preparations treated with the bacterial concentrate, a fragmented muscle layer is visualized, in comparison with the control group the stain was absorbed quite weakly (Figure 11b). Thin collagen fibers in the preparation are discomplexed, weakly stained with picrofuchsin to shades of pink (Figure 11d).

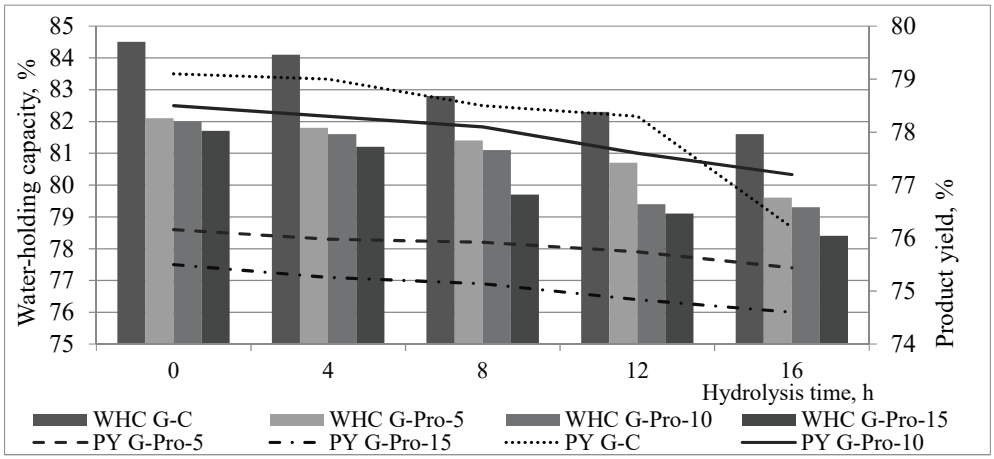


Figure 7. Water-holding capacity (WHC) and product yield after heat treatment of chicken gizzards

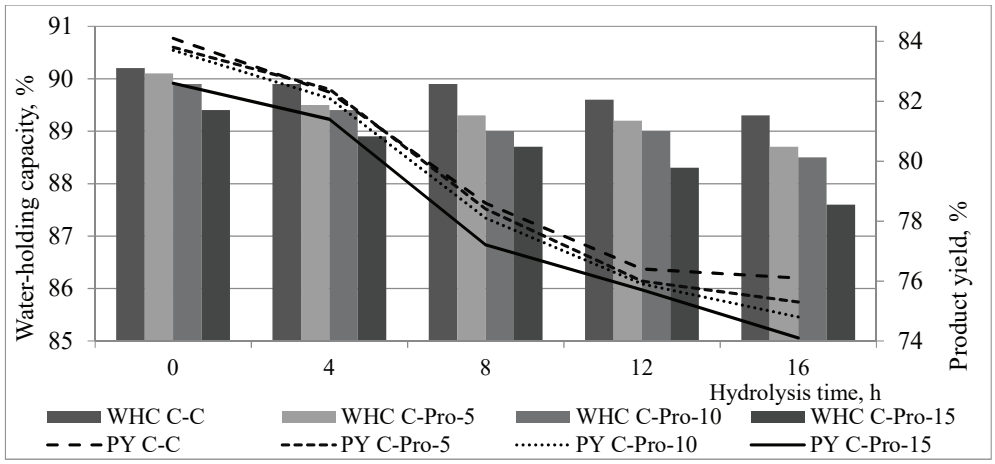


Figure 8. Water-holding capacity (WHC) and product yield after heat treatment of chicken combs

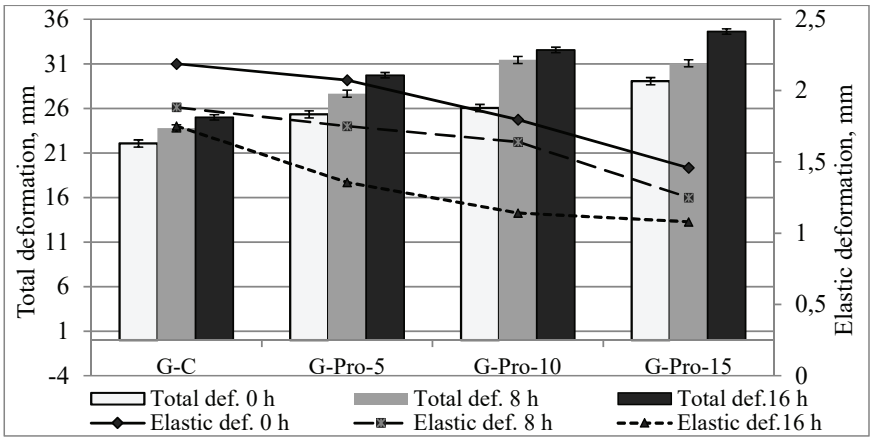


Figure 9. Dynamics of deformation characteristics during heat treatment of brood chickens gizzards

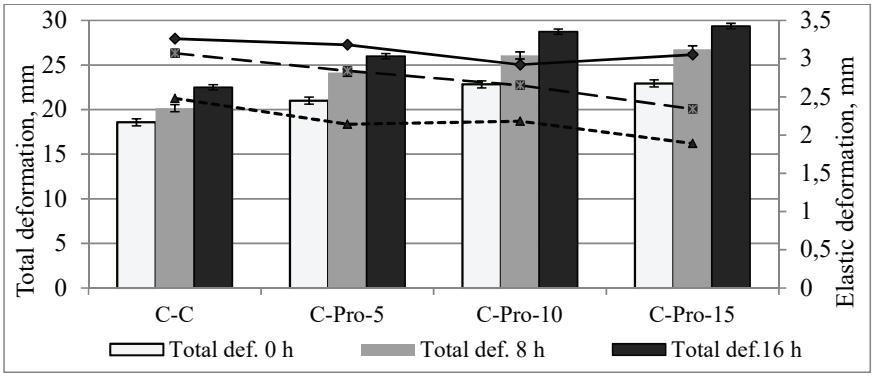
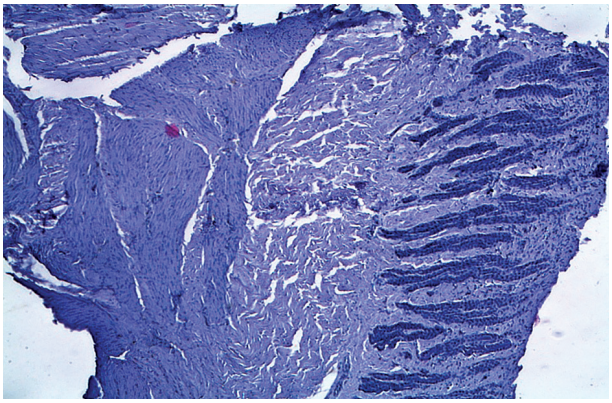


Figure 10. Dynamics of deformation characteristics during heat treatment of chicken combs

The gizzards of brood chickens. Hematoxylin-eosin staining



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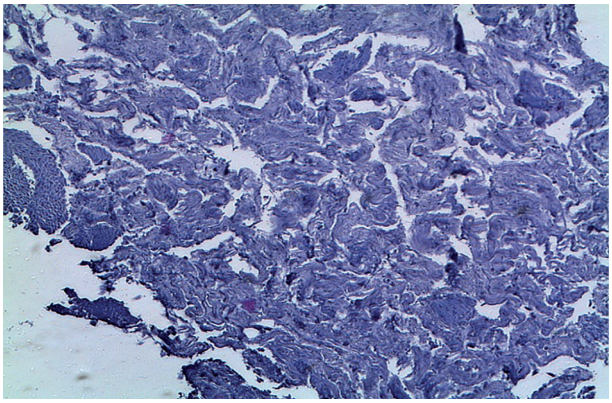
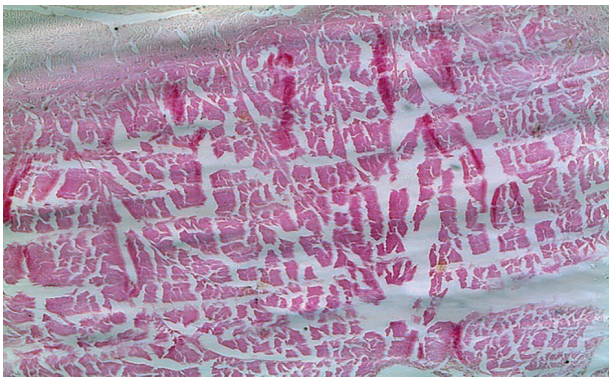


b

The gizzards of brood chickens. Van Gieson staining



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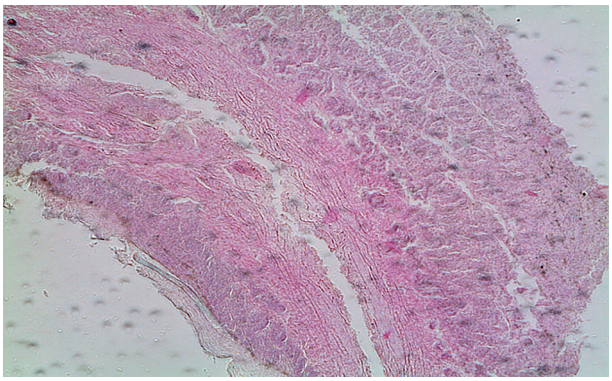
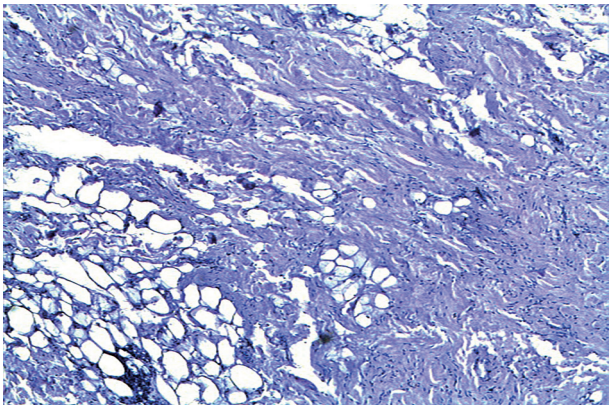


d

Combs of brood chickens. Hematoxylin-eosin staining



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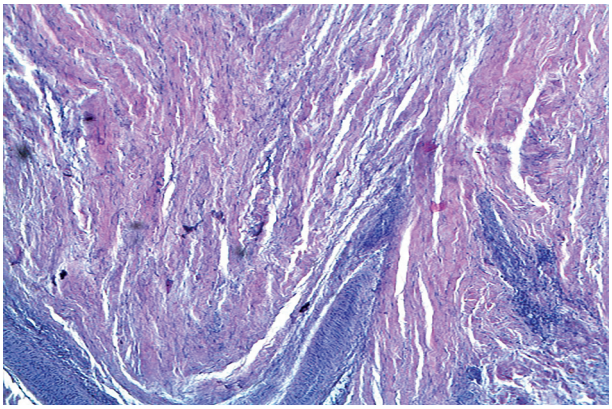

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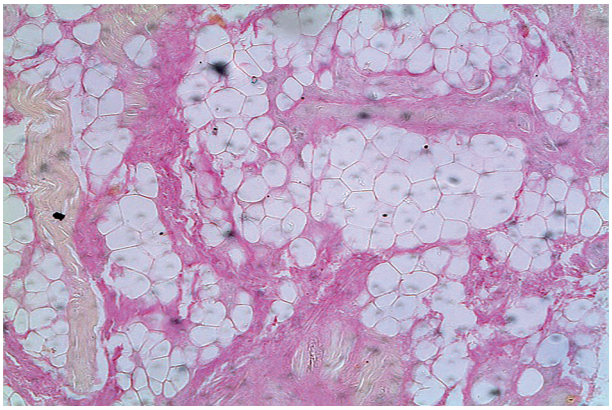
Combs of brood chickens. Van Gieson staining



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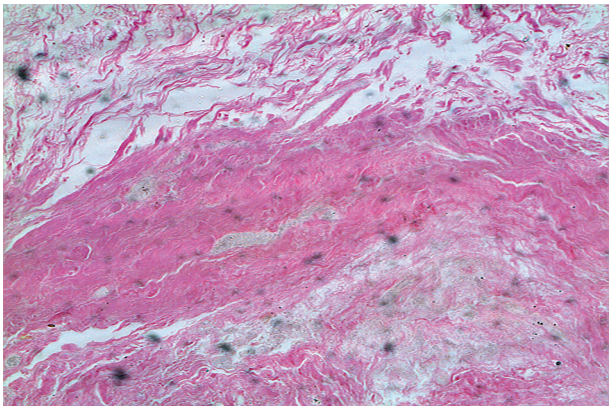

h

Figure 11. Results of histological examination of gizzards and combs of brood chickens: gizzards of brood chickens stained with hematoxylin-eosin: a) G-C, b) G-Pro-15; gizzards of brood chickens stained by Van Gieson method: c) G-C, d) G-Pro-15; combs of brood chickens stained with hematoxylin-eosin: e) C–C, f) C-Pro-15; combs of brood chickens stained by Van Gieson method: g) C–C, h) C-Pro-15

Histological preparations of the combs in the control sample are represented by fragments of folded skin with a thin *stratum corneum*. Cells and their nuclei are clearly visible, with good absorption of histological stains (Figure 1le). The extracellular matrix is represented by randomly located collagen fibers. In the deep layers, the layers of fat tissue are visible, which are separated by thick connective tissue septa, consisting mainly of compactly grouped collagen fibers. The areas free of collagen fibers (bundles of smooth muscle fibers) are colored yellow with picric acid (Figure 1lg).

In the samples of combs hydrolyzed with propionic acid bacteria, in comparison with the control sample, the attention is drawn to the reduced absorption of histological dyes, as well as the "blurring" of the connective matrix and the boundaries of cellular elements (Figure 1lf). When stained with picrofuchsin according to Van Gieson method, the test samples show a diffuse pinkish-crimson staining in all fields of view (Figure 1lh).

As a result of a morphometric study, the regularity was established for a decrease in both the specific area of connective tissue and the diameter of collagen fiber bundles for samples of combs and gizzards hydrolyzed by enzymes of the bacterial concentrate. A decrease in muscle fiber thickness was also noted in gizzard samples (Table 2). The data obtained are due to the proteolytic activity of enzymes of propionic acid bacteria, which are capable of hydrolyzing both muscle and connective tissue proteins. Also, the change in the structure of muscle and collagen fibers is influenced by lactic and propionic acids, which are the products of the metabolism of propionic acid bacteria. As noted by authors Aktas & Kaya (2001), lactic acid is harmful to animal tissues [22].

Conclusion

When the by-products of brood chickens are processed with propionic acid bacteria, this enzymatic processing caused significant changes in the structural and mechanical, functional and technological parameters, as well as in microstructure of the samples. The values of moisture-binding capacity, water-holding capacity were significantly decreased, while the flexibility and general deformation of fermented gizzards and combs of brood chickens increased due to intensive hydrolysis of proteins chains being exposed to action of microbial enzymes. These regularities must be taken into account when using the biotechnological method of preliminary enzymatic processing of raw meat materials during production of various meat foods.

Table 2. Results of morphometric analysis of gizzards and combs of brood chickens

Parameters	G-C	G-Pro-15	C–C	C-Pro-15
Specific area of connective tissue (%)	61.43 ± 1.922	33.80 ± 1.981	59.22 ± 3.067	43.77 ± 1.742
Average thickness of collagen fiber bundles (μm)	15.36 ± 3.056	6.65 ± 0.953	19.90 ± 3.006	7.18 ± 2.492
Average thickness of myocytes (μm)	15.59 ± 1.856	9.98 ± 1.395	—	—

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EXPOSURE ASSESSMENT TO ESSENTIAL ELEMENTS THROUGH THE CONSUMPTION OF CANNED FISH IN SERBIA

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Keywords: *exposure assessment, Monte Carlo analysis, essential elements, estimated daily intake, canned fish*

Abstract

The aim of this study was to provide a quantitative exposure assessment to essential elements through the consumption of canned fish in Serbia. This objective was fulfilled by analyzing content of essential elements in canned fish and by using data from a food consumption survey. Consumption survey of canned fish was designed and performed to general principles and EFSA guidelines on data collection of national food consumption. The questionnaire was performed on 1,000 respondents during 2018. Determination of copper, zinc and iron levels were performed on 454 canned fish and seafood samples divided into four groups (canned tuna, canned sardines, canned other sea fish and canned seafood) during five consecutive years (2014–2018). This study showed significant association between sex, BMI and weight and consumption patterns. Obtained average weekly consumption of canned fish confirms our assumption that consumption of canned fish is significant in Serbia. Zinc and iron were found in all 454 samples (100%), and copper in 222 samples (48.9%). The average obtained concentration in all samples were 1.268 mg kg^{-1} for Cu, 5.661 mg kg^{-1} for Zn and 9.556 mg kg^{-1} for Fe. The highest concentration for all three minerals were found in canned sardines (Cu — 6.49 mg kg^{-1}, Zn — 37.2 mg kg^{-1} and Fe — 21.8 mg kg^{-1}). Obtained mean exposure to intake of copper, zinc and iron from canned fish was 1.2241 μg/kg bw/day, 5.4634 μg/kg bw/day and 9.2231 μg/kg bw/day, respectively. Exposure of Serbian population to zinc, copper, and iron through consumption of canned fish is less than recommended daily reference intakes and there is no risk of reaching toxic levels by consuming fish.

Introduction

It is considered that eating fish has beneficial impact on human health. It is reach in omega-3 fatty acids, highly digestible good quality proteins containing all the essential amino acids and liposoluble vitamins (A and D), as well as vitamin B_{12} [1]. Amounts of essential elements in fish, such as zinc, copper, and iron, are significant [2]. Although copper, zinc and iron have important biochemical functions, their excess can cause adverse health effects, as well as deficit. Copper and zinc are present in numerous metalloenzymes and co-factors [3]. Copper is present in numerous oxidative stress-related enzymes and enzymes involved in redox system [4]. However, copper generates superoxide and hydroxyl radicals, which are toxic and excessive exposure can lead to cellular damage. Copper intoxication could be related to Wilson's disease and development of Alzheimer's disease [5,6]. Zinc is involved in signal conversion and gen expression. If there is excess or deficit of zinc in the organism, it may affect cell function and multiplication, threatening cell survival, which may lead to disease [7]. Iron has an important role in major metabolic processes in the body, such as oxygen transfer, electron transfer and DNA synthesis [8]. Excess levels of iron in human body can lead to liver damage, and can affect pancreas, heart, and lungs. It can lead to hormonal irregularities, diabetes mellitus, pancreatic hypertrophy and other health disorders [9].

Whether fish can be considered a significant source of zinc, copper, and iron? To obtain such an information it is needed to perform exposure assessment to these elements through fish consumption. Levels of exposure of population depends on fish consumption pattern and levels of these essential elements in fishery products in the local markets. Since Serbia does not have a developed fish production industry, mainly imported fish is consumed. Canned fish is cheaper, and it can be assumed that it is widely consumed. There is publication of essential elements exposure of Serbian population from canned fish consumption, but using SORS (Statistical Office Of Republic Serbia) data [10]. SORS includes only official data of imported fish, which may differ from real consumption. However, to estimate real exposure of the population to essential elements through canned fish consumption, it is needed to perform valid canned fish consumption survey. In order to obtain relevant data on food consumption, EFSA has issued a guide [11] which provides general principles on the collection of data on food consumption at the national level.

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The aim of this study was to provide a quantitative exposure assessment to essential elements through the consumption of canned fish in Serbia. This objective was fulfilled by analyzing content of essential elements in canned fish in period of five years (2014–2018) and by using data from a food consumption survey performed during 2018.

Objects and methods

Consumption of fish and seafood

Consumption survey of canned fish was designed and performed to general principles and EFSA guidelines on data collection of national food consumption [11]. The questionnaire was performed on 1,000 respondents. The tested population was similar in gender and age to the Serbian population. Average body weight of all interviewees was 71.3 kg which corresponds to recommendation given by EFSA to take 70 kg as average body weight of an adult European citizen, when unknown [12]. Demographic profile of tested population is given in Table 1. The questionnaire was done anonymously during 2018 and all respondents were informed of the purpose of the survey. After excluding the respondents that do not consume canned fish (155), for further analyses left 845 fully answered questionnaires.

Table 1. Demographic profile of the sample (N=845)

Gender	Male	366 (43.3%)
	Female	479 (56.7%)
Age	Less than 24 years	105 (12.43%)
	25–34 years	154 (18.22%)
	35–49 years	237 (28.05%)
	50–64 years	240 (28.40%)
	Over 65 years	109 (12.90%)
Weight	Below 70 kg	446 (52.8%)
	Above 70 kg	399 (47.2%)
BMI	14.5 ≤ BMI ≤ 24.9	528 (62.5%)
	BMI ≥ 24.9	317 (37.5%)
Average body weight [kg]		71.3 ± 10.4
Average weekly consumption [g]		228.8 ± 210.9

n — represents the number of respondents; (%) represents their share in the sample.

The questionnaire consisted of three sections. In the first section respondents were asked about general demographic information (sex, age, weight and height, and BMI was calculated later as BMI = weight/height^2). The second section included questions about frequency of consumption of canned fish. In the third section the respondents were asked to recall their consumption of canned fish in the last seven days and to state the amount of consumed canned fish (in grams) in four defined categories (canned tuna, canned sardines, other canned sea fish or canned seafood). By choosing 7-day recall instead of 1-day recall, authors tried to avoid bias from dietary habits, according to EFSA recommendations.

Samples

Analyses were performed on 454 canned fish and seafood samples divided into four groups (canned tuna, canned sardines, canned other sea fish and canned seafood) during five consecutive years (2014–2018).

Chemicals and Standards

The chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany): nitric acid 65% and hydrogen peroxide 30% (for analysis EMSURE® ISO). Deionized water (electrical resistivity 18.2 MΩ cm^{-1}) was obtained using the Simplicity® water purification system (Merck Millipore, Burlington MA, USA). For the quantification of copper, zinc, and iron certified standards were used (Certipur®, Merck, Darmstadt, Germany) in concentrations of 1000 μg mL^{-1}. For quality control of the analytical procedure the certified reference material DORM-2 (dogfish muscle, NRC Canada) was used.

Sample preparation

After homogenization, test portion of about 0.5 g were used for further analyses. Into polytetrafluoroethylene (PTFE) vessels with test portions, 7 mL of nitric acid and 2 mL of hydrogen peroxide were added. Samples were mineralized in a microwave closed digestion system (Ethos Touch, Milestone, Italy). The following temperature program was used: heating up to 180 °C for 15 min, followed by heating up to 220 °C for 15 min, and then heating up to 240 °C for 10 min, with a maximum power of 1000 W. After digestion, solutions were quantitatively transferred into 50 mL volumetric flasks and diluted with deionized water.

Instrumentation and analytical procedure

Content of copper, zinc and iron was determined according EN14084:2003 [13]. Flame atomic absorption spectrometer (932 plus, GBC, Australia) was used.

Method validation and assurance of the quality of the results

Validation of analytical procedure was performed according ISO/IEC17025:2017 [14] requirements, by determination of the following parameters: linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) (Table 2). Certified reference material (DORM-2, NRC Canada) was used for determination of accuracy and precision. Linearity is expressed as a correlation coefficient (r^2), accuracy as recovery, and precision as relative standard deviation in repeatability conditions (RSD_r), and in reproducibility conditions (RSD_R). LOD and LOQ were expressed as the analyte concentration corresponding to 3 times and 10 times, respectively, the standard deviation (SD) of 10 sample blanks.

Table 2. Validation parameters

Parameter	Linearity (R^2)	Accuracy (recovery)	Precision		LOD (mg kg^{-1})	LOQ (mg kg^{-1})
			RSDr	RSD_R		
Cu	1.00	95%	4.7%	5.9%	0.30	1.00
Zn	1.00	90%	4.3%	6.6%	0.25	1.00
Fe	0.99	102%	4.4%	8.4%	0.30	1.00

Assurance of the quality of the results were provided by analyzing CRM (DORM-2, NRC Canada) within every sample set. The obtained results were monitored through control charts. If recovery of CRM was within satisfactory range, results of all samples in the sample set were accepted, if it was not, all samples were analyzed again.

Exposure estimation

Obtained results from the analyses of levels of copper, zinc and iron in canned fish and results from the canned fish consumption survey were combined to estimate exposure of Serbian population to these elements through eating canned fish. Estimation was calculated according following equation [15]:

$$EDI = \frac{\sum_{i=1}^{n} F_i}{7} \times \frac{1}{bw} \times C_t$$

EDI is the estimated daily intake of Cu/Zn/Fe [μg/kg bw/day]. F_i is the amount of canned fish consumed weekly [kg]. Body weight (bw) is taken to be 70 kg as recommended by EFSA for adult European citizen (EFSA, 2012a). C_t is the concentration of Cu/Zn/Fe [μg/kg].

To assess exposure, with including the variability of the population, the Monte Carlo simulation, as one of the most common methods [16], was used.

Statistical methods

Canned fish consumption patterns of demographic groups (defined by sex, age, weight, and BMI) were compared by Chi-square test for association. An independent sample t-test was used for analyzing average consumption with statistical significance of 0.05.

For estimation the intake of copper, zinc and iron from canned fish consumption, the Monte Carlo analysis of 100,000 simulations was used. Calculating levels of Cu/Zn/Fe was based on their mean values (Table 4). According FAO/WHO [17] it can be assumed that an individual exposure over time is equal to this level.

Minitab was used for probability distribution fitting for body weight and weekly intake of canned fish and for Monte Carlo simulation. For fitting of the probability distributions visual analysis was equally considered [18].

Results and discussion

Consumption of canned fish

Frequency consumption patterns for canned fish obtained from consumption survey are given in Table 3. Obtained average weekly consumption of canned fish is 228.8 g (Table 1). It confirms our assumption that consumption of canned fish is significant in Serbia.

Relationships between canned fish consumption patterns and demographic characteristics of the sample were tested (Chi-square test for association). On average, depending on the demographic group, consumption on a weekly basis was confirmed by 26.3% to 62.0% of interviewed consumers. This study showed significant association between sex, BMI and weight and consumption patterns ($p < 0.05$). Male respondents, respondents with weight over 70 kg and with BMI >24.9 eat canned fish more frequently than females, consumers with weight below 70 kg and with "ideal" BMI. There were no

Table 3. Frequency of consumption of canned fish (tuna, sardine, sea fish, seafood)

Gender	On a weekly basis	On a monthly basis	Once a year or less	Total
Female	126 (26.3%)	49 (10.2%)	304 (63.5%)	479 (100%)
Male	227 (62.0%)	33 (9.0%)	106 (29.0%)	366 (100%)
$\chi^2 = 114.557$; $p < 0.05$				
Below 70 kg	134 (30.1%)	43 (9.6%)	269 (60.3%)	446 (100%)
Above 70 kg	219 (54.9%)	39 (9.8%)	141 (35.3%)	399 (100%)
$\chi^2 = 58.189$; $p < 0.05$				
BMI (14–5–24.9)	201 (38.1%)	48 (9.1%)	279 (52.8%)	528 (100%)
BMI<14.5 / BMI>24.9	152 (47.9%)	34 (10.7%)	131 (41.3%)	317 (100%)
$\chi^2 = 10.589$; $p < 0.05$				
Below 35	103 (39.9%)	23 (8.9%)	132 (51.2%)	258 (100%)
35–49	104 (43.7%)	26 (10.9%)	108 (45.4%)	238 (100%)
Above 50	146 (41.8%)	33 (9.5%)	170 (48.7%)	349 (100%)
$\chi^2 = 1.834$; $p > 0.05$				

Table 4. Concentration of essential elements in canned fish

		Canned Tuna	Canned sardine	Canned sea fish	Canned seafood	ALL
Copper	Number of samples (positive samples)	276 (69)	122 (105)	54 (47)	2 (1)	454 (222)
	Mean [mg kg^{-1}]	0.769	2.100	1.931	1.565	1.268
	Range [mg kg^{-1}]	0.5–3.08	0.5–6.49	0.5–3.11	0.5–2.63	0.5–6.49
Zinc	Number of samples (positive samples)	276 (276)	122 (122)	54 (54)	2 (2)	454 (454)
	Mean [mg kg^{-1}]	4.824	7.382	6.016	6.395	5.661
	Range [mg kg^{-1}]	1.92–8.7	2.9–37.2	2.61–8.64	4.11–8.68	1.92–37.2
Iron	Number of samples (positive samples)	276 (276)	122 (122)	54 (54)	2 (2)	454 (454)
	Mean [mg kg^{-1}]	8.401	11.607	10.848	9.005	9.556
	Range [mg kg^{-1}]	1.3–13.9	2.71–21.8	2.11–15.4	6.91–11.1	1.3–21.8

statistically significant association between age groups ($p > 0.05$).

Levels of copper, zinc, and iron in canned fish

Copper, zinc, and iron determination were done in 452 samples of canned fish and 2 samples of canned seafood. Obtained concentrations of copper, zinc and iron in canned fish and seafood are given in Table 4.

Zinc and iron were found in all 454 samples (100%), and copper in 222 samples (48.9%). Values of copper below the LOQ were substituted with a constant value of LOQ/2 for further analyses, as it is recommended [19]. Monte Carlo uncertainty analysis of 95% CI of the mean values shows that such results have little effect on the upper percentile exposures [20]. The average obtained concentration in all samples were 1.268 mg kg^{-1} for Cu, 5.661 mg kg^{-1} for Zn, and 9.556 mg kg^{-1} for Fe. The highest concentration for all of three minerals were found in canned sardines (Cu - 6.49 mg kg^{-1}, Zn — 37.2 mg kg^{-1}, and Fe — 21.8 mg kg^{-1}), as well as the highest average concentrations (Cu 2.100 mg kg^{-1}, Zn 7.382 mg kg^{-1}, and Fe 11.607 mg kg^{-1}).

There are two published investigations of levels of Cu, Zn and Fe in canned fish from Serbian local markets. Popovic et al. [10] performed analyses in 207 samples of canned tuna, sardines and mackerel and Novakov et al. [21] in 98 samples of canned tuna, sardines and sprouts. Popovic et al. [10] found the highest average concentration for all the three minerals in canned sardines, which was in line with our results. Found levels (Cu – 1.28/1.37 mg kg^{-1} oil/tomato sauce, Zn — 15.1/14.05 mg kg^{-1}, and Fe — 13.8/16.78 mg kg^{-1}) were lower than ours for Cu, but significantly higher for Zn and Fe. On the other hand, Novakov et al. [21] reported higher levels of Cu and Zn in canned tuna (2.60 mg kg^{-1}, 21.96 mg kg^{-1}, respectively) than in canned sardines (2.49 mg kg^{-1}, 18.21 mg kg^{-1}, respectively), but higher level of Fe in canned sardines (21.98 mg kg^{-1}) then in canned tuna (20.36 mg kg^{-1}). All results are significantly higher than ours, except for Cu, which is in line for canned sardines and little higher in canned tuna.

Authors from other countries reported different levels of the elements. Lower levels of Cu in canned sardines are reported by authors from Croatia (0.88 mg kg^{-1}) [22], Spain (0.513–0.898 mg kg^{-1}) [23], Turkey (1.024 mg kg^{-1}) [24], Iraq (0.7–2.1 mg kg^{-1}) [25], and Nigeria (0.01 mg kg^{-1}) [26], but results from USA (0.83 mg kg^{-1}) [27], and Brazil (1.31–2.25 mg kg^{-1}) [28] are in line with our results. In canned tuna, authors from Spain (0.483 mg kg^{-1}) [23], Turkey (0.604 mg kg^{-1}) [24], and USA (0.25 mg kg^{-1}) [25] found levels lower than ours. Reported levels of Zn in canned sardines are higher than ours from Croatia (18 mg kg^{-1}) [22], Turkey (23.267 mg kg^{-1}) [24], USA (11.45 mg kg^{-1}) [27], and Brazil (16.16–36.09 mg kg^{-1}) [28], and lower than ours from Spain (4.329–6.767 mg kg^{-1}) [23], and Nigeria (0.09–4.49 mg kg^{-1}) [26]. Regarding canned tuna, levels of Zn reported by authors from Turkey (10.802 mg kg^{-1}) [24] and Lebanon (7.49 mg kg^{-1}) [29] are higher, from Spain (2.27 mg kg^{-1}) [23] is lower, and from USA (4.78 mg kg^{-1}) [27] is in line with our results. Levels of Fe found in canned sardines by authors from Croatia (19 mg kg^{-1}) [22], Turkey [24], Iraq [25] and Brazil [28] are higher (22.162 mg kg^{-1}, 20–30 mg kg^{-1} and 20.96–88.83 mg kg^{-1}, respectively), and from Nigeria [26] (8.04–48.18 mg kg^{-1}), and USA [27] (12.7 mg kg^{-1}) in line with ours. In canned tuna reported level for Fe by authors from USA (15.8 mg kg^{-1}) [28] is higher, and from Turkey (8.105 mg kg^{-1}) [24] is in line with our result.

Exposure assessment

According to our research on the level of minerals in canned fish and eating habits of the Serbian population, mean estimated daily intake of copper, zinc and iron in canned fish were 1.2241 μg/kg bw/day, 5.4634 μg/kg bw/day, and 9.2231 μg/kg bw/day, respectively (Table 5). Estimated total daily intake of the three minerals after a Monte Carlo analysis of 100,000 simulations is shown in Figures 1a-c.

Table 5. Estimated daily intake of copper, zinc and iron in canned fish

	Copper [μg/kg bw/d]	Zinc [μg/kg bw/d]	Iron [μg/kg bw/d]
Mean	**1.2241**	**5.4634**	**9.2231**
5th percentile	**0.454**	**2.043**	**3.373**
1st quartile	**0.763**	**3.404**	**5.746**
3rd quartile	**1.549**	**6.913**	**11.669**
95th percentile	**3.586**	**16.201**	**26.936**
95% confidence interval of mean	**1.2203–1.2279**	**5.4465–5.4803**	**9.1946–9.2516**

All values are derived from a Monte Carlo simulation.

Similar investigation of contribution of Cu, Zn and Fe from fish to dietary intake in Serbia was performed by Popovic et al. [10]. Investigation included canned fish (tuna, sardines, and mackerel in oil and in tomato sauce). Results were expressed as%RDA for men and women for each type of fish separately. Reported EDI for Cu were in range 0.28–0.46%RDA, EDI for Zn were in range 0.21–0.57%RDA and EDI for Fe were in range 0.14–0.64%RDA. The authors concluded that levels of Cu, Zn and Fe in canned fish do not represent any health risk, and even do not represent important dietary source of Cu, Zn and Fe. Reported EDI of Cu (1.671 μg/kg bw/day) and Zn (6.629 μg/kg bw/day) in fish from Spain, were slightly higher than our results [30].

Provisional maximum tolerable daily intake (PMTDI) established by JECFA for Cu is 0.5 mg/kg bw/day [31], for Zn is 0.3–1 mg/kg bw/day [32] and for Fe is 0.8 mg/kg bw/day [33]. According Regulation EU EC [34], recommended daily reference intakes for Cu, Zn and Fe for adults are 1 mg (i. e. 14.286 μg/kg bw/d), 10 mg (i. e. 142.857 μg/kg bw/d) and 14 mg (i. e. 200.000 μg/kg bw/d), respectively. According to our results mean estimated daily intake of the minerals from canned fish is below recommended levels (0.175% RDA Cu, 7.805% RDA Zn and 18.446% RDA Fe). Moreover, in the «best case scenario», if we take into account the values for the 95th percentile, the population does not get enough of these minerals through canned fish. Obtained values for 95th percentile are 0.025% RDA for Cu, 0.011% RDA for Zn and 0.013% RDA for Fe.

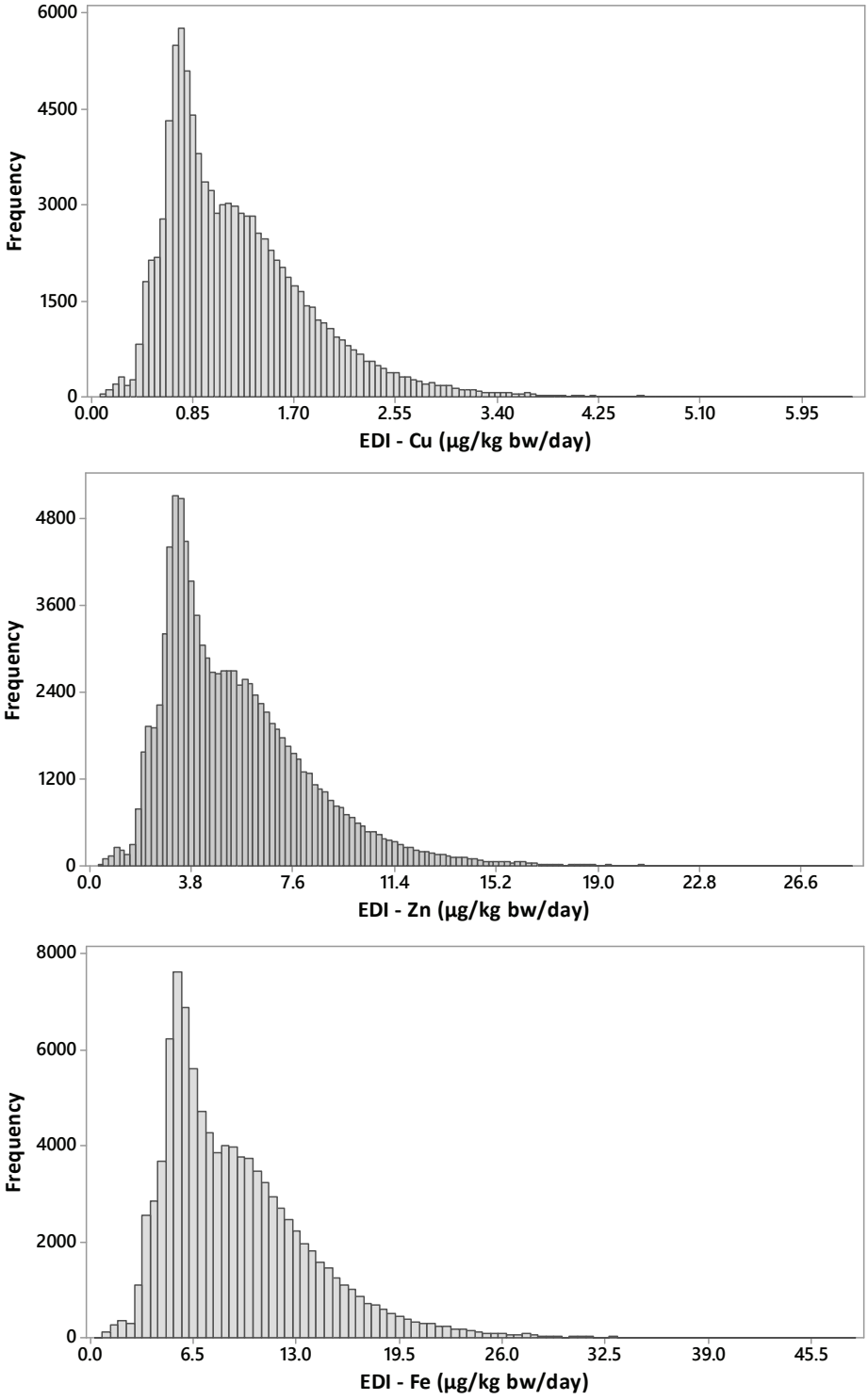

Figure 1. Estimated total daily intake of Copper, Zinc, and Iron after a Monte Carlo analysis of 100,000 simulations

Obtained results indicates that there is no risk of exposure to the toxic values of copper, zinc and iron through canned fish consumption and, moreover, canned fish does not represent source of these three essential minerals.

Conclusion

Consumption of canned fish is significant in Serbia (228.8 g/w). On average, depending on the demographic group, consumption on a weekly basis was confirmed by 26.3% to 62.0% of interviewed consumers. Male respondents, respondents with weight over 70 kg and with BMI > 24.9 eat canned fish more frequently than females, consumers with weight below 70 kg and with "ideal" BMI. There were no statistically significant association between age groups.

Copper was detected in 49% of tested samples, where zinc and iron were found in all the tested samples. The highest levels of all three essential elements were found in canned sardines.

Exposure of Serbian population to zinc, copper, and iron through consumption of canned fish is less than recommended daily reference intakes and there is no risk of reaching toxic levels by consuming fish.

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COMPARATIVE STUDY OF TECHNOLOGIES FOR EXTRACTION OF BIOLOGICALLY ACTIVE SUBSTANCES FROM THE RAW MATERIAL OF ANIMAL ORIGIN

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Keywords: *extraction, pH, pancreas, low molecular weight proteins, porcine*

Abstract
Technologies of isolation and concentration of biologically active substances, developed in the middle of the 20th century, need adjustment and adaptation to modern conditions both to increase the activity of substances and for greater economic efficiency. The aim of the research is the comparison of dynamics of biologically active compounds extraction from porcines pancreas in two methods: the saline method based on 0.9% sodium chloride solution, and the acidic method based on 2.4% trichloroacetic acid solution. Also the purpose of research is to assess the possibilities for further optimization of technologies. The total protein concentration based on the biuret reaction in the samples taken during the extraction, as well as the calculation and analysis of the point degrees and rates of extraction are chosen as the controlled parameters. Local maxima of the protein yields into the extractant media at the 60th, 135th and 255th minute were recorded during saline extraction; and at the 75th and 135th minute during acid extraction. Also the proteomic profile of the extracts was studied. Wide range of compounds with molecular weight of less than 52 kDa was found in extracts based on physiological saline solution, and protein substances of whole presented range of molecular weights in trichloroacetic acid based extracts were considered. The predominance of low molecular weight protein fraction of interest was noted also in this method of extraction in comparison with the other methods of extraction. According to the UniProt database, we assume availability of probable compounds with a molecular weight of less than 30 kDa in the purified acidic extract. The presence of some proteins absent in the final saline extract was noted. The acidic erythrograms showed a weak degrading effect of both types of extracts on the membranes of rat erythrocytes, as well as the cytoprotective effect of acidic ultrafiltrates (less than 3 kDa). The obtained results prove a better efficiency of trichloroacetic acid extraction method used for obtaining a mixture of a wide range of compounds, including biologically active substances of low molecular weight.

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Introduction

The study and optimization of technologies for isolation of biologically active compounds from animal raw materials are today one of the most important aspects of modern biotechnology. Along with the fast development of the analytical chemistry and biochemistry spheres, the technological execution of methods, aimed to supervision and control of the observed processes, requires timely improvement to match the progress. In this regard, the permissible limits of sensitivity of the detection devices are improved, devices are designed with functional possibility to run combined experiments (for example, HPLC with a diode-matrix UV-visible spectrophotometric detector (DMD), fluorimetric MS-detector, capillary electrophoresis with DMD [1], low molecular weight electrophoresis combined with mass spectrometry of inductively coupled plasma [2]). The databases of discovered compounds are being replenished more and more (for example, refer to the UniProt protein database) [3]. The early studies, replicated on the currently obsolete equipment, were as accurate as it was possible for that time. Technologies based on such researches are still used today. By the way, these technologies often feature high cost, low efficiency and high labor intensity. In order to potentially reduce the economic costs of production, as well as to reduce the volume of waste, the dynamics of the process flow shall be studied in order to identify possible ways to optimize the methods.

In the 1920s insulin, pancreatin, glucagon, obtained from animal pancreas extracts, were patented. Later, the development of technologies for production of certain medical preparations of enzymes and hormones, extracted from porcine pancreas, went on. Pigs were used as a source of a whole range of important biologically active substances, and in the 30s of the last century, traditional approaches to the extraction of some specific compounds or their mixtures from the pancreas were developed. Those achievements serve as the basis of modern technologies [4,5]. Due to the rapid development of the technological execution of necessary compounds isolation, it can be assumed that the potential of this raw material is not fully achieved.

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Among the variety of extraction methods used to obtain biologically active substances from porcine pancreas, one can distinguish method of saline solutions (sodium and calcium salts), method of acidic solutions based on weak and strong acids, method of alcoholic solution based on ethyl alcohol, incl. the method of bismaceration [6]. Each of the method presented above has its specific advantages. The main advantage of physiological saline extraction is the absence of aggressive agents and the proximity of the conditions to natural, which matters for the recipient organism. Acid extraction by trichloroacetic acid (TCA) provides an acidic reaction medium, which contributes to inactivation of the main proteolytic enzymes of the pancreas and inhibits autolysis. Alcohol extraction allows achieving a better degree of the active substance extraction due to the partial disintegration of tissues exposed to organic solvent (ethanol). The disadvantage of technologies that include the use of acids or organic solvents is the necessity to remove them from the obtained ready extract. Despite the obvious difficulties related to this task, those technologies are still often used in production of medical preparations based on an extract of the pancreas, since they allow obtaining a higher yield of specific compounds. Alcohol extraction technology, which is often used to obtain insulin, can be considered as potential for further study in the prospect of comparing it with the methods discussed in this article.

At this stage it is of scientific interest to compare the methods of saline extraction and acid extraction. First of all, this is reasoned by very different conditions of the process described above, namely — the acidity of the medium, duration of the extraction, nature of the extractants, and the purification stage. The technologies under consideration also allow running the process at low temperatures for a long time, which has a positive effect on the results of the analysis of the protein composition and provides a better yield of native low molecular weight compounds.

The aim of the article is to study and compare the saline and acidic method of extraction of biologically active compounds of protein range from porcine pancreas, as well as to determine the efficiency of the considered technologies for obtaining a highly active complex of substances rich in low molecular weight components.

The absence of aggressive conditions during extraction process in physiological saline medium implies a higher content of protein substances in the extracts, which are also rich in the low-molecular fraction, which was partially replenished during the extraction as a result of the action of proteases. A high activity of low molecular weight fraction in the acid ultrafiltrate is expected. This is primarily caused by decrease in proteolytic enzymes activity in an acidic medium, as well as by the additional stage of purification from ballast inactive proteins.

Objects and methods

The object of the study was porcine pancreas *Sus scrofa*, sampled at Limited Liability Company "Pushkinskiy Dvor" (Russia, Pushkino). The raw materials were subjected to extraction by two methods: aqueous saline (physiological saline) and acidic (trichloroacetic acid), followed by study of obtained extracts and their ultrafiltrates.

The raw materials were frozen and stored at a temperature of minus (40 ± 2) °C with further thawing immediately before the study at a temperature of (22 ± 2) °C. Before extraction, the pancreas was also subjected to fine grinding with the help of a meat grinder (Kenwood, England): mesh of 100 holes with a diameter of 3 mm. The extraction scheme is shown below in Figure 1.

Saline extraction [7] was conducted in 0.9% sodium chloride solution by way of mixing of the finely chopped raw materials and an extractant in hydromodule in ratio of 1:5. The process lasted for 330 minutes. After completion of the extraction, the obtained solution was centrifuged in a Centrifuge CM-6M (ELMI, Latvia) at 3500 rpm for 8 min. at a temperature of (22 ± 2) °C. Then, the supernatant liquid, separated from the sediment, was saved for further analyses. To study the extraction process, samples were taken throughout the entire process: at 0, 5, 10, 15 minutes; and further every 15 minutes till to 330th min. Each sample was centrifuged for 5 minutes immediately after its sampling at a temperature of 4 °C on a Centrifuge 5427R (Eppendorf AG, Germany) at a speed of 3500 rpm; the supernatant liquid was frozen.

In case of acid extraction [8,9], the finely chopped raw material was loaded into the processor bowl together with an extractant — 2.4% trichloroacetic acid solution with a hydromodule of 1:3.15. The acid extraction technology also included the stage of the final extract purification from ballast proteins: primary centrifugation was run in a Centrifuge 5427R (Eppendorf AG, Germany) at 6,861 rpm for 10 minutes with cooling the extract to 4 °C; the process also included the precipitation with sodium hydroxide solution at pH 6.2–7.2. The final centrifugation was run at 3,500 rpm for 10 minutes on a Centrifuge CM-6M (ELMI, Latvia) at a temperature of (22 ± 2) °C. Extraction was conducted in the same laboratory installation, that was used for saline extraction, for 2 hours 15 minutes with sampling at 0, 5, 15 minute and then every 15 minutes till to 135 min. Each sample was centrifuged at 6,861 rpm for 10 minutes. when cooled to 4 °C. The supernatant liquid was frozen at a temperature of minus (40 ± 2) °C.

In both cases, the extraction process was conducted by the laboratory unit in LDU (Labotex, Russia) along with cooling down to (4 ± 2) °C in order to avoid autolysis, at a stirring speed of 400 rpm. The supernatant liquids of the purified extracts obtained after centrifugation were frozen at a temperature of minus (40 ± 2) °C and stored for no more than a month.

To obtain a highly active low molecular weight fraction of the extracts, the extracts underwent ultrafiltration on filters with a permeability limit of 3 kDa (Amicon, Ireland) as the final stage of the technology process. The process was carried out on a Centrifuge 5427R (Eppendorf AG, Germany) at a speed of 11,481 rpm for 20 minutes at a temperature of 4 °C.

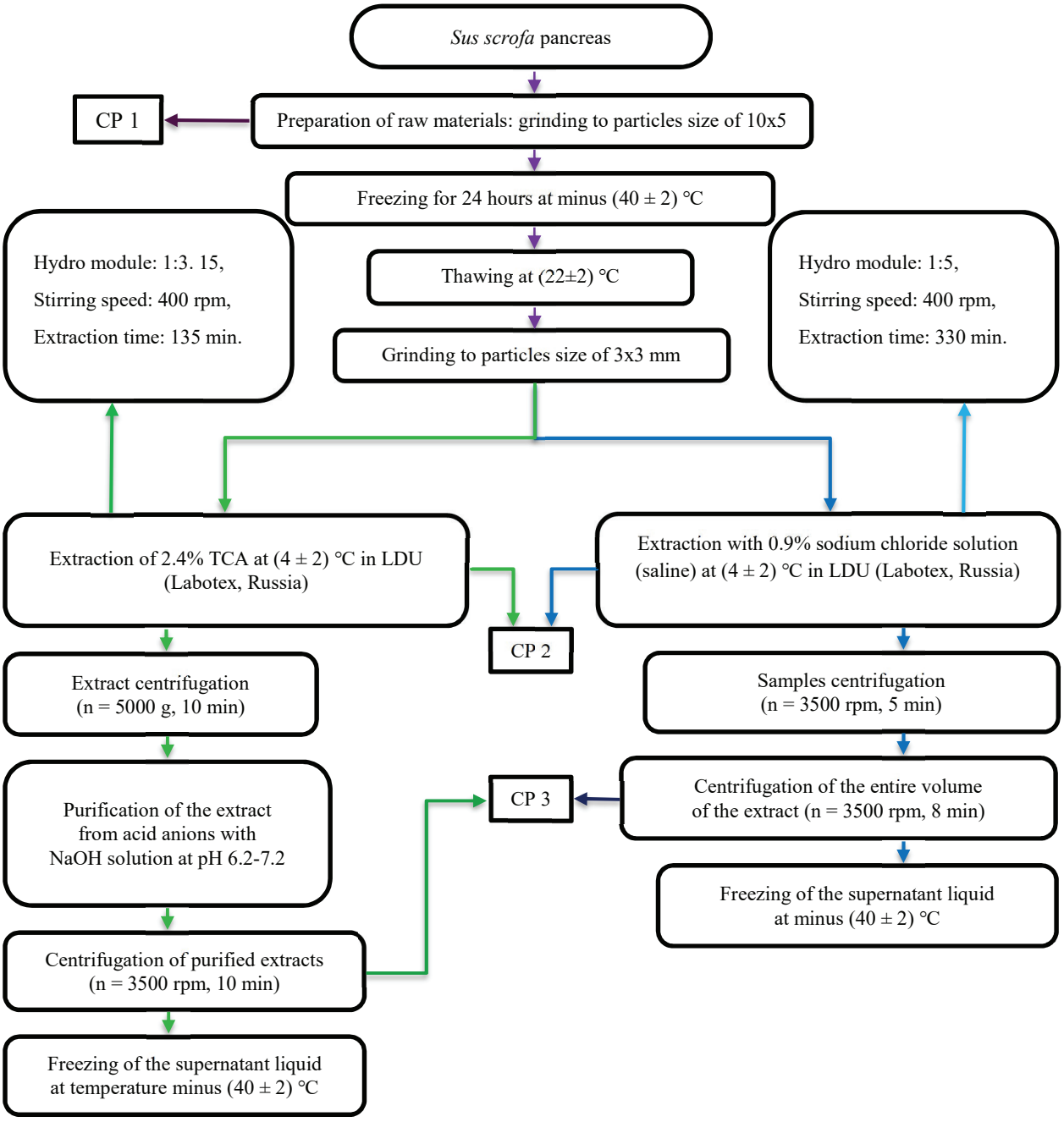

Figure 1. Scheme of extractions based on saline and trichloroacetic acid
Legend: CP1 — control point for native pancreas sampling to obtain a comparative sample (CS); CP2 is the control point for sampling of extracts to study the dynamics of both extraction methods; CP3 — control point of the final extract sampling: after centrifugation of the total volume of the extract — for saline extraction; after purification and secondary centrifugation — for acid extraction

In the samples taken during the extraction, the total protein concentration, the total proteolytic activity of enzymes, and the dynamics of the yield of protein compounds were analyzed by the proteomic profile. The membranotropic effect of biologically active compounds in primary purified extracts, as well as in their ultrafiltrates were studied.

Sample of the raw material (*pancreas native* — further PN) were prepared in a known way [7].

The total protein concentration and ultrafiltrates in the extracts were measured on the basis of the biuret reaction according to the Kingsley-Weixelbaum method [10]: 600 µl of the biuret reagent was incubated with the test sample, and after 10 minutes the optical density of the solution was measured at 540 nm by a photometer BioChem SA (HTI, USA).

On the basis of parameters of protein yield to the extractant the basic characteristics of the process like degree and rate of extraction were determined.

The degree of extraction was determined as the ratio of the extracted substance (protein) mass to the total mass of raw material and was calculated by the following formula:

$$D = \frac{M}{m} \times 100\,\% \qquad (1)$$

where

M — is the mass of the extracted substance, g;

m — is the total mass of the initial raw material in the mixture, g.

The point rate of extraction was determined by change in protein concentration (extracted substance) along the vector of time. It was calculated at each moment of the extraction process. Calculations were conducted by the formula:

$$v = \frac{\Delta c}{\Delta t} = \frac{dc}{dt} \quad (2)$$

where

c — is concentration of protein in extract, g/l;

t — is corresponding time of the extraction, min.

The total proteolytic activity of the final extracts was determined by Leilian-Folgard method, which included heating in a test flask up to 37 °C in a water bath of 20 ml of 5% alkaline casein solution with 10 ml of the test sample for 60 minutes. In a control flask, a solution of the same composition, immediately after mixing was precipitated first with a 0.2 N hydrochloric acid solution, and then with a 15% sodium sulfate solution. The precipitated fallout was filtered off. After one hour of sample exposure in a test flask, the same operations were carried out. Then 10 ml of the obtained filtrate was transferred into clean flasks and titrated with 0.1 N sodium hydroxide solution in presence of 1% cresol red solution till obtaining the saturated bright crimson color. The proteolytic capacity was calculated from the difference in the volumes of sodium hydroxide required for titration of the experimental sample and control sample.

Membranotropic activity of biologically active compounds in extracts and ultrafiltrates was determined by constructing acidic erythrograms of erythrocyte membrane degradation [11, 12]. 10 ml of the test sample with a protein concentration of 1000 ng / ml on the basis of 0.9% sodium chloride solution that was introduced into a test flask. The control flask contained pure physiological saline. In both cases, 20 μL of blood from rats' tails vein was added to the flasks, the contents were mixed for uniform contact of active compounds with erythrocytes and left to incubate at room temperature for 1 hour. After expiration of this time, 2 ml of both samples were transferred into clean flasks and mixed with 2 ml of 0.004 N sodium chloride-based hydrochloric acid solution. The dynamics of changes in optical density was recorded by SF-2000 spectrophotometer (Spektr, Russia) at 650 nm every 15 seconds from the moment of mixing until the end of hemolysis (when the optical density values reach a plateau), i. e. for at least 10 minutes.

The proteomic profile of the extracts was studied on the basis of electropherograms obtained by Laemmli method, i. e. by one-dimensional denaturing electrophoresis in 12.5% polyacrylamide gel in presence of SDS. The process was run in a VE-10 chamber (Helicon, USA) at a temperature of (22 ± 2) °C at 60 V, until the samples reached the boundary of the separating gel, then at 130 V until the electrophoresis was completed. Standards (Fermentas, Lithuania) were used as comparison referential markers.

The analyzed samples were primary stained with *Coomassie Brilliant Blue G-250* solution. Later the stain was removed by 10% acetic acid.

To increase the sensitivity level of this method, silver staining was performed according to Bloom's method by alternate incubation of the samples in solutions of sodium hyposulfite, of silver nitrate and sodium carbonate.

Density diagrams were obtained by the software *ImageJ* (National Institutes of Health, USA) [13] based on results of studying the proteomic profile of extracts selected during the extraction process at control points. The results were visually presented on the basis of automatic densitometric analysis of the scanned electropherograms. In order to avoid errors in study of various extractions electropherogram, the same parameters were set up. The image was converted to 8-bit type, then an area of 1280x1938 pixels was selected on the track. The molecular weight of the fractions was determined by correlating of the corresponding peaks on the density diagram of the test sample with the peaks of the standards [14,15].

The software STATISTICA 10.0 was used for statistical processing of the total protein content. The final results were calculated and presented as "mean ± standard error" (M ± SE). Significant differences were checked using one-way analysis of variance ANOVA followed by Tukey's test. The deviations, when P values didn't exceed 0.05, were considered as statistically significant [16].

Results and discussion

When measuring the total protein concentration (Figure 2) in the samples taken during the saline extraction, a sharp increase in the values from 0 to 5 minutes was observed. And the further decrease in the protein content in the samples was observed at 90th min. (21.2 ± 2.7 g/l), at 165th min. (19.6 ± 0.4 g/l), at 240th min. (19.7 ± 0.2 g / l), at 270th min. (19.5 ± 0.3 g/l). The observed maximum concentration values are explained by release of a new fraction of compounds into the extractant, for example, at 60th min. (23.1 ± 0.9 g/l), which together with the concentration value for 135th min. (23.3 ± 0.6 g/l) was the highest throughout the entire process of extraction. Minor increases in concentration were also observed at 195th and 255th min. In general, it is necessary to note the smooth way of change in the samples protein content, starting from 5th min. of the process.

TCA extraction featured a smooth increase in total protein content up to 75th minute, where it reached a maximum and amounted to 15.6 ± 0.4 g/l. A further slight decrease in concentration led to coming of protein amount, released to extractant, to a plateau. This plateau values were was observed until the very end of the extraction process.

According to the diagram of dependence of the total protein concentration on time of extraction, in case of saline extraction a higher yield of protein into the extractant is evident throughout the entire process. From the first moments of the process, a sharp increase in the amount of protein was observed in comparison with a smooth increase of protein concentration in case of acid extraction. Both methods of extractions feature an oscillatory process of the protein content changing in the extracts.

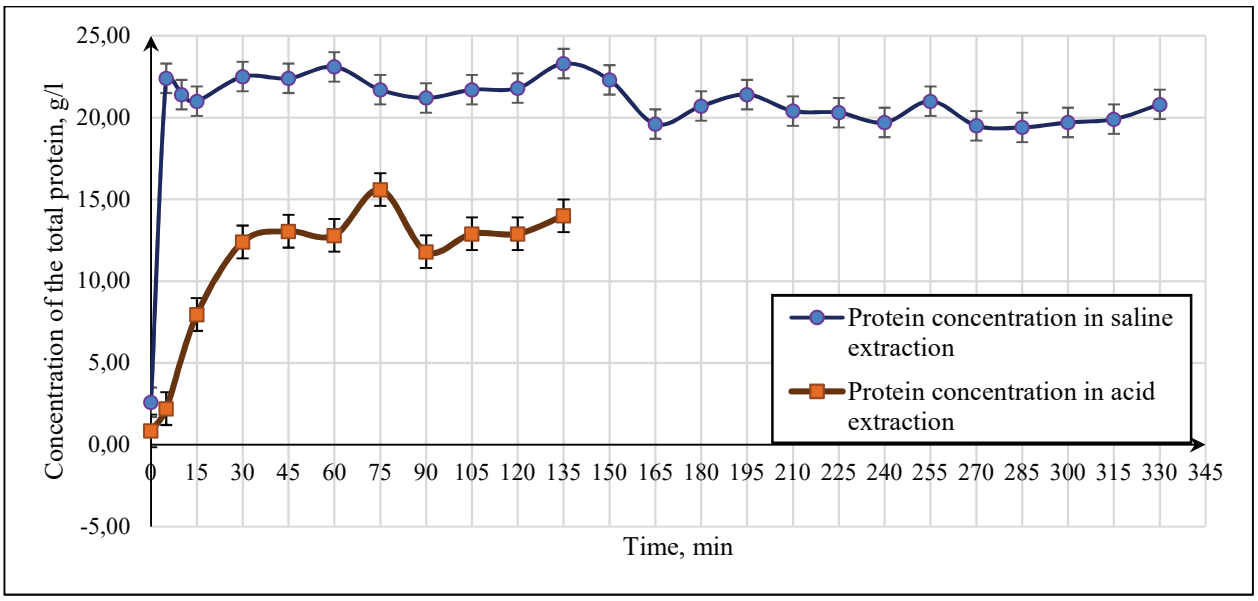

Figure 2. Diagram of the dependence of the total protein concentration in acid and saline extracts on the duration of the process

As result of processing the data on total protein concentration in the extracts, the degree (D) and point rate (v) of extraction were calculated for both methods. Diagram of the dependence of rate of proteins release into the extractant are shown below in Figure 3. It is necessary to note the high rate of saline extraction till the 5th minute, in contrast to TCA extraction, in which the rate gradually increased up to the 15th minute and then began to decrease uncritically. In both cases, the process showed an oscillatory change in the rate within the range of 0 g/(l*min), i. e. in general the release of protein into the extractant took approximately constant values with minor deviations. Thus, there was a slight decrease in rate of extraction at the 165th and 270th minutes in saline extraction, and at the 90th minute in case of TCA extraction.

The data on degree of extraction are shown above in the Table 1. It is necessary to note the correlation with the data on the total protein content in the extracts. The final degree of extraction of the protein component in the saline variant exceeded 2.25 times the value of D for acid extraction. It proves that the saline solution is more efficient as an extractant in terms of this regard. The highest degree of extraction (Table 1, light green line) was observed from the 30th till 150th minute, and at the 75th minute. (Table 1, red line) for extracts based on 0.9% sodium chloride solution and TCA, respectively.

The study of proteolytic activity of enzymes in the final extracts showed a higher value in the case of saline solution extraction and amounted to 94,276 U/ml in comparison with 33,748 U/ml for the TCA-based sample. The data indicate the best enzymatic activity in the saline extract, which is primarily associated with mild environmental conditions (neutral pH, the absence of denaturing agents that can negatively affect the active centers of enzymes, etc.). The low proteolytic capacity of the acid extract indicates an insufficient degree of purification of active enzymes from the inhibitory action of TCA, even after the completion of additional stages of purification. The reason may also be the removal of enzymes fraction together with the ballast proteins

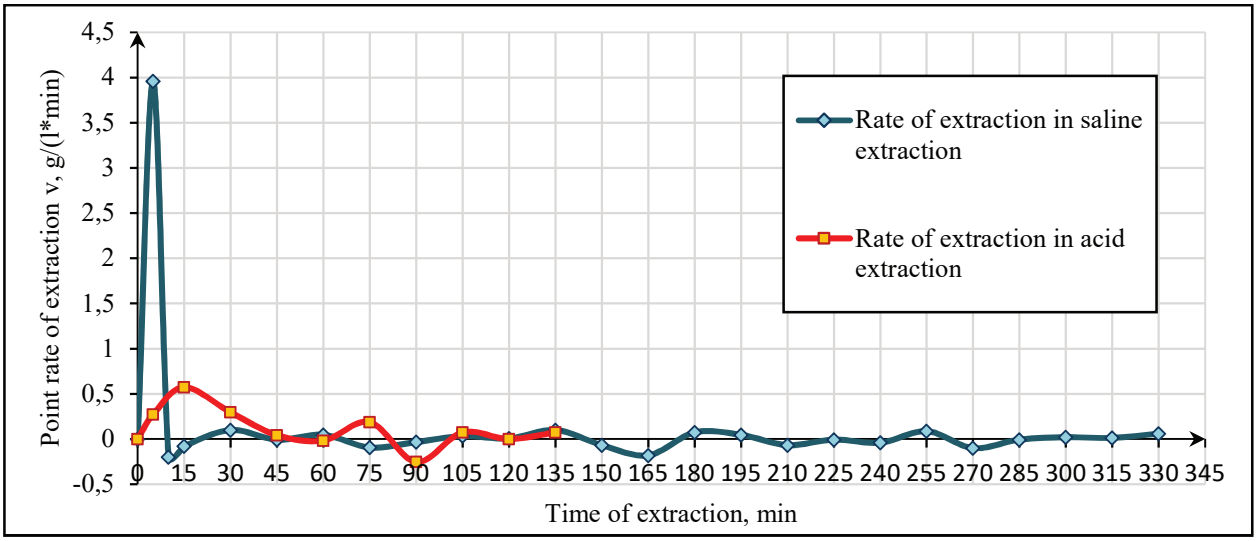

Figure 3. Diagram of the change in the point rate of extraction for saline extraction and for acid extraction

Table 1. Extraction rate of protein-peptide fraction in the process of saline and acid extraction

Saline extraction													
D, %	1.30	11.17	10.63	10.40	11.11	11.03	11.33	10.61	10.34	10.55	10.56	11.25	10.73
Time, min	0	5	10	15	30	45	60	75	90	105	120	135	150
D, %	9.40	9.90	10.20	9.69	9.61	9.30	9.88	9.14	9.06	9.17	9.23	9.62	
Time, min	165	180	195	210	225	240	255	270	285	300	315	330	
Acid extraction													
D, %	0.27	0.70	2.52	3.91	4.10	4.01	4.86	3.66	3.98	3.97	4.28		
Time, min	0	5	15	30	45	60	75	90	105	120	135		

precipitated at the stage of neutralization and centrifugation. It is necessary to keep in mind that a decrease in proteolytic activity may be a positive factor, indicating that many large and medium-sized proteins, that avoided the undesirable process of autolysis, are preserved in their native form.

To obtain information on the biological activity of the low molecular weight fraction, ultrafiltration was conducted. The data on the total protein concentration in the final investigated extracts and their ultrafiltrates are presented below in the Table 2.

Table 2. Total protein concentration in extracts before and after ultrafiltration

Extraction variation	Total protein concentration, g / l	
	Final extract	Ultrafiltrate
Saline	23.3 ± 0.6	6.03 ± 0.1
Acidic	10.8 ± 0.1	8.04 ± 0.2

The results of the analysis of erythrograms obtained with acid inhibition of rat blood erythrocytes after its incubation with extracts (Figure 4) and their low-molecular-weight fractions (Figure 5) proved a short-term hemolysis and a tendency for a smooth decline in all samples.

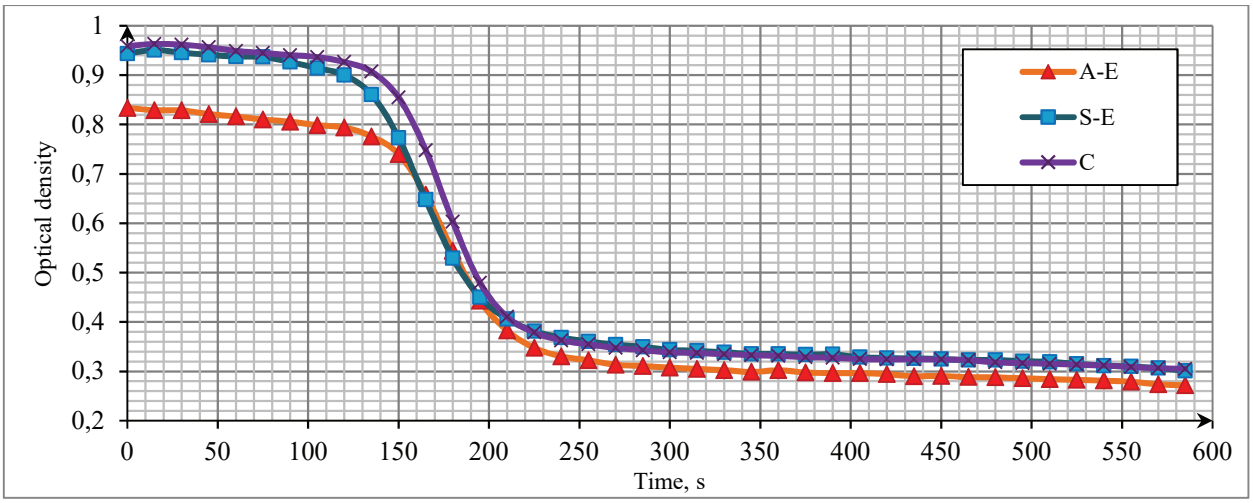

Figure 4. Acidic erythrograms of erythrocytes incubation with the original extracts.
Legend: A-E — curve of incubation of erythrocytes with TCA extract; S-E — curve of incubation of erythrocytes with saline extract; C — curve of incubation of erythrocytes with saline solution without the sample

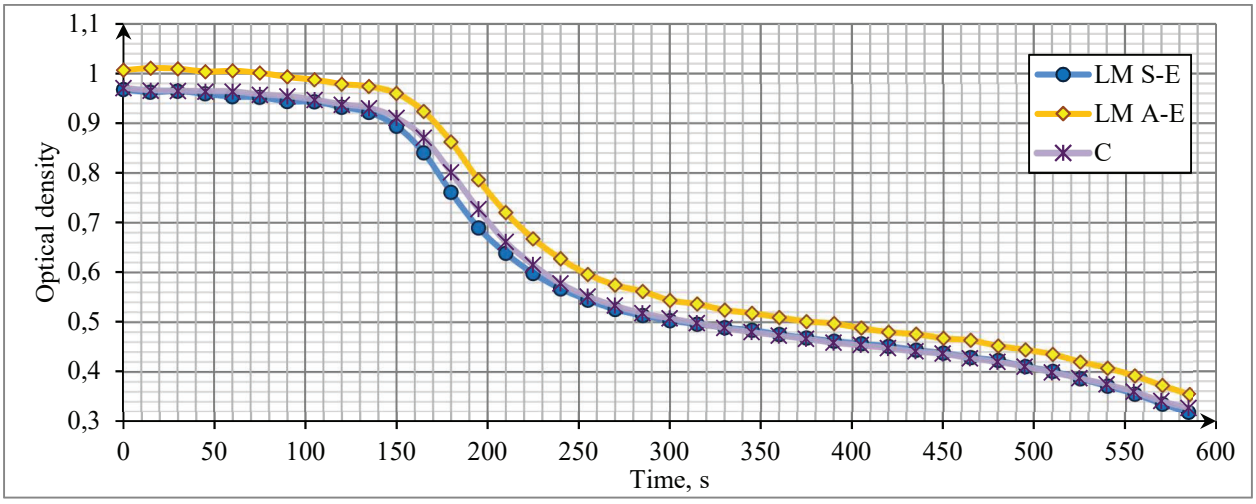

Figure 5. Acidic erythrograms of incubation of erythrocytes with ultrafiltrate extracts.
Legend: LM A-E — incubation curve of erythrocytes with low molecular weight fraction of TCA extract; LM S-E — incubation curve of erythrocytes with low molecular weight fraction of saline extract; C — curve of incubation of erythrocytes with saline without a sample

Analysis of the diagram typical for incubation of erythrocytes with primary extracts (Figure 4) revealed particularly low optical densities for the TCA extract, which is explained by the presence in the sample of a large number of biologically active compounds that can penetrate through the membrane and accelerate the process of its degradation. In addition, this hemolysis curve is also spanned below the control sample curve, which confirms the overall negative effect of the extract on biological membranes. The results of determining the optical density of erythrocytes in the case of incubation with a saline extract were similar to the values in the control sample at the very beginning of the process and at the end of hemolysis. The decline in the diagram, that indicates the process of direct cells destruction, was found below the control curve and almost coincided with the values for the acid extract, which indicates a high degree of membrane degradation. Based on the results of the analysis, it can be concluded that the primary extracts are unable to provide cytoprotective effect on biological membranes, which is more profound in the TCA-based sample. In this regard, it is possible to assume the possible presence of highly active compounds in the samples, capable to transport substances through the membrane, which indirectly leads to its destruction.

A similar process was observed in the case of saline ultrafiltrate (Figure 5). The incubation diagram with erythrocytes also coincided with the control curve, which proves a weak membranotropic activity of the low molecular weight fraction in this sample. An obvious difference was observed when considering the acid ultrafiltrate curve. High values of the optical density of the sample based on TCA confirmed the cytoprotective effect of biologically active substances released into the extract, as well as an increase of erythrocyte membrane preservation level being exposed to degrading agent — hydrochloric acid.

Analysis of the proteomic profile of the final extracts (refer to Figure 6) showed the range of protein compounds on electropherogram at the final point of saline extraction (the 330th min. — Figure 6, No. 1) and track of the raw material (Figure 6, No. 2) corresponding to the pancreas sample, which was not subjected to extraction. Compounds within the range of 52 kDa and less were found in the extracts, which were discussed in detail in our previous research [7]. It should be noted that the fractional bands in this extract are more saturated than the primary electropherogram of acid extraction (Figure 6, No. 3, 4). In case of acid extraction only a few slightly expressed protein fractions were observed in the range of more than 250 kDa, 69–70 kDa, 52 kDa, 22–23 kDa and 15 kDa. The visual analysis of the native track of purified acid extract found no difference from the track of extraction 135 minutes long. The staining with silver (Figure 6, No. 5) revealed a wide profile of protein compounds in whole presented range of molecular weights. In the track of the purified extract (refer to Figure 6, No. 6), some medium-molecular fractions were absent: 70–95 kDa, 32–48 kDa; the fraction 15–16 kDa became less saturated, and the intensity of the low-molecular fraction of weight of less than 8 kDa increased slightly.

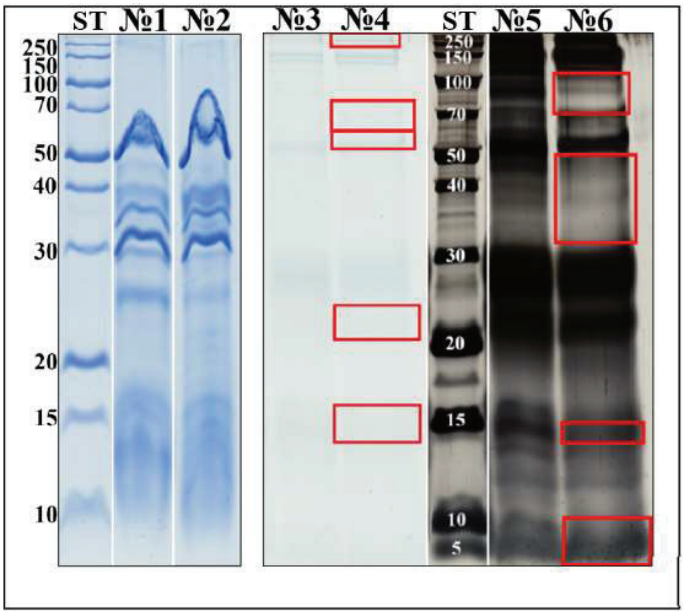

Figure 6. One-dimensional electropherogram of porcine pancreas extracts
Legend: ST — molecular weights standards: 250, 150, 100, 70 50, 40, 30, 20 15, 10 and 5 kDa; No. 1 — track of the endpoint of saline extraction; No. 2 — track of the sample not subjected to extraction (PN); No. 3 — track of the endpoint of acid extraction; No. 4 — track of purified acidic extract; No. 5 — silver-stained acid extraction endpoint track; No. 6 — silver stained track of a purified acid extract. Significant positions of fractional bands or changes in fractional composition are marked red

The densitometric study of native electropherograms of acid extracts in the ImageJ software brought little information, since only 5 weak peaks were identified at the endpoint of extraction and in the purified extract too (Figure 7, No. 3, 4). After silver staining, a greater number of fractions were found on the corresponding tracks diagram within entire presented range of molecular weights. This range featured prevalence of fractions over 100 kDa, 83–90 kDa, 70–75 kDa, 43–65 kDa, 18–32 kDa, 14–16 kDa, less than 11 kDa. After purification of the extract some of the protein compounds were removed, and that provided better separation of the peaks in these specified ranges of masses. Thus, peaks at 137–140 kDa, 110 kDa, 100 kDa, 70 kDa, 52 kDa, 41–42 kDa, 27–31 kDa, and 21 kDa were clearly distinguishable. The absent peaks in the density diagram (49–50 kDa, 33–34 kDa, 16 and 11 kDa) most likely corresponded to the removed ballast proteins.

During saline extraction (Figure 7, No. 1) and analysis of the sample not subjected to extraction (Figure 7, No. 2), a large number of similar peaks were noted: a profound peak at 52 kDa, a row of clear minor peaks with varying degrees of manifestation within the range 32–47 kDa, at 30 kDa, the peaks at 24–25 and 29 kDa were expressed better in case of the saline extraction, as well as the low molecular weight fraction of 10–15 kDa which was better visible too.

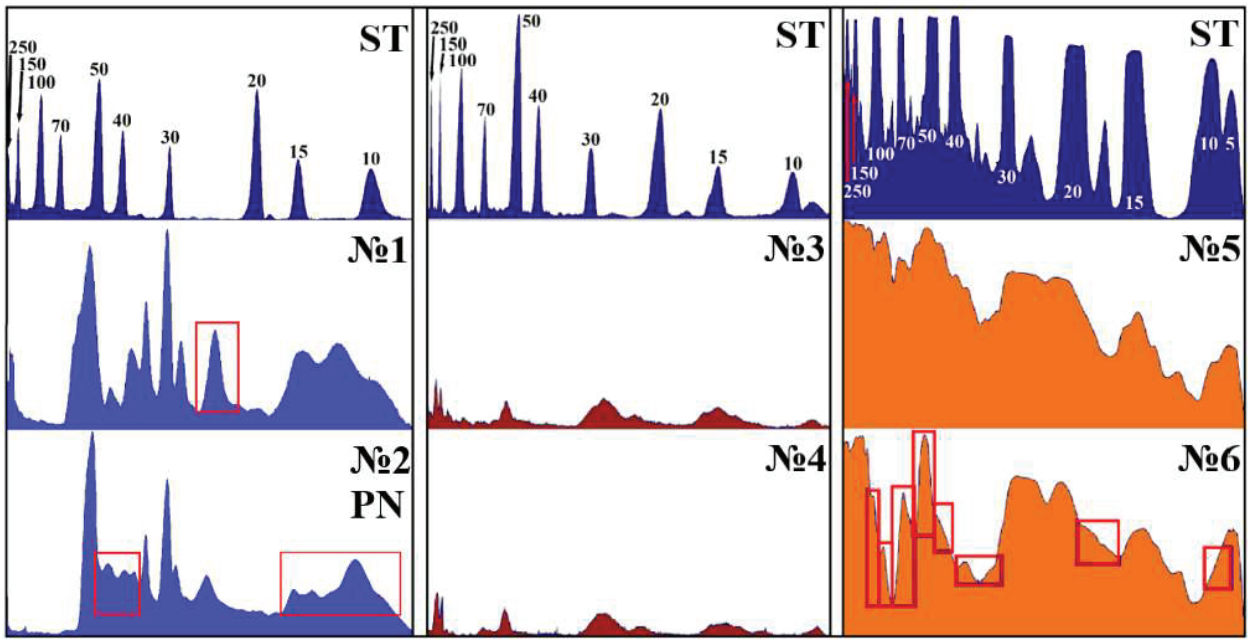

Figure 7. Histograms of the density of the protein fractions of the pancreas extraction.
Legend: ST — molecular weight standards: 250, 150, 100, 70 50, 40, 30, 20 15, 10 and 5 kDa; No. 1 — track of the endpoint of saline extraction (330 min); No. 2 — track of the sample not subjected to extraction (PN); No. 3 — track of the endpoint of acid extraction (135 min); No. 4 — track of purified acidic extract; No. 5 — silver stained acid extraction endpoint track; No. 6 — silver stained track of a purified acid extract.
Significant changes in the fractional composition of the extracts are marked red

Analysis of electropherogram and corresponding density diagram by ImageJ software showed a wide range of compounds in all types of extracts. In case of acid extraction the high molecular weight proteins prevailed, which was detected by silver staining. The saturation of the protein bands prevailed in the saline extract and the track of the initial PN sample, which protein bands correlated with the previously presented data on the total protein content.

The analysis of dynamics of the biologically active protein substances extraction from porcine pancreas showed high values of the total protein concentration during saline extraction along with a wide range of compounds throughout the entire process. In case of acidic extracts the protein content values were lower; however, the proteomic profile of the samples taken during the process was characterized by a greater variety of fractions within the whole presented range of molecular weights. First of all, this may be explained by dependence of solubility [17, 18] and activity of many enzymes [19] secreted by the porcine pancreas on the acidity of the medium. Thus, as the pH approaches the isoelectric point of a protein, its solubility decreases when it reaches its minimum at pI value [20]. This indicator has long been established for many hydrolytic enzymes of the pancreas of cattle and pigs. It has been determined that most of the main enzymes are secreted by the organ under normal conditions in the form of pancreatic fluid. To the maximum extent, isoenzymes of α-amylase I and II, for example, precipitate at pH 6.5 and 6.1, trypsin at pH 10.2–10.8, chymotrypsin at pH 8.1, ribonuclease at pH 9.6 [6,21–23]. In this regard the acidic environment created by TCA provides better solubility and, accordingly, a greater variety of compounds in extracts. In saline samples, due to physiological solution, a neutral reaction of the medium is established, which leads, to varying degrees, to a decrease in the yield of some compounds into the extractant. The lower concentration of total protein in acid extracts samples is also associated with inhibition of many digestive enzymes, since the maximum activity of pancreatic lipase is observed at pH 8–9, trypsin 7–9, chymotrypsin 7.8–8.0, pancreatic elastase 8.5, carboxypeptidase A 8.0 [9,24–27]. Thus, a higher yield of pancreatic proteins, most of which are enzymes [28], is observed in case of neutral or slightly alkaline medium, since these pH values account for maximum activity of enzymes; this phenomena was also observed in case of saline extraction. On the other hand, high values of the protein concentration in these extracts can be caused by mild extraction conditions as those mild conditions provided extraction of active substances together with biologically active compounds of ballast proteins. The ballast proteins were removed from the extract in case of acid extraction by an additional purification stage.

Throughout the entire experiment, in both methods the oscillatory nature of the degree and rate of protein extraction from pancreas was observed. Decrease in these values at some moments of the process is explained by a decrease in amount of proteins released to the extractant or by their fermentative breakage as a result of autolysis caused by action of extracted proteolytic enzymes [29–32]. Decrease in proteases activity can be achieved by dropping down of the process temperature (which was done in this research), as well as by acidifying the extractants as indicated above. When using saline as an extractant, an instant increase in the extraction rate was observed due to absence of obvious inhibitory agents. For obvious reasons the extraction

of TCA was accompanied by smooth increase in rate of protein release into the extractant. Based on the obtained results, it is possible to note the greater dependence of the extraction method efficiency on availability of inhibiting factors, as well as on the type of the extractant.

The fraction of interest with molecular weight of less than 30 kDa in the final saline extract was researched in early works [7]. Using the UniProt database [3] the following low molecular weight compounds were presumably detected in the purified acid extract: chymotrypsin C (28.9 kDa), a member of family of chymotrypsin-like elastases 1 (28.8 kDa) and 2A (28.7 kDa), glutathione S — transferase omega-1 (27.4 kDa), kininogenase (27.2 kDa), proglucagon (21 kDa), secretin (14.6 kDa), trefoil factor 2 (13.8 kDa), somatostatin (12.7 kDa), colipase (12.1 kDa), inhibitor of serine protease of Kazal-type 4 (9.6 kDa) and 1 (6 kDa), precursor of pancreatic hormone (7.3 kDa) etc. Due to smaller amount of compounds in the saline extract, many of the above specified types of substances were not found there.

Conclusion

The study of dynamics of extraction based on 0.9% sodium chloride solution showed a higher concentration of total protein throughout the entire process with a wide range of compounds with molecular weight of less than 52 kDa. In TCA extraction, the protein content in the analyzed samples was lower than in the first extraction method; however, analysis of the proteomic profile of the samples showed a greater variety of compounds in the entire presented range of molecular weights. The low molecular weight fraction prevailed in acid extracts both before ultrafiltration (according to results of electropherograms study) and after ultrafiltration (based on analysis of protein content in the ultrafiltrates).

Using of the drawn acidic erythrograms enabled to detect the capability, peculiar for primary extracts, to destroy the biological membranes. The low molecular weight fraction of the acid extract possessed the highest cytoprotective effect, which proved the presence of highly active compounds in the extract.

When comparing the saline and acid extraction methods, the applicability of both modifications for obtaining a mixture of proteins with different molecular weights is noted. In this case, greater efficiency is observed in extraction with trichloroacetic acid. In result of this method it is possible to obtain a complex of low-molecular-weight highly active biological compounds.

It is necessary to note that the processes, considered in this work, require a comprehensive approach and are the subject of further extensive research. It is necessary not only to trace the dynamics of the process, but also to select target markers-compounds, which could be used to determine the efficiency of extraction, and also to study the potential for isolating of some specific compounds while maintaining of their high activity. The study of other extractants, for example, alcohol or sulfuric acid, is also considered a promising direction of researches.

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DEVELOPMENT OF REGRESSION MODEL OF PROTEINS ATTACKABILITY PROCESS IN MEAT FOOD (*IN VITRO*)

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Abstract
In the presented article the authors consider the issues of development of regression model for process of food digestion by proteolytic enzymes in human body. The authors use correlation analysis. They analyze the main nutritional values and physical and chemical properties of meat products, the modes of heat treatment of semi-finished lamb products. The essential parameters and features are determined to find the dependence between the factor values and efficient values of the basic raw material, which affect the quality of the technological processes and, in general, the finished product. The regression model equation is mathematically calculated by methods of solving K. Gauss linear equations. The standard deviations of parameters are calculated, the initial data are normalized; the matrices of the pair correlation coefficients, lower and upper limits of their values are compiled. Equations of the mathematical regression model of meat proteins attackability by proteolytic enzymes — in vitro (pepsin, trypsin) are developed. It is proved that the obtained equation represents a regression model of the process of meat food proteins attackability by enzymes (pepsin, trypsin and chymotrypsin), depending on the determined 3 essential factors (weight of a meat piece, duration of frying, collagen content in lamb meat). Also this equation reflects the process of lamb digestibility in a digestive tract of a human body.

Introduction

The rate of proteins digestion in the gastrointestinal tract, or attackability of the proteins in the composition of meat dish by proteolytic enzymes is one of the important factors that determine the biological value of food products [1–5].

Nowadays the theory and methods of correlation analysis can be successfully applied to study mathematical problems, relations between phenomena and features in various fields of science, technology and national economy. Correction relation is considered as established when a row of function values correspond to the same value of the argument. The features that characterize this relation are divided into factor and efficient. The features that affect a certain result are called factor features. And the features that respond to factor features are called efficient [6].

Correlation-regression analysis is often used in scientific research. When processing the big amounts of statistical data, correlation analysis quantifies the strength of link between two or more quantitative variables. Regression or correlation analysis describes the link between variables, while the correlation provides a numerical way to measure the level or strength of link between two variables [7,8].

Here are the examples of some researches that cover the application of correlation-regression analysis in various fields of science. The research [9] assessed the capacity of an interactive dual-energy X-ray absorptiometer (DEXA) installed on the slaughter line of a meat processing plant to determine the composition of lamb carcasses. 607 lamb carcasses from 7 slaughter groups were scanned by DEXA device and later were scanned by computed tomography device to determine the ratio of fat, lean meat and bone in carcasses. The results of those test across whole data range showed high accuracy of body fat percentage forecasting by computed tomography device, with coefficient of determination (R^2) = 0.89, compared to 0.69 for lean meat and 0.68 for bones in carcass, that showed less accuracy. Accuracy in the seven groups was also high in comparison with the mean values of bias 0.66, 0.83 and 0.51.

The researches [10] carried out by the Danish Research Institute of Meat are conducted to develop the forecasting models for the shelf life of meat products. Expiration dates of chilled meat were simulated for beef cuts (850 samples), pork cuts (1500 samples) and chicken (1080 samples), minced beef and minced pork (680 samples of each type of minced meat) and for bacon (1080 samples). In this case, the samples of meat and meat products were packed in modified atmosphere into vacuum bags in combination with microwave treatment at various storage temperatures. Research showed that forecasting models serve as tools able to assess the importance of temperature and packaging changes for the shelf life of various meat products. In the research [11] volatiles were studied during roasting beef at a temperature of 180 °C. 70 volatile substances were identified, including non-aromatic, homocyclic and heterocyclic compounds. A significant positive regression model was constructed to forecast the storage of toluene, benzol acetaldehyde, 2-formylfuran, pyrazine, 2.6-dimethylpyrazine, 2.3-dimethylpyrazine, 2-acetylthiazole, and

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2-formyl-3-methylthiophene. For calculation of aging time a linear and logarithmic regression model was chosen.

In a research [12] the authors studied the influence of the carcass parts weights (thigh, chest, wing, back, stomach, heart) on whole carcass weight of white turkeys (Big-6). Data were analyzed with the help of regression analysis based on ridge regression and factor analysis. Both regression models were found to be suitable for turkey carcass weights forecasting. However, the ridge regression method was preferred, as it showed higher R^2 value and explains carcass weight in a better way.

In the study [13] three varieties of wheat (PKB Talas, BG Merkur and PKB Lepoklas) harvested in 2009 and 2010 were studied. The correlations between the morphological and yield parameters of plants were studied: the number of shoots, the number of spikelets on a wheat head, the number of grains per wheat head, the weight of 1,000 grains and mass of grain per wheat head. Taking into account the parameters of all three varieties, high and positive correlations were found between the number of grains per wheat head and the weight of grain per wheat head(> 0.78), the number of spikelets per wheat head and the number of grains per wheat head (> 0.79), as well as the number of spikelets per wheat head and grain weight per wheat head (> 0.73). Regression analysis was conducted only as an addition to the correlation formula, and was presented in the form of charts showing the link between the studied dependent and independent characteristics.

Researchers in the study [14] constructed a quadratic polynomial that explains the link of three variables (fermentation temperature, X_1; amount of inoculation, X_2; and concentration of solid substrate, X_3) on yield of monacolin K.

The goals of the study [15] were to define: (1) the effect of fertilizers, the environment and their interactions on the thousand grains weight (TGW), hectolitre weight (HW) and grain yield (GY) of winter triticale variety and (2) the correlation between these characteristics in various environments. Negative and significant correlation was found between GY and TGW (minus 0.392) in 2015, positive highly significant correlation was found in 2013 (0.648) and 2014 (0.493).

Recently, researches have appeared which study the effects of meat consumption of health of population in the modern world, including issues related to the consumption of saturated fat [16]. Regression analysis was used to determine the effect of stearic acid on total cholesterol (TC) level in blood plasma and low-density lipoprotein (LDL) cholesterol when people of various ages consume fatty foods is described in researches [17,18,19].

With an increase in standards of living and well-being of peoples of Central Asia, and in particular, the Republic of Uzbekistan, in the diet of population the consumers' demand for natural food products and a wide range of lamb meat dishes cooked in traditional ways increases. In accordance with modern concepts of nutritional science, food must have an attractive appearance and high taste, and also food shall be biologically complete, i. e. the food shall contain all necessary essential amino acids and other important food components in optimal proportions. Those food components are well digested in a human body by digestive enzymes [20,21].

The basis of enzymatic hydrolysis of meat proteins according to method of A. A. Pokrovsky and I. D. Ertanov [5] is the conditions when the availability of attacked peptide bonds in a meat is determined not only by its physical and chemical parameter of proteins, but also by characteristics related to the structure and chemical composition of the basic food product. It is important and necessary to conduct scientific research in this sphere using mathematical methods of analysis, as they prove the effectiveness of the approaches, the possibility of determining the calculated parameters of the biological and nutritional value of food products and comparing them with the FAO data.

The purpose of our study was to build a regression model of the most important processes of food digestion by proteolytic enzymes (pepsin, trypsin), using correlation analysis and expressing the analytical dependence of the efficient characteristic (Y-process of meat proteins attackability) on factor characteristics $x_i, i = \overline{1, n}$.

To achieve this goal the following tasks were solved: based on 20-fold physical and chemical analyzes to obtain the necessary digital parameters of the analyzed main product (meat) and based on results of obtained data, calculate their position and dispersion characteristics, determine the influence of significant factors — (modes of heat treatment, quantitative content of imperfect proteins) for efficient features (quality of semi-finished meat products).

Objects and methods

To conduct the scientific research we selected and prepared sample materials, as well as chose experimental methods for testing. The object of research and study was: meat productivity, quality of meat and raw fat of fat-tailed sheep of "Jaydara" breed, popular in the foothill regions of the Republic of Uzbekistan. Experimental studies analyzed 14 parameters of meat product quality, including the parameter fully covered in this article.

The process of hydrolysis (digestion of proteins *in vitro*) was run in the Department of Food Technology laboratory on a special 3-cells device that provides continuous mixing and dialysis of the samples, and the products of their breakdown were analyzed by micro-methods that allow simultaneous analysis of a significant number of samples under study [22].

The meat product containing about 150 mg of protein (N2 x 6.25 or x 5.75) is placed in the inner vessel of the specified device, then 15 ml of 0.02 N solution of HCl with pH 1.2 is added into the vessel. In order to comply with the isoionic process, it is also necessary to add 60 ml of

the same solution to the outer vessel. The test samples are incubated in the thermostat at a temperature of 37 °C.

The index of the proteins attackability of meat semi-finished products by proteolytic enzymes (pepsin, trypsin) was estimated by build-up of hydrolysis products in individual samples. The calculations were made according to the corresponding formulas.

For statistical data processing and construction of 1) matrix of pair correlation coefficients; 2) the regression equations, we used "Data Analysis" in a spreadsheet processor MS Excel.

Results and discussion

In order to conduct the mathematical analysis, preliminarily we studied the process of proteins attackability in lamb meat food cooked in various modes and duration of heat treatment, taking into account the unequal content of connective tissue proteins in meat (collagen and elastin), as well as the content of pure hydroxyproline in the samples under study [21].

As a result of physical and chemical analyzes data were obtained, which were subsequently presented as initial data, where:

X_1 — is weight of meat pieces (weight, g);
X_2 — deep fry temperature (°C);
X_3 — duration of frying (min);
X_4 — collagen content in meat (%);
X_5 — elastin content in meat (%);
X_6 — oxyproline content in meat (mg,%),
Y — degree of attackability (mg of hydrolyzed protein).

The layout of the initial data table is compiled on the basis of laboratory analysis values and is presented below in Table 1.

Table 1. Initial data

№, n/n	X_1	X_2	X_3	X_4	X_5	X_6	Y
1	250.7	200	108	2.03	0.68	49.63	93.8
2	245.8	190	95	2.53	0.77	50.10	83.6
3	260.0	195	86	2.82	0.88	65.10	81.1
4	235.6	185	95	3.05	1.17	174.7	68.1
5	248.0	170	107	2.01	0.70	48.75	94.3
6	198.5	176	98	2.23	0.76	49.90	89.4
7	170.4	211	87	2.47	0.81	52.37	87.5
8	189.3	183	95	2.11	0.71	51.20	91.9
9	157.0	165	103	1.97	0.64	47.94	97.4
10	165.8	178	92	2.28	0.75	52.68	90.5
11	235.2	174	125	1.19	0..63	42.67	140.11
12	200.0	198	135	1.05	0.59	40.30	143.3
13	185.0	194	120	1.39	0.70	44.17	135.4
14	230.0	209	115	1.48	1.17	60.38	120.7
15	179.5	150	123	1.43	1.05	54.86	122.0
16	158.7	153	130	1.32	0.68	45.25	133.4
17	195.4	151	130	1.28	0.65	43.87	134.6
18	185.6	167	125	1.31	0.70	50.11	130.8
19	148.0	160	127	1.23	0.61	45.79	135.3
20	170.0	193	112	1.50	1.03	56.15	120.2
$\overline{X}_k$	200.425	180.1	110.4	1.834	0.784	56.296	109.6705

Based on the initial data, the parameters of the multiple regression equation were calculated as follows:

— The arithmetic mean value of each parameter is determined according to the formula (1):

$$\overline{X}_k = \frac{\sum_{i=1}^{n} x_{ki}}{n} \quad (1)$$

where k — is number of factors (in our case $k=1, 2 \ldots 6$); $\overline{X}_k$ — arithmetic mean of the k^{th} factor; x_{ki} — value of the i^{th} measurement of the k^{th} factor (i=1, 2…n); n — number of trials.

The obtained values are presented in the last line of the Table 1 and are highlighted in orange.

— The standard deviation of each parameter was determined by the formula (2):

$$C_{xr} = \sqrt{\frac{\sum_{i=1}^{n}\left(x_{ri} - \overline{x}_k\right)^2}{n-1}} \quad (2)$$

where C_{xr} — is the mean-square deviation of the k^{th} factor.

$C_{x1}=35.67$ $C_{x2}=18.74$ $C_{x3}=15.84$ $C_{x4}=0.59$
$C_{x5}=0.18$ $C_{x6}=28.5$ $C_{xy}=23.89$

Further, the initial data were normalized (Table 1) according to the following formula:

$$x^* = \frac{x_{ki} - \overline{X_k}}{C_{xn}} \quad (3)$$

where x^* — is rationing the value of the kth factor.

After normalizing the data the following results were obtained (Table 2).

Table 2. Values of normalized initial data

№, n/n	X_1	X_2	X_3	X_4	X_5	X_6	Y
1	1.41	1.06	–0.15	0.33	–0.58	–0.23	–0.66
2	1.27	0.53	–0.97	1.17	–0.08	–0.22	–1.09
3	1.67	0.80	–1.54	1.66	0.53	0.31	–1.20
4	0.99	0.26	–0.97	2.05	2.14	4.15	–1.74
5	1.33	–0.54	–0.21	0.30	–0.47	–0.26	–0.64
6	–0.05	–0.22	–0.78	0.67	–0.13	–0.22	–0.85
7	–0.84	1.65	–1.48	1.07	0.14	–0.14	–0.93
8	–0.31	0.15	–0.97	0.46	–0.41	–0.18	–0.74
9	–1.22	–0.81	–0.47	0.23	–0.80	–0.29	–0.51
10	–0.97	–0.11	–1.16	0.75	–0.19	–0.13	–0.80
11	0.98	–0.33	0.92	–1.08	–0.85	–0.48	1.27
12	–0.01	0.96	1.55	–1.32	–1.07	–0.56	1.41
13	–0.43	0.74	0.61	–0.75	–0.47	–0.43	1.08
14	0.83	1.54	0.29	–0.60	2.14	0.14	0.46
15	–0.59	–1.61	0.80	–0.68	1.47	–0.05	0.52
16	–1.17	–1.45	1.24	–0.87	–0.58	–0.39	0.99
17	–0.14	–1.55	1.24	–0.93	–0.74	–0.44	1.04
18	–0.42	–0.70	0.92	–0.88	–0.47	–0.22	0.88
19	–1.47	–1.07	1.05	–1.02	–0.96	–0.37	1.07
20	–0.85	0.69	0.10	–0.56	1.36	–0.01	0.44

— The pair correlation coefficients can be calculated according to the formula (4) or using the spreadsheet processor "Data Analysis" in MS Excel.

$$R_{Ks} = \frac{1}{n}\sum_{i=1}^{n} x_{ki}^{*} \cdot x_{si}^{*} \quad (4)$$

where R_{Ks} — is coefficient of correlation dependence between factors *k* and *s*.

The results of calculation are presented in the form of a matrix of pair correlations (Table 3).

Table 3. Matrix of pair correlation coefficients

	Y	X_1	X_2	X_3	X_4	X_5	X_6	
Y	1							X_1
X_1	–0.38	1						X_2
X_2	–0.33	0.39	1					X_3
X_3	0.94	–0.27	–0.45	1				X_4
X_4	–0.97	0.41	0.36	–0.93	1			X_5
X_5	–0.33	0.22	0.29	0.30	0.35	1		X_6
X_6	–0.50	0.28	0.13	–0.34	0.57	0.64	1	

Analyzing the data presented in the Table 3, it is obvious that the coefficients of pair correlation between Y and factors X_1, X_2, X_5 feature rather low values (all coefficients in absolute value are below 0.4), and it means that the link is quite weak. The moderate link is observed between Y and X_6 (correlation ratio is minus 0.50). Strong link is defined between Y and X_3, X_4 (correlation coefficients to modulo exceed 0.9). In addition the strong link is observed between variables X_3 and X_4 (correlation coefficient is minus 0.93), moderate link is determined between variables X_4 and X_6 (correlation coefficient is 0.57), X_5 and X_6 (correlation coefficient is 0.64) and it means that these coefficients can be collinear.

After finding the pair correlation coefficients, the partial correlation coefficients were determined by the following formula:

$$r_{yx_i/x_j} = \frac{r_{yx_i} - r_{yx_j} \cdot r_{x_i x_j}}{\sqrt{\left(1 - r_{yx_j}^2\right) \cdot \left(1 - r_{x_i x_j}^2\right)}} \quad (5)$$

As result the following values are obtained:

Rx1x2	–0.290	**Rx1x3**	–0.368	**Rx1x4**	0.075	**Rx1x5**	–0.334	**Rx1x6**	–0.288

Correlation ration is low everywhere

Rx2x1	–0.209	**Rx2x3**	0.309	**Rx2x4**	0.107	**Rx2x5**	–0.255	**Rx2x6**	–0.303

Correlation ratio is low everywhere

Rx3x1	0.938	**Rx3x2**	0.937	**Rx3x4**	0.369	**Rx3x5**	0.932	**Rx3x6**	0.943

Correlation ratio is high (to modulo ober 0.9), except for Rx3x4. In this case the correlation is low.

Rx4x1	–0.969	**Rx4x2**	–0.971	**Rx4x3**	–0.789	**Rx4x5**	–0.970	**Rx4x6**	–0.967

Correlation ratio is high everywhere

Rx5x1	–0.275	**Rx5x2**	–0.260	**Rx5x3**	–0.161	**Rx5x4**	0.039	**Rx5x6**	–0.015

Correlation ratio is low everywhere

Rx6x1	–0.448	**Rx6x2**	–0.492	**Rx6x3**	–0.557	**Rx6x4**	0.292	**Rx6x5**	–0.403

Correlation ratio is low for Rx6x4, moderate for others.

To establish a connection between all factors and the resulting characteristic, the multiple regression coefficient is found:

$$R_{yx_1x_2} = \sqrt{\sum \beta_i \cdot r_{yx_i}} \quad (6)$$

As a result of the calculations, we found that the coefficient of multiple regression is 0.98, which proves a strong correlation between the entire set of factors and the result.

The unadjusted coefficient of multiple determination shows that 96% of the variation in the result are explained by the variation of the factors presented in the equation.

The adjusted coefficient of multiple determination defines the correlation ration, taking into account the degrees of freedom of the total and residual variances and is calculated by the following formula:

$$R_{corr}^2 = 1 - (1 - R^2) \cdot \frac{n-1}{n-m-1} \quad (7)$$

The adjusted multiple regression coefficient is 0.94, i. e. practically equal to 1, i. e. the regression equation explains the variation of attackability.

To build a linear multiple regression model, "Data Analysis" in MS Excel (Figure 1) was used.

	df	*SS*	*MS*	*F*	*Significance F*			
Regression	6	18,2349	3,0392	51.6415	0,0000			
Residue	13	0,7651	0,0589					
Total	19	19,0000						
	Coefficients	Standard error	t-statistics	P-value	Lower 95%	Upper 95%	Lower 95,0%	Upper 95,0%
Y-crossing	0.0000	0.0542	0.0000	1,0000	-0.1172	0.1172	-0.1172	0.1172
XI	-0.0493	0.0774	-0.6373	0.5350	-0.2166	0.1179	-0.2166	0.1179
X2	0.0900	0.0743	1.2112	0.2474	-0.0706	0.2506	-0.0706	0.2506
X3	0.3419	0.2840	1.2040	0.2501	-0.2716	0.9554	-0.2716	0.9554
X4	-0.6659	0.3222	-2.0668	0.0593	-1.3620	0.0301	-1,3620	0.0301
X5	-0.0197	0.0840	-0.2347	0.8181	-0.2011	0.1617	-0.2011	0.1617
X6	0.0102	0.1299	0.0789	0.9383	-0.2704	0.2909	-0.2704	0.2909

Figure 1. Regression analysis data

Using the values in the "Coefficients" column (Figure 1), we obtain the linear multiple regression equation in the standardized form:

$$\check{y} = -0.049x_1 + 0.09x_2 + 0.342x_3 - 0.666x_4 - 0.02x_5 + 0.01x_6 \quad (8)$$

The analysis of the data presented in Figure 1 allows concluding about the significance of the regression equation, as $F_{table}(51.64) > F_{obser}(0.00)$ with probability $1 - \alpha = 0.95$.

Using the particular of F-test of Fisher, we assessed the feasibility of including the factors x_i in the multiple regression equation after the other factors:

$$F_{x_i} = \frac{R^2 - R^2(x_{i+1}, x_n)}{1 - R^2} \cdot (n - m - 1) \quad (9)$$

where $R^2(x_{i+1}, x_n) = \sum \beta_i r_{yx_i}$.

We came to conclusion that it is feasible to include x_1, x_3, x_4 into the regression equation.

To switch from standardized values to natural values, the following formula is used:

$$A_i = \frac{C_y}{C_{xi}} \cdot b_i \quad (10)$$

Where A_i is the natural coefficient of the equation; C_y is the standard deviation of the factor; C_{xi} the standard deviation of the parameter.

Thus the regression equation in natural values looks as follows:

$$y = 89.41 - 0.033x_1 + 0.516x_3 - 26.792x_4 \quad (11)$$

This equation represents a regression model of the process of meat proteins attackability by enzymes (pepsin, trypsin and chymotrypsin), depending on the defined 3 essential factors (mass of a piece of meat; duration of frying; collagen content in meat) and reflects the process of digestibility of meat products in a human digestive tract.

It can be seen from the equation that while the mass of a meat piece increases by 1 unit, its attackability decreases by 0.033 units. While the duration of frying meat increases, the protein attackability also increases by 0.516 units. While the collagen content in meat increases, attackability decreases by 26.792 units.

Conclusion

As a final summary of the research it is possible to conclude that the analyzes, mathematical calculations, statistical analysis of reliability of the obtained regression equation prove the possibility of successful application of correlation-regression analysis for the calculations of biological and nutritional value assessment — i. e. the processes of protein breakdown in various finished lamb culinary products by proteolytic enzymes (*in vitro*).

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MEAT SUPPLY CHAIN IN THE PERSPECTIVE OF UN SDGS

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Keywords: *meat and meat products, supply chain, United Nations, sustainability development goals*

Abstract

This paper presents an overview of the meat supply chain in the perspective of main UN sustainable development goals (SDGs). To perform this overview, meat supply chain was presented with five main stakeholders (livestock farmers, slaughterhouses, meat processors, retailers and consumers). As this chain is specific, four SDGs have been revealed as most important, as follows: SDG6 — Clean water and sanitation; SDG7 — Affordable and clean energy; SDG12 — Sustainable consumption and production; SDG13 — Climate action. Discussion and literature review was performed for each of the four UN SDGs. In addition, other UN SDGs of interest for this supply chain have been briefly presented.

Introduction

The Sustainable Development Goals (SDGs) comprise of 17 goals deployed into 169 targets and 232 indicators that aim to strive the World to a more sustainable future. They were defined by the United Nations General Assembly in 2015 by leaders of almost 200 countries [1]. Although generic, food systems have been identified as one of key sectors that need to be tackled in order to achieve the UN SDGs by 2030. During July 2021, a Food Systems pre-summit was held in Rome where participants from all around the world discussed how to improve national pathways and address different impacts of food systems associated with UN SDGs "based on the best science and reflecting local and national realities within a global context" [2]. In September 2021, the UN Secretary-General António Guterres will assemble the Food Systems Summit. It is expected that the Summit will initiate a variety of actions related to all 17 SDGs, focused on healthier, more sustainable, and equitable food systems [3]. The Food and Agriculture Organization (FAO) of the United Nations has identified eight (out of 17) UN SDGs that are directly associated with food systems, as follows: SDG1 — No Poverty; SDG2 — Zero Hunger; SDG6 — Clean water and sanitation; SDG7 — Affordable and clean energy; SDG12 — Sustainable consumption and production; SDG13 — Climate action; SDG14 — Life below water and SDG15 — Life on land [4]. Other nine UN SDGs are indirectly related to food systems.

When it comes to meat and meat supply chains, many studies identify this sector as one of main environmental polluters in the food system [5]. The livestock sector's has severe environmental emissions on air, water and soil, joint with needs for natural resources such as water and energy [6]. Meat slaughtering and processing additionally put pressure on the environment from emissions into the environment and/or from the consumption of all kinds of natural resources [7]. To summarize, all environmental impacts of this chain influence three dimensions: (i) climate change in respect to the global warming potential; (ii) consumption of natural resources and (iii) pollution of the environment caused by waste water discharge and waste disposal [8]. In parallel, global consumption of meat has increasing in terms of its overall production and consumption as a result of growing world's population and consumption of meat per capita [9]. In relation to its sustainability pillars, meat production and consumption directly affect the economy, the society and the environment [10]. To better understand the meat supply chain, one of approaches is to present it from a "farm to fork" perspective [11]. In that sense, this chain consists of five main links: (i) livestock farms (ii) slaughterhouses (iii) meat processing plants (iv) customers / retail and (v) meat consumers / households [8].

The objective of this paper is to analyze meat supply chains from the perspective of four UN Sustainable development goals, as follows: SDG6 — Clean water and sanitation; SDG7 — Affordable and clean energy; SDG12 — Sustainable consumption and production; SDG13 — Climate action. In addition, a short discussion regarding other UN SDGs has been provided.

Objects and methods

To perform an overview on connecting UN SDGs and the meat supply chain, at first glance it was obvious that a literature review with combinations of various key words (meat production / meat supply chain vs specific UN SDGs) in various scientific databases will raise different results. Since this topic is dispersed through different types of scientific publications (research and review articles, book chapters, conference papers, editorials, etc.), main focus was to identify four key UN SDGs clearly connected with the meat supply chain. Therefore, the focus of this overview was to shed light on SDGs no.6, no.7, no.12 and no.13 and their connection with the meat supply chain.

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Results and discussion

SDG6 — Clean water and sanitation and the meat supply chain

The need for clean, accessible water is one of the UN SDGs since the world is striving for sufficient fresh water opposed to water scarcity, poor water quality and inadequate sanitation that has a negative severe impact on food security throughout any food supply chain [12]. The meat industry requires adequate supply of potable water for both meat processing and hygiene and sanitation to ensure meat and meat products are not contaminated. Therefore, one of key targets associated with meat and meat products is how to produce more using less water [4].

Meat slaughtering and meat processing are the two links in the meat supply chain that require potable water while they also discharge significant volumes of pollutant wastewater [13]. However, the entire chain is a big user as water is important for live animals at farms and when entering the slaughterhouse as well as for hygiene and sanitation of slaughterhouses, meat processing plants and retail, and finally for use at households for meat preparation [8, 14].

Water used in the meat supply chain comes from different sources, such as ground or surface water and is extensively used for numerous technological purposes in different quantities satisfying requirements outlined in water safety and quality standards [15]. Also, in some meat processing products it is a primary ingredient like in the case of hams and sausages. In these cases, water needs to be of highest "potable" quality. When used for non-processing purposes such as boiler feed water, fire-extinguishing water, pasteurizing, heating or cooling medium, quality of such water is medium to high [16]. As wastewater from meat industry may be a big environmental polluter, it is important to protect all water sources and treat wastewater with similar care [15].

SDG7 — Affordable and clean energy and the meat supply chain

Different types of energy are used throughout the meat supply chain basically for machines and equipment, for controlling temperature regimes (heating / refrigerating) and for transportation purposes [14, 17]. Its source is deployed in terms of clarifying consumption from electric energy, thermal energy and other sources of energy, such as types and quantities of fossil fuels.

Advanced housekeeping practices in the meat sector can obtain improvements while additional savings can be made through promoting energy efficiency associated with equipment and heat recovery systems [18, 19]. A promising approach in reducing energy consumption and related energy costs is through energy management [20]. Some types of organic solid waste classified as biomass can facilitate energy recovery instead of their disposal [21]. Biomass to energy conversion are routes to energetic and economic benefits [21, 22]. Recovering methane from manure is another potential of energy improvements in the meat sector [19]. This type of waste can also be used as a secondary fuel for thermal energy [23]. The main goal of thermal disposal of (organic) wastes is its conversion to safe materials, as well as in reducing its weight and volume [24].

SDG12 — Sustainable consumption and production and the meat supply chain

From 1961 to 2011, global meat consumption almost doubled from 23.1 kg per capita per annum to 42.2 kg per capita per annum [25]. Regarding expected population growth, total consumption will increase yearly by almost 1.5% [26]. Major contributor to global meat production originates in the pig sector while the poultry sector is the fastest growing livestock sector as it's a source of healthy high protein and low-fat type of meat [27–29]. Consumption of animal-based proteins has increased during a half-century period worldwide (1961–2011), from 61 g per capita per day up to 80 g per capita per day [25].

However, not only from a nutritional point of view, consumption of meat is also studied in terms of its environmental impact [30]. The FAO has coined a new term "sustainable diet" associated to diets with optimal healthy and low environmental impacts [31]. As a result, some authors claim that main trigger in "sustainable diets" is to avoid meat and meat products due to their severe environmental impacts. However, global warming potential of meat consumption compared to other products is not so much higher as one would assume analyzing some media and literature. Also, most authors associate climatic impact of food with both production and consumption [30], with limited number of papers that analyzed impact of meat consumption [32]. Finally, in line with various dietary habits that exclude consumption of meat and meat products such as veganism, vegetarianism, raw foodism, or fruitarianism, still a large majority of people eat meat regularly or occasionally [33]. Such environmental pressure on changing dietary habits is still more a scientific que than an every-day routine. One of a few studies that analyzed replacement of animal origin food with plant-based substitutes revealed some potentials in changing dietary habits in parallel with decreasing environmental impacts [34].

SDG13 — Climate action and the meat supply chain

Speaking about climate action (SDG13), agriculture is one of sector that will play a big role in responding to climate change [4]. Based on the Paris Agreement [35], two main actions arise: (i) limiting the global warming to below 2 °C above pre-industrial levels and to pursue efforts in limiting the increase of temperature to 1.5 °C [36] and (ii) preventing these threats to food systems [37]. Main challenge is how to produce sufficient amounts of food for the world's population from the perspective of observing interaction between climate change and food production. The impact of meat production is twofold in terms that meat production has an impact on climate change and *vice versa*, climate change has an impact on meat production [38].

Analysis of meat on climate change can be observed from life-cycle assessment studies, such as analysis of pork, beef and chicken meat production [33]. These studies confirm that carbon footprint (expressed as carbon-dioxide equivalent — CO_{2e}) is the main predictor of evaluating climate change impact of the meat supply chains [39]. For calculating CO_{2e}, it is necessary to measure all greenhouse gasses emissions [40]. In parallel, ozone depletion potential expressed as CFC-11 or R11 equivalents is an additional indicator used to measure the potential for reducing the protective stratospheric ozone layer [41]. This indicator indirectly affects climate change and is associated with maintaining cold chains and using refrigerants for chilling / freezing [42]. These cold chains are vital for keeping meat safe since inadequate temperatures inhibit growth of harmful microorganisms [43].

Households are responsible for preparing meat but also for discarding meat waste [44]. Reasons for discarding meat waste are expired date and rotten taste and/or smell [45]. In order to maintain meat safety, control of the cold chain joint with expiring date care are very important [38] as consumers are the weakest link in cold chains.

Temperature increase joint with climate variability affect quality of feed [46] causing an increase of mycotoxins in crops used for feed [47]. As an example, maize represents a typical crop used in feed production where presence of fumonisins is directly correlated with chronic exposure [48]. Second threat observed at farm levels are animal diseases caused by temperature rise such as death of animal further causing growth of pathogens, parasites and various vector-borne diseases [46].

Heat stress causes additional water needs for animals. Their response are dietary changes (less feed / more water) and changes in reproductive and productive effectiveness [49]. These changes lead to energy disbalances and reduction of animal weight [50] causing decrease of meat production by growth and carcass weight [49], economic losses [51]. Finally, reproduction efficiency during heat stress affects animal fertility [52], embryo development and pregnancy rate [53]. It is assumed that temperature rise may cause up to 30% of biodiversity loss of both plants and animals [54]. Depending on the region, highest risks linked with livestock and breed elimination are with chicken, followed by pigs and cattle [46].

Indicators associated with UN SDGs

To calculate impacts associated with selected UN SDGs, it is common to introduce and calculate environmental performance indicators (EPIs) defined as a "measurable representation of the status of operations, management or conditions related to environmental aspects" [55]. To evaluate meat production, it is important to define a functional unit (FU) in which the impacts are presented and to define formula as this is the basis for all further comparisons [11]. In the meat supply chain, the most common FUs are one kg of livestock [56, 57], one kg of carcass [58, 59] and one kg of meat / meat products [60].

In every meat supply chain, it is common to calculate consumptions and discharges per meat FUs such as water consumption per FU and wastewater discharge per FU directly associated with SDG6 [14, 19, 42] or energy-to-meat ratio, associated with SDG7. As presented above, global warming potential is recognized as an environmental indicator associated with the meat chain [39], linked with SDG13. The GWP is calculated for each link of the meat supply chain.

Table 1 depicts main indicators associated with four SDGs and the meat supply chain [15, 33].

Considering the link of the four UN SGDs and the meat supply chain, Figure 1 depicts the most influential UN SDGs in terms of its severity and time-scale associated with all five links in the meat chain. The most severe and long lasting stage is at farms with SDG13 as the most influential. Slaughtering and meat processing are activities that lasts short (related to one FU) but the overall impact of slaughtering and meat processing on water consumption and wastewater discharge is high (SDG6). Within retails, meat can be stored for a long period of time, but the impact is not so high, mainly associated with energy consumption for maintaining the cold chain (SDG7). Finally, lowest impact is associated with meat consumers / households where meat is often consumed within 7 days from purchasing. This activity is mostly associated with SDG12.

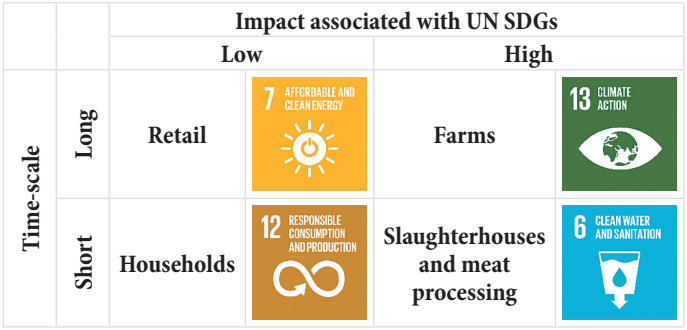

Figure 1. Four UN SDGs and their impacts on the five links in the meat supply chain

Other UN SDGs and the meat supply chain

Adapting to climate change for small — scale livestock farms is needed to enhance food security (SDG2 — Zero Hunger) and reduce poverty (SDG1 — No Poverty) of all types of small farmers [61, 62]. This is pronounced since 80 percent of extremely poor people live in rural areas depending on various aspects of agriculture — farming, fisheries and forestry [4]. Improvement of food (meat) trade is in direct correlation with making accessible and affordable safe and healthy meat (SDG2 — Zero Hunger; SDG3 — Good health and well-being). Considering that three billion people receive 20 percent of their daily animal protein intake from fish, it is obvious that sustainable management of oceans, seas and marine resources in important for the fish supply chain, namely sustainable fisheries [4]. Modernization of meat processing can indirectly impact this

Table 1. Indicators associated with four SDGs and the meat supply chain

UN SDG	Indicator	Formula [unit]
6 CLEAN WATER AND SANITATION	Consumption of water per FU	$\frac{Consumption\ of\ water\ [L]}{FU\ [kg\ of\ livestock]}$
		$\frac{Consumption\ of\ water\ [L]}{FU\ [kg\ of\ carcass]}$
		$\frac{Consumption\ of\ water\ [L]}{FU\ [kg\ of\ meat\ product]}$
	Reuse of water [%]	$\frac{Water\ reuse\ [L]}{Water\ reuse\ [L]+Water\ consumption\ [L]} \times 100$
	Water quality index (WQI)	Ranking the WQI from 1–100 (depending on the legislation / methodology applied and parameters measured). The 'rule of the thumb' is 'the higher the WQI, value, the better the quality' of water.
	Discharge of wastewater per FU	$\frac{Discharge\ of\ wastewater\ [L]}{FU\ [kg\ of\ livestock]}$
		$\frac{Discharge\ of\ wastewater\ [L]}{FU\ [kg\ of\ carcass]}$
		$\frac{Discharge\ of\ wastewater\ [L]}{FU\ [kg\ of\ meat\ product]}$
	Wastewater quality index (WWQI)	Ranking the WWQI from 1–100 (depending on the legislation / methodology applied and parameters measured). The 'rule of the thumb' is 'the higher the WWQI value, the higher the quality of wastewater'.
	Water efficiency [%]	$\frac{Water\ consumption\ [L]}{Wastewater\ discharge\ [L]} \times 100$
7 AFFORDABLE AND CLEAN ENERGY	Consumption of energy per FU	$\frac{Consumption\ of\ energy\ [MJ]}{FU\ [kg\ of\ livestock]}$
		$\frac{Consumption\ of\ energy\ [MJ]}{FU\ [kg\ of\ carcass]}$
		$\frac{Consumption\ of\ energy\ [MJ]}{FU\ [kg\ of\ meat\ product]}$
13 CLIMATE ACTION	Global warming potential	$GWP = \sum_{i}^{n} GWP_i \, x \, m_i \, [kgCO_{2e}]$ m_i — mass of emitted gas (kg); GWP_i — global warming potential of the emitted gas.
12 RESPONSIBLE CONSUMPTION AND PRODUCTION	ALL OF THE ABOVE	

Legend: *FU* — Functional unit (livestock, carcass, meat / meat product); *GWP* — Global warming potential

supply chain, with more use of digital technologies, optimization and innovation of processing, including Food Industry 4.0 supported by efficient infrastructures [63]. This may be connected with SDG9 — Industry, motivation and infrastructure.

Finally, all stakeholders connected with the meat supply chain (legal authorities, inspection services, academia) should act as partners in improving this chain aligning to the motivation of achieving SDG 17 — Partnerships for the goals. A good example is the technical committee ISO/TC34 'Food products' [64] declaring that they contribute to the following SDGs (SDG1 — No Poverty, SDG2 — Zero Hunger, SDG3 — Good health and well-being, SDG5 — Gender equality, SDG8 — Decent work and economic growth, SDG10 — Reduced inequalities, SDG11 — Sustainable cities and communities, SDG12 — Responsible consumption and production, SDG13 — Climate action, SDG15 — Life on land and SDG16 — Peace, justice and strong institutions) with published food related standards.

Conclusion

Considering meat customers (retail / consumers), meat producers (farms / slaughterhouses / processors) and UN SDGs as cornerstones of an interactive triangle, the area within elevates a perspective of improvement opportunities in terms of sustainable production and consumption associated with SDG12. It is expected that this will pave the way in supporting sustainable technologies as well as sustainable diets promoting both sustainable and nutritional values associated with all types of meat and meat products and leaving the (meat) consumers with a free choice. In parallel, striving towards SDG17 — Partnerships for the goals enables deeper fulfillment of all UN SDGs and all stakeholders in the meat supply chain continuum. It is obvious that further attempts are needed to pave the way for fulfilling the UN SDGs' targets and exceeding expectation of all meat supply chain stakeholders.

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RESEARCH OF THE OVERALL CHEMICAL AND AMINO ACID COMPOSITION OF MEAT FROM YOUNG ANIMALS OF NEW SHEEP GENOTYPES

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Abstract
The paper presents the results of the investigation of the overall chemical composition (mass fraction of moisture, fat, protein and ash) and the amino acid composition of mutton from the experimental animals of new genotypes: Sovetsky Merino x Dzhalginsky Merino and Sovetsky Merino x Stavropolsky breed. As a control, meat of purebred Sovetsky Merino was studied. The experimental and control rams were slaughtered at the age of 8 months by the conventional technology. Meat samples for laboratory examination were taken a day after slaughter. The results of the analysis of the overall chemical composition showed that lamb meat of new genotypes had lower moisture content and higher content of fat and protein compared to the control. Analysis of the amino acid composition of meat proteins of genotypes Sovetsky Merino x Dzhalginsky Merino and Sovetsky Merino x Stavropolsky breed revealed lower content of essential amino acids compared to the control. Comparison of amino acid scores showed that the amino acid composition of meat proteins from the experimental animals was limited by valine and lysine, respectively. It was concluded by the results of the investigations of the amino acid composition of proteins that the biological full value of proteins of mutton from new genotypes was 22.9% and 30.3% lower than that of the control.

Introduction

Mutton has the specific chemical composition of muscle and fatty tissues, physico-mechanical and taste peculiarities [1–4]. Compared to pork, mutton has the higher content of protein but less fat. Mutton contains on average 18–20% of protein, 10–12% of fat, 0.85–0.95% of ash and 63–68% of water.

Mutton muscle tissue proteins contain all essential amino acids, which are balanced in the ratio that is most optimal for the human body. They include lysine, tryptophan, methionine, leucine, isoleucine, valine, threonine and phenylalanine. Lysine, tryptophan and methionine are considered most important [5].

Mutton is only slightly inferior to beef and pork in terms of the content of essential amino acids. The biological value, including the amino acid composition of meat from animals raised in various keeping conditions and with various feeding rations can be different [6,7].

Sheep are very mobile, energetic and strong animals adapted to traveling long distances in different terrains. In contrast to pork, mutton has the higher content of myoglobin, which is necessary to supply muscle tissue with oxygen; therefore, the muscle tissue color is more saturated and varies from red to brick-red [8,9].

One of the main benefits of mutton is its hypoallergenicity, which is beneficial when using this raw material in food production for child nutrition. Mutton and its components are widely used for production of specialized gerodietetic foods because of their high nutritional value and unique functional properties [10].

Analysis of the meat chemical composition gives the full picture of its quality characteristics. The meat chemical composition is determined by many factors: species, age, fatness, breed and gender of an animal, as well as other traits. Among various meat types, mutton and especially lamb have high palatability, good assimilability and dietetic properties [11].

Mutton is a valuable component of human nutrition being a source of animal protein. By the balance of fatty acids and amino acids, content of vitamins and minerals, it is not inferior to beef; and by caloricity, it is even superior [12,13,14,15].

Meat from lambs at the age of 6–8 months is considered the best. During the first 8 months of life, the most intensive deposition of the most valuable meat part (animal protein) takes place. As animals grow older, the carcass weight increases mainly due to fat deposition, which reduces the biological value of meat and economic efficiency of its production [16,17,18,19].

Over the last years, a special emphasis has been placed on meat direction of sheep husbandry, in particular, raising and selling of lambs in the year of birth to produce lamb meat [20].

Modern selection in sheep husbandry is based on the rational use of the existing genetic resources of breeding animals, effective and timely reproduction of the herd, production of animals with desired physiological and morphological characteristics and optimal level of economic traits [21,22].

The progress of the agricultural production to a great extent depends on rates of genetic improvement of existing

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productive and biological characteristics of farm animals and creation of new ones.

A degree of manifestation of these traits determining quantitative and qualitative properties of manufactured products from sheep and their economic significance depend on hereditary traits of sheep, paratonic conditions and technological methods for production of this product type [23,24,25,26,27].

The most important criteria for profitability of sheep husbandry are factors determining the genetic potential of mutton production [28,29].

In Russia, the further development of sheep husbandry should be aimed towards the rational use of the genetic potential of animal breeds that allows optimal use of feed, energy and financial resources to obtain high quality and ecologically pure products [30,31,32].

The growth in mutton production in our country is one of the reserves for increasing meat resources [33]. The variety of breeds and genetically isolated groups of sheep with the various degree of manifestation of individual productivity indicators and biological peculiarities create unlimited possibilities to increase production and improve quality of mutton.

Genetic diversity of local breeds can facilitate preservation of products that are economically necessary to improve sheep [34].

One of the ways to increase mutton output is creation of new sheep breeds and improvement of existing ones characterized by earliness and high meat productivity [35].

An increase in output of meat products in the country requires the search for ways of problem solution both by the selection genetic methods and by the technological methods. An important role in this regard is played by national breed resources that can ensure high realization of the genetic potential of productivity provided that necessary environmental conditions are created [20,36].

Nowadays, sheep breeding is performed mainly by peasant farm enterprises [37].

Sheep productivity is formed under the action of a genotype and environmental conditions. Therefore, the task of achieving high productivity of sheep comes down to the full use of the genetic potential with creation of the favorable environmental conditions [38,39,40].

Meat productivity of sheep is closely linked with the genetically conditioned heritability programmed for a certain potential of a sheep breed or type, as well as with the level of their feeding, keeping and handling system [41,42,43,44].

In foreign countries, mutton production specializes largely in intensive raising and fattening of lambs and their slaughter at the age of 8–9 months. The leading lamb meat producers are China, Australia, United Kingdom, France, USA and Bulgaria, where high quality lamb meat is produced when slaughtering fattened lambs. This meat is characterized by the high palatability and nutritional value, and is in great demand in the European and Japanese markets [45].

In Russia, the existing sheep population adapted to local climatic conditions mainly has the wool and wool-and-meat direction of productivity. Sheep of meat direction of productivity account for less than 10% [46,47].

The most common sheep breed in our country is the fine wool breed Sovetsky Merino, which is resistant of the extreme climatic factors. In the process of breeding of this variety, two intrabreed directions of productivity were developed, namely, wool and wool-and-meat.

Sheep of the wool direction have low indices of meat productivity and meat quality due to the fact that their genetic and biological potential was used, first of all, for production of wool, sheepskin and so on. Processing of such animals for meat has low efficiency. Meat packing plants that process such sheep often bear losses, especially, when processing lean mutton.

According to the experience of foreign countries, a promising direction is an improvement of the genetic structure of a herd by methods of complex assessment and early diagnostics of sheep productive qualities [48,49].

One of the ways to increasing meat productivity of sheep and mutton quality is two-way and three-way crossing. Practice of animal husbandry proves that superiority of hybrid animals over the parental forms prevails in an individual trait but not in the complex of traits [50].

Crossing is used on a large scale in our country and foreign countries such as Australia, USA, Germany, New Zealand and others. This is linked with the desire of sheep breeders to increase slaughter and meat quality of animals at the present stage [51].

In the modern conditions, the sheep husbandry direction oriented towards production of mutton and lamb meat has assumed great importance. The task of selection is to create such sheep. To perform this task it is necessary to know about the modern requirements for sheep husbandry products, including mutton, accumulated data on sheep and methods for implementation of these data into practice [22].

Prerequisites for sheep husbandry development that are formed in several regions of the country, including the Southern Federal District, together with social significance of this branch will facilitate its revival and sustainable development [52].

With that, there is deficiency of high quality mutton, which is evident from high prices that are not lower (and in many regions are higher) than prices on beef, which is still deficient.

The development of scientifically substantiated requirements for industrially suitable sheep and mutton by the meat processing industry is aimed towards the growth in meat productivity of animals and quality of produced meat, an increase in profitability of sheep breeding enterprises and meat processing plants, provision of the population with high quality mutton products, which is quite topical today. For example, similar requirements for pigs and meat [53], which were timely developed by VNIIMP

in 2007, facilitated, among other things, full provision of the population with domestic pork.

The aim of this study was to investigate quality indicators of mutton from animals of new genotypes to develop scientifically substantiated requirements for industrially suitable sheep and mutton.

Objects and methods

The object of the research was an average sample of meat from the whole ovine carcass.

The overall chemical composition of mutton was determined using the following methods: moisture mass fraction by GOST 33319–2015[1], fat mass fraction by GOST 23042–2015[2], protein mass fraction by GOST 25011–2017[3], ash mass fraction by GOST 31727–2012[4].

The amino acid composition of mutton was analyzed by GOST 34132–2017[5].

The amino acid score, a chemical method for detection of protein quality in foods, is based on comparison of the amino acid composition of products under study with the amino acid composition of the standard (ideal) protein.

The amino acid score for each of the amino acids was detected by the equation:

$$AS = \frac{C_{tested} \times 100\,\%}{C_{standart}}$$

where:

AS is the amino acid score, %,

C_{tested} is the content of the essential amino acid in 1 g of the tested protein, mg,

$C_{standard}$ is the content of the essential amino acid in 1 g of the standard protein, mg.

Simultaneously with analysis of the amino acid score, a limiting essential amino acid for a particular protein with the lowest score was determined.

Results and discussion

The study was carried out on hybrid young rams of the following genotypes:

Sovetsky Merino x Dzhalginsky Merino (SMxDzh, exp. 1) and Sovetsky Merino x Stavropolsky breed (SMxSb, exp. 2). As a control, purebred young rams of the Sovetsky Merino (SM) breed were used.

Young animals were raised up to the age of 8 months in the peasant farm enterprise Smorodin V. I. of the Tselinsky district of the Rostov region.

The experimental and control animals were slaughtered in the meat packing plant in the village of Razvilnoe (Peschanokopsky district, Rostov oblast).

Average samples of meat from the whole ovine carcasses were taken a day after slaughter from each of chilled carcasses held in refrigerating chambers at a temperature of 0–4 °C and humidity of 90%.

The overall chemical composition and the amino acid composition of the meat samples were analyzed in the laboratory conditions of the V. M. Gorbatov Federal Research Center for Food Systems of RAS (Moscow) and its North-Caucasian Branch (Rostov-on-Don).

The results of the overall chemical composition of meat from lambs of new genotypes are presented in Figures 1–4.

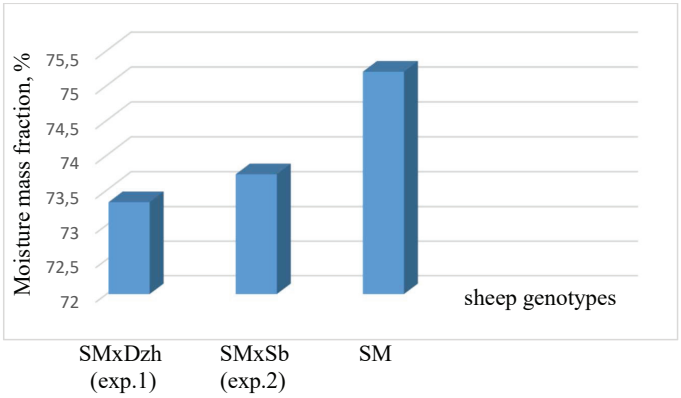

Figure 1. Moisture mass fraction in meat from sheep of new genotypes, %

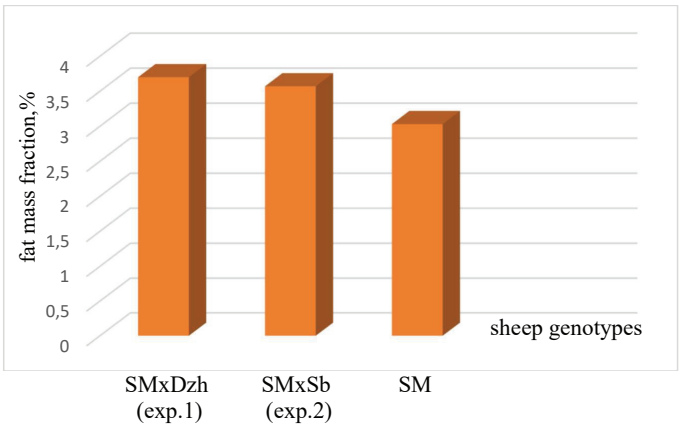

Figure 2. Fat mass fraction in meat from sheep of new genotypes, %

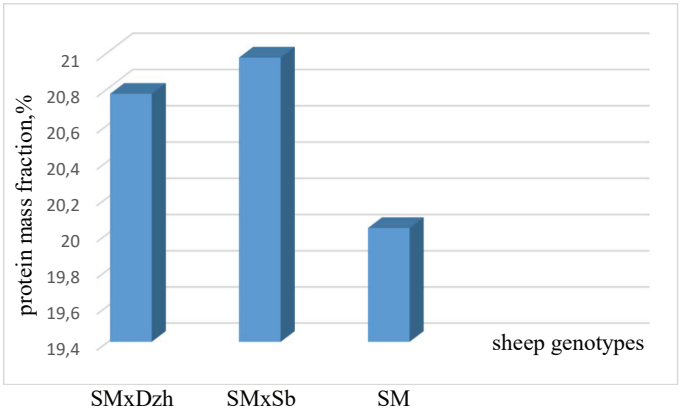

Figure 3. Protein mass fraction in meat from sheep of new genotypes, %

[1] GOST 33319–2015. "Meat and meat products. Method for determination of moisture content". Moscow: Standartin-form, 2018. — 14 p. (In Russian)

[2] GOST 23042–2015 "Meat and meat products. Methods of fat determination". Moscow: Standartinform, 2019. — 8 p. (In Russian)

[3] GOST 25011–2017 "Meat and meat products. Protein determination methods". Moscow: Standartinform, 2018. — 14 p. (In Russian)

[4] GOST 31727–2012 (ISO 936:1998) "Meat and meat products. Determination of total ash". Moscow: Standartinform, 2019. — 11 p. (In Russian)

[5] GOST 34132–2017 "Meat and meat products. Determination of amino acids composition of animal protein". Moscow: Standartinform, 2017. — 16 p. (In Russian)

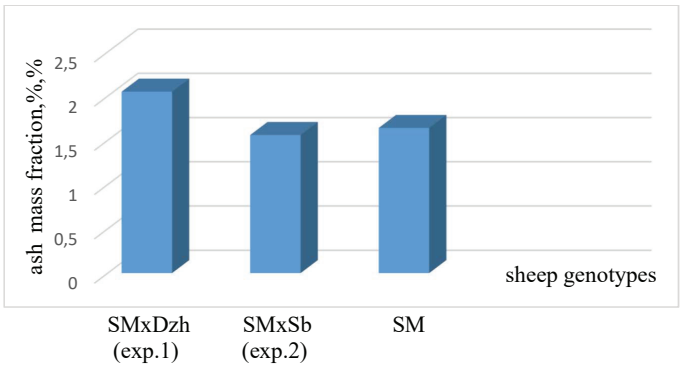

Figure 4. Ash mass fraction in meat from sheep of new genotypes, %

It was established that compared to the control, meat from sheep of new genotypes (in experiments 1 and 2) had:

- lower moisture content by 1.87% and 1.47%, respectively (Figure 1);
- higher fat content by 0.67% and 0.54%, respectively (Figure 2); the maximum fat content in mutton was in experiment 1 (3.70%);
- higher protein content by 0.74% and 0.94%, respectively (Figure 3); the maximum protein content in mutton was in experiment 2 (20.97%).

Meat from sheep of new genotypes (Figure 4) had the higher ash content (by 0.41%) in experiment 1 and lower ash content (by 0.08%) in experiment 2 compared to the control.

The mean values of the amino acid composition of meat from young animals of new genotypes are presented in Table 1.

Table 1. Mean values of the amino acid composition of proteins in meat from young rams of new genotypes

Amino acids	Content, g/100g meat			Content, g/100g protein		
	SM	SMxD zh	SMxSb	SM	SMxDzh	SMxSb
Essential amino acids	8.00	6.70	6.00	40.0	32.4	28.7
including:						
Valine	1.00	0.70	0.70	5.00	3.4	3.3
Isoleucine	1.30	0.90	0.90	6.5	4.3	4.3
Leucine	1.60	1.10	0.90	8.0	5.3	4.3
Lysine	1.00	0.80	0.70	5.0	3.8	3.3
Methionine	0.60	0.50	0.30	3.0	2.4	1.5
Threonine	1.50	1.60	1.50	7.5	7.7	7.2
Tryptophan	0.30	0.30	0.30	1.5	1.4	1.5
Phenylalanine	0.70	0.80	0.70	3.5	3.8	3.3
Non-essential amino acids	12.00	14.10	14.90	60.0	67.9	71.3
including:						
Alanine	1.40	1.00	0.90	7.0	4.8	4.3
Arginine	1.00	0.70	0.50	5.0	3.4	2.4
Aspartic acid	1.90	2.60	3.50	9.5	12.5	16.8
Histidine	0.60	0.70	060	3.0	3.4	2.9
Glycine	1.50	1.90	180	7.5	9.1	8.6
Glutamic acid	2.90	4.60	5.10	14,5	22.2	24.4
Proline	0.80	0.70	0.70	4.0	3.4	3.3
Serine	1.10	1.20	1.20	5.5	5.8	5.7
Tyrosine	0.50	0.40	0.40	2.5	1.9	1.9
Cysteine	0.30	0.30	0.20	1.5	1.4	1.0
Amino acids in total	20.0	20.8	20.9	100.0	100.0	100.0

The results of the analysis of the amino acid composition in mutton from sheep of new genotypes showed that the content of the essential amino acids in protein of meat from the experimental animals of the genotypes SMxDzh and SMxSb was lower than that in protein of meat from purebred SM (control) by 7.6% and 11.3%, respectively.

The amino acid scores for each of the essential amino acid calculated according to the method described above are presented in Table 2.

Table 2. The amino acid score of protein in meat from young rams of new genotypes

Amino acid	Content in the ideal protein, mg/g	Content in mutton protein, mg/g			Amino acid score (AS), %		
		SM	SMxD zh	SMxSb	SM	SMxDzh	SMxSb
Isoleucine	40	65	43	43	162.5	107.5	107.5
Leucine	70	80	53	43	114.3	75.7	61.4
Lysine	55	50	38	33	90.9	69.1	60.0
Methionine + cystine	35	45	38	25	128.6	108.6	71.4
Phenylalanine + Tyrosine	60	60	57	52	100.0	95.0	115.4
Tryptophan	10	15	14	15	150.0	140.0	150.0
Threonine	40	75	77	72	187.5	192.5	180.0
Valine	50	50	34	33	100.0	68.0	66.0
Limiting acid					lysine	valine	lysine

It can be seen from Table 2 that:

1. Proteins of all experimental (SMxDzh and SMxSb) and control (SM) mutton samples had the imbalanced essential amino acid content compared to the "ideal" protein recommended by FAO/WHO.
2. The amino acid compositions of proteins in the experimental mutton samples were not balanced by the following four amino acids: SMxDzh by valine, phenylalanine, leucine and lysine; SMxSb genotype by valine, methionine, leucine and lysine compared to the "ideal" protein.
   The amino acid composition of the control mutton sample (SM) was not balanced only by one amino acid (lysine).
3. The amino acid composition of proteins in meat from the SMxDzh genotype was limited by valine (AS=68.0%), the SMxSb genotype by lysine (AS=60.0%) and purebred young animals by lysine (AS=90.9%).
4. The biological full value of proteins from the experimental mutton samples obtained from new genotypes of sheep (SMxDzh and SMxSb) was lower than in the control sample (SM) from the purebred animals by 22.9% and 30.9%, respectively.

Several information sources indicate that mutton protein is not lysine deficient [54,55]; on the contrary, its content is significantly higher than the level recommended by FAO/WHO (more than 170%).

In an attempt to explain the obtained results of the investigation and quite a low content of lysine in the protein

from all three sheep genotypes, we proceed from the fact that they all belong to the wool direction of productivity and are classified with fine-fleece sheep.

It is known that the genetic and biological potential of fine-fleece sheep is directed mainly towards "production" of fine wool according to the previous selection work.

We link the low lysine content in meat from lambs of the wool direction of productivity, to which Sovetsky Merino belong, with the increased use of this "building material" during coat formation in young animals, which is of top priority for this direction of productivity.

The main protein component of ovine wool is collagen, which is a protein with the complex amino acid composition containing essential amino acid lysine among others.

It was found that ovine wool achieves its optimal quality at the age of about 12 months.

In lambs, changes in several quality indicators (for example, strength) occuring in wool are associated with changes in collagen during animal growth.

Recent studies [56] established that ovine wool of young purebred and hybrid animals of the précoce breed at the age of 8 months contains collagen with the high level of lysine, which decreases by the age of 12 months as animals grow. For example, the content of lysine in protein from animals of the meat-and-wool type at the age of 8 months is 7.15%-9.00%, at the age of 12 months 5.10%-5.50%. Similarly, it is 7.25%-8.52% and 6.04%-6.40% in animals of the wool-and-meat type at the age of 8 months and 12 months, respectively.

There is no doubt that a decrease with advancing age in the lysine expenditure on formation of hair collagen in animals enables increasing availability of this essential amino acid for construction of other proteins of the body, including meat (muscle) proteins.

Extrapolation of data obtained by sheep breeders when studying the amino acid composition of wool from young purebred and hybrid sheep of the précoce breed allows us to explain a low level of lysine in meat from young rams in our experiment and assume that the lysine deficiency in meat can decrease with age.

The result of the human selection activity, that is artificial selection, is not the only reason for this phenomenon. In our opinion, all this is linked with the natural selection.

Lambing takes place, mainly, in the second half of winter — at the beginning of spring. There is less than a year before the following winter. To survive during winter, lambs have to prepare well: grow, become stronger, gain superficial fat and, first of all, strong, warm wool cover.

In the course of the natural selection, individuals that had not prepared to winter died and those that had acquired necessary quality of fleece due to mutations survived. The genetic potential was directed exactly towards this with mobilization of all resources for this top priority task.

In connection with the new revealed circumstances, it was necessary to check our assumption by the experimental way.

Taking into account the available information about the fact that the deficiency of lysine, which is abundant in nuclear proteins (protamines and histones) causes retardation of the growth in the protein biosynthesis (and it is actually observed as our experiments show), it was necessary to do an experiment on inclusion of this amino acid into the feeding ration of animals not only for improvement of the biological full-value of meat proteins but also for possible acceleration of the growth of young sheep and improvement of meat productivity.

Industrial production of synthetic amino acids and their rational theoretically substantiated use in rations of farm animals is considered to be one of the most topical problems [57].

Inclusion of synthetic amino acids into the ration of growing young sheep in amount of 6–8g of lysine and 3–4 g of methionine per one feeding unit facilitates the enhancement of the redox processes, increase in productivity and other indicators [56].

In particular, it is possible to use the feed additive "Lysine sulfate" containing the active substance lysine in the quantity of not less than 70%, as well as vitamins, microelements and other amino acids (methionine + cystine 0.35%; methionine 0.30%; threonine 0.56%; tryptophan 0.10%; arginine 0.56%; isoleucine 0.39%; leucine 0.63%; valine 0.53%; alanine 0.75%; glycine 0.50%; serine 0.31% and aspartic acid 0.80%).

Lysine will facilitate assimilation of phosphorus, calcium and iron by the body, an increase in the hemoglobin content in blood, assist the digestive processes, improve biological value of food protein and rations in general.

Conclusion

The following conclusions were made based on the results of the analysis of the chemical and amino acid composition of meat from young animals of new genotypes:

1. Compared to the control, meat from sheep of the new genotype (SMxDzh, experiment 1) had:
   — lower moisture content by 1.87%;
   — higher fat and protein content by 0.67% and 0.74%, respectively;
   — lower content of essential amino acids by 7.6%;
   — amino acid composition of proteins limited by valine (AS = 68.0%);
   — lower biological full value of proteins from mutton of the new genotype by 22.9% compared to the control;
2. Compared to the control, meat from sheep of the new genotype (SMxSb, experiment 2) had:
   — lower moisture content by 1.47%,
   — higher fat and protein content by 0.54% and 0.94%, respectively;
   — lower content of essential amino acids by 11,3%;
   — amino acid composition of proteins limited by lysine (AS = 60.0%);
   — lower biological full value of proteins from mutton of the new genotype by 30.9% compared to the control.
3. Analysis of the amino acid composition of protein from all experimental and control meat samples indicate a deficiency of lysine compared to the "ideal protein".

4. It was assumed that the low content of lysine in young animals is linked with the peculiarity of hair coat formation at the age of 8 months, which is characterized by an increased use of lysine for building wool collagen.
5. A method was proposed for improvement of lamb quality by using the feed additive "Lysine sulfate", which allows checking this hypothesis experimentally.

The obtained results of the analysis of the overall chemical and amino acid composition of meat from lambs of new genotypes will be used for the development of the scientifically substantiated requirements for industrially suitable sheep and mutton.

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QUALITY OF CARCASSES AND MEAT FROM MALE AND FEMALE RABBITS

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Keywords: *oryctolagus cuniculus, meat quality, colour, cooking loss, tenderness, gender*

Abstract
Meat from rabbits offers high nutritive properties as it has high levels of essential amino acids, minerals and vitamins. Rabbit meat is also regarded lean, since it contains less fat than many other meat types. The composition of rabbit meat lipids is also favourable due to higher contents of unsaturated fatty acids compared to other types of meat. The aim of the present study was to examine and compare meat quality from male and female rabbits. A total of 24 rabbits (12 males and 12 females) were used. The rabbits were slaughtered at an age of 17–18 weeks. The following parameters were studied: slaughter traits, colour, pH, cooking loss, tenderness, total fat content and water holding capacity (WHC). Interestingly, the ultimate pH was significantly lower in male compared to female rabbits ($p = 0.008$). Moreover, cooking losses were higher in the meat from male rabbits ($p = 0.001$). No other relevant differences were found between meat from male and female rabbits suggesting that sex might be regarded as a minor factor in the production of rabbit meat at the chosen slaughter age.

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Introduction

Nowadays, interest in a healthy and sustainable lifestyle and particularly a healthy diet is constantly growing. A Healthy diet became a priority in sustainable production and consumption because of the increasing number of scientific evidences linking quality and quantity of food consumption to human health. Consumer's demands for food products with low content of saturated fat pushed industry and researchers to find alternative food sources and continue investigations on underestimated traditional food sources. Rabbit meat was repeatedly suggested as a functional food and as an attractive part of a healthy, diet, mainly due to the high nutritional value [1,2]. Indeed, rabbit meat is characterized by high contents of proteins and oleic acid, and a low-fat content [3–5]. Rabbit meat is also characterized by lower calory content (approximately 618 kJ/100 g fresh meat) compared to red meats [6]. Additionally, rabbit meat consumption might be important role in fighting food shortage in some countries. New rabbit-based products are continuously being developed to satisfy the consumer's needs and promote rabbit meat on the market [5]. Another important issue to consider is the low environmental impact from raising rabbits compared to raising of cattle and some other livestock species. Thus, it can be regarded as is a sustainable choice compared to red meat [7]. However, compared to other species, our knowledge on the effects of slaughter age and rabbit sex on productive performance, slaughter traits and carcass quality is still limited. Knowledge on the relationship between rabbit welfare and meat quality is also fragmentary. Similar to other types of meat, quality of rabbit meat is affected several factors, including age and sex of the animals, genetic propensity of the animals, rearing conditions and nutritional status [8–10]. Animal welfare and health status are also important determinants of the final meat quality [9].

The objectives of the present study were to examine and compare meat quality from male and female rabbits. The following parameters were studied: slaughter traits, colour, pH, cooking loss, tenderness, total fat content and water holding capacity (WHC).

Objects and methods

Animals raising and slaughter

An equal number of male and female rabbits of a crossbreed of Champagne d'Argent and New Zealand red ($n = 24$) were raised at a conventional farm located in Avesta Krylbo, Sweden. The animals were fed 1–1.5 dl of the commercial feed Kalvstart from Edel (Table 1) per animal and day and had free access to water and hay. The rabbits were also occasionally provided with fresh branches, grass, fruits and vegetables. The male rabbits were housed in a 10×4 m pen, while the female rabbits were kept in a 7×3 m pen.

The rabbits were slaughtered at an age of 17–18 weeks on the farm at the same day, using a captive bolt pistol for anesthetization, followed by severing of the carotid arteries and jugular vein. The body weights were recorded after

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bleeding. The carcasses were placed in a cold room and pH (pH1) was measured 1 hour after slaughter using a Knick Portamess 913 X pH (Berlin) pH-meter with a penetrating electrode. The pH-meter was adjusted to muscle temperature at each measurement. The meat was then packed into plastic bags and transported to a laboratory at Swedish University of Agricultural Sciences for further analyses. Colour and ultimate pH were investigated on fresh meat within 24 hours after slaughter, carcasses were stored at 4 °C overnight. The samples for all other analyses were stored at minus 20 °C until analyses.

Table 1. Composition of the feed Kalvstart from Edel

Item	Value	Units
Energy	13	MJ
Protein	200	g
Fat	45	g
Starch	270	g
Neutral detergent fiber	255	g
Dry matter	87	%
Calcium	11	g
Phosphorus	8	g
Magnesium	3.0	g
Vitamin A	10000	IE
Vitamin D	2800	IE
Vitamin E	60	mg
Copper	7	mg
Selenium	0.5	mg

The experiment on animals, including their housing, welfare, care and all manipulations, were run in compliance with the Council Directive 86/609/EEC of November 24, 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes.

Measurements of meat colour and pH

The colour was measured in three sites on the surface of the inside of the loin using Chroma Meter CR-300 Minolta 1991 (Singapore). To evaluate the colour, we used L*a*b* system, where the L* value designates lightness, while a* and b* are colour coordinates (+a* = redness, minus a* = green, +b* = yellow, minus b* = blue). Ultimate pH was determined 24 hours post mortem using a Knick Portamess 913 X pH (Berlin) pH-meter with a penetrating electrode. The pH-meter was adjusted to muscle temperature.

Fat content of meat

For determination of fat content, the fat was extracted as previous described [11] with slight modifications. Briefly, approximately 2 g of rabbit meat was chopped and homogenized with 15 ml 3:2 (v/v) hexane: isopropanol, (HIP) using Ultra-turrax (T25 basic, IKA-Werke, Germany). The samples were transferred to a Teflon tube, and 12 ml of Na_2SO_4 were added, the tubes were well shaken and subsequently centrifuged at 1079 g for 5 minutes using a Sorvall Super T21 (SL-50T rotor, Newtown, Connecticut, USA.). Fat content was determined gravimetrically from the separated upper phase after evaporation of the Hexan.

Water holding capacity (WHC)

WHC was analysed by putting the samples between two filter papers (Munktell 00A 125mm Ø) under a weight of 2000 g for 10 min. Final weight of the sample was recorded as compared to initial weigh before the press was applied. WHC was calculated as following:

$$WHC = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Initial weight}} \quad (1)$$

Cooking loss and tenderness

To evaluate the cooking loss, the loin samples were thawed in 4 °C overnight, kept in room temperature for 3 hours and weighted prior to cooking in a water bath (Tectron-Bio 100, Barcelona) in vacuum bags, at 70 °C for 120 min with constant circulation. Then, the samples were cooled under running water for 30 min. The samples were removed from the vacuum bags, dried with paper and weighted. Cooking loss was calculated as following:

$$\text{Cooking loss (\%)} = \frac{(\text{Weight prior to cooking} - \text{Weight post cooking})}{\text{Weight prior to cooking}} \quad (2)$$

The tenderness was evaluated using the Warner-Bratzler shear test. Right and left longissimus dorsi muscles were dissected and three pieces from each muscle were cut along the fibre direction in 10 x 10mm. The shear force was measured using TA.HDi Texture analyser (Hamilton, MA) with a shear blade. The load cell was 50 kg and the speed 50–100 mm/min. Both maximum and total forces were recorded.

Statistical analyses

Statistical analysis was performed with the Statistical Analysis System, version 9.4 (SAS Institute, Cary, NC, USA). The Shapiro–Wilk and Kolmogorov–Smirnov tests as well as visual evaluation of the data were used to investigate normality of data distribution. Eventual sex-related difference in measured parameters were estimated using the general linear model (GLM). The relationships between measured parameters were estimated using Pearson's correlation coefficients. The level of statistical significance was set at $p<0.05$.

Results and discussion

The major characteristics of meat from male and female rabbits are presented in Table 2. The relationships between the measured traits are presented in Table 3. Majority of characteristics were similar in male and female rabbits ($p>0.05$; Table 2).

The rabbits in the present study were slaughtered at an age of 17–18 weeks. One of the limitations of the study is that maturity stage was not evaluated. Since rabbits of both

sexes often are slaughtered at the same age, male and female rabbits might be at different maturity stages, which might result in difference carcass weight and other related characteristics. In many species, males have a higher growth potential compared to females. However, weights of male and female rabbits (at least prior to adult weight) are usually similar. This was also demonstrated in several previous studies on rabbits [10,12]. The slaughter weights in the present study did not differ between male and female rabbits. Slaughter weight at 17–18 weeks (119–126 days) was higher than found by Viliene et al [13] in Californinan rabbits raised up to 112 days, which might be due to difference in breed but also due to a longer rearing period. Carcass yield was higher in the study by Viliene, which however might be affected by different cutting methods, as traditions might be different in different countries.

Interestingly, the pH 24 hours post slaughter (ultimate pH) was significantly lower in male compared to female rabbits ($p = 0.008$). In contrast, no significant differences between male and female rabbits were observed in other studies [14,15]. The ultimate pH in the present study ranged from 5.6 and 6.2. It was previously suggested that this pH might indicate that rabbit meat has a poorer shelf life than meat of other types [16]. Generally, the ultimate pH is affected by various factors, including pre- and post-slaughter conditions, type of muscle and individual animal characteristics. A low ultimate pH leads decreased WHC, increased drip losses and greater cooking losses. Indeed, in the present study, cooking losses were higher in the meat from male rabbits ($p = 0.001$) and the ultimate pH was negatively correlated with cooking losses ($p = 0.007$). However, no sex-related differences in WHC were observed ($p = 0.252$). The same is true for tenderness ($p = 0.262$) and fat content ($p = 0.849$). Similar results were found previously by Daszkiewicz [17], were WHC differed between breed but not due to sex.

The colour of meat is related to pH. In the present study, the ultimate pH was negatively correlated to b* values (yellowness) ($p = 0.002$). The same results were previously observed on broiler breasts [18].

Table 2. Meat characteristics from male and female rabbits

Parameters	Male rabbits, n = 12	Female rabbits, n = 12	p-value
Carcass weight (kg)	1.7 ± 0.07	1.7 ± 0.07	0.972
Slaughter ratio (%)	52.5 ± 0.83	53.1 ± 0.68	0.586
pH 1 h post mortem	6.5 ± 0.07	6.5 ± 0.07	0.582
Ultimate pH	5.8 ± 0.05	6.0 ± 0.05	0.008
L*	44.8 ± 0.54	45.2 ± 0.54	0.615
a*	minus 0.6 ± 0.44	minus 1.7 ± 0.44	0.087
b*	18.6 ± 0.26	17.9 ± 0.26	0.289
Water-holding capacity (%)	16.2 ± 1.01	14.5 ± 1.01	0.252
Cooking loss (%)	14.6 ± 0.47	10.1 ± 0.45	0.001
Tenderness (force, N)	10.4 ± 0.76	11.6 ± 0.76	0.262
Fat%	1.1 ± 0.05	1.1 ± 0.06	0.849

Data are presented as least square mean and standard error, obtained from the GLM model with the fixed effect of sex.

The relationships between WHC and intramuscular fat have been extensively studied. Many studies have indicated a relationship between intramuscular fat content and the sensory traits such as "tenderness" and "juiciness" in meat of different types [19,20]. In the present study, fat contents were similar in meat from male and female rabbits and were not related to any of other parameters (data not shown). However, some other studies observed that the total amount of adipose tissue in females tended to be higher compared to males [21,22]. A discrepancy between the results from different study might be due to the different meat types and analytical methods.

Table 3. Pearson correlation coefficients between the ultimate pH, colour characteristics, water-holding capacity, cooking loss and tenderness of rabbit meat

	L*	a*	b*	Water-holding capacity	Cooking loss	Tenderness
Ultimate pH	minus 0.28	minus 0.38	minus 0.59**	minus 0.26	minus 0.55**	minus 0.01
L*		minus 0.47*	0.78***	0.43*	0.15	0.23
a*			-0.09	0.04	0.32	minus 0.16
b*				0.31	0.38	0.21
Water-holding capacity					0.45*	minus 0.07
Cooking loss						minus 0.14

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

The comparison of our results with other studies shows that significant difference might be found in different breeds but not sex [17].

Practically, our results imply that rabbits of both sexes can be used for meat production without any relevant effects on carcass characteristics and meat quality. However, more research should be focused on the effects of other parameters of rabbit quality such as diet and stress-related factors, including raising conditions and transport.

Conclusion

Generally, our study showed no or irrelevant differences in meat from male compared to female rabbits, in contrast to other type of meat, such as beef or pork, where the sex-related difference are more notable. Thus, sex might be regarded as a minor factor which affect production of rabbit meat from with crossbreed Champagne d'Argent and New Zealand red slaughtered at 17–18 weeks. Additionally, small and no differences in majority of slaughter traits and meat quality parameters suggest that it is not necessary to raise male and female rabbits to a separate age. Other factors such as breed, age/slaughter weight, transport to slaughter and feeding regime should be further studied.

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AN EFFECT OF FOOD ADDITIVES ON MICROBIOME

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Keywords: *food additives, sweeteners, microbiome, microbiota*

Abstract

The paper presents a review of available data about an effect of food additives on the human microbiome and lists the main physiological functions of the gut microbiome. The process of the human microbiome evolution is examined. The relationship between the emergence of a disease and the microbiome composition, as well as the main factors influencing the gut microbiome composition are described. The main food additives used today are listed, their key features are discussed and their structural formulas are given. The information about their effect on the human body through an influence on the microbiome composition is presented. The data on an effect of polysorbate 80, carboxymethylcellulose, sodium sulfite, nisin, potassium sorbate, sodium benzoate, sodium nitrate, essential oils, titanium dioxide and different sweeteners on the microbiome are analyzed. It is explained what microbial communities are suppressed and what communities gain advantages in multiplication when consumers eat food with one or another food additive. The consequences of alterations in the microbiome for the consumer's body are examined. Conclusions were made about the necessity of additional studies about an effect of food additives on the composition of the human microbiome.

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Introduction

A type and diversity of consumed food significantly affect the human microbiome composition. Changes in the food composition can lead to alterations in the metabolic pathways and immune processes in the consumer's body [1]. The human gastrointestinal tract is inhabited by various symbiotic microorganisms. They colonize mainly the colon; with that, a ratio of the microbiome cells to cells of the host organism is 1:1 [2]. It is believed that microbiota is in the mutually beneficial relationships with its host taking part in various metabolic and immune processes [3]. The complex microbial ecosystem is closely linked with the host health [3,4]. Alterations in the gut microbiome composition can be associated with metabolic disorders, inflammation and even with neurological diseases [5,6]. The microbiome composition alters along the gastrointestinal tract forming specific regional communities [7]. It is agreed today that the microbiome composition includes, at least, 1000 species [8]. With that, *Firmicutes* and *Bacteroidetes* are two predominant phyla representing Gram-positive and Gram-negative bacteria colonizing the mammalian gastrointestinal tract. They account for 90% of total bacterial counts in the intestine [9]. A ratio between the number of members of these two phyla can vary depending on the individual peculiarities of the host organism, but total proportions are similar in the majority of people [10]. Among other members, the human microbiome also includes *Fusobacteria*, *Verrucomicrobia, Actinobacteria, Proteobacteria* and several species of archaea [9]. Moreover, researchers emphasize the importance of the presence of *Bifidobacterium*, *Clostridium*, *Ruminococcus*, *Lactobacillus*, *Streptococcus*, *Bacteroides* and *Escherichia* [11]. It is believed that the diet that includes increased consumption of legumes, cereals, fruit and vegetables is beneficial for the consumer's body [12,13]. There is a trend towards an increasing presence of artificial sweeteners in our diet [14]. Nowadays, an effect of food additives on the consumer's body has been comprehensively studied. However, food additives can also affect the body indirectly by influencing the gut microbiome [4,15]. Experiments on animals show that food additives can have an adverse effect on the colon and cardiovascular system [1]. It was demonstrated that food emulsifiers such as polysorbates and carboxymethylcellulose can increase the intestine permeability, alter the microbiota composition, and facilitate penetration of Escherichia coli through epithelium [16].

In the Russian Federation, the use of food additives is regulated by Federal Laws № 29-FZ[1], № 52-FZ[2] and Technical Regulations of the Customs Union TR CU029/2012 "On the safety for food additives, flavorings and technological aids"[3]. The list of food additives permitted in Russia is approved by the Ministry of Health of the Russian Federation and the state control of their quality is performed by the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor).

[1] Federal Law No. 29-FZ on food quality and food safety. (In Russian)
[2] Federal Law No. 52-FZ on sanitary and epidemiological well-being of the population. (In Russian)
[3] TR CU029/2012 Technical Regulations of the Customs Union "On the safety for food additives, flavorings and technological aids" Retrieved from https://docs.cntd.ru/document/902359401. Accessed April 15, 2021. (In Russian)

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The available data on artificial sweeteners show disorders of the metabolic processes in rodents due to the disturbance in the microflora balance [17,18]. Therefore, it is important to study an effect of food additives on the gut microbiota composition [19]. Nowadays, there is limited knowledge about an effect of food additives on the human gut microbiota as available studies were carried out mainly on animal models [20].

Interrelation between the gut microbiome composition and diseases

Disturbance of the balance in the gut microbiome composition is closely linked with the development of many human diseases, such as obesity, diabetes, cardiovascular and inflammatory pathologies [5]. It was shown that an increase in the number of *Streptococcus* and *Enterobacteriaceae* in the intestine is typical for the atherosclerotic cardiovascular diseases [21]. Also, the number of *Faecalibacterium prausnitzii* and *Lactobacillus* in patients with type 2 diabetes mellitus was significantly lower than in healthy individuals. The number of *Bifidobacterium* was significantly higher than in healthy individuals [22]. Probably, imbalance of the gut microbiota composition influences the cancer development [1]. For example, the increased number of *Bacteroides massiliensis* was observed in patients with prostate cancer, while the number of *Eubacterium rectale* and *Faecalibacterium prausnitzii* was lowered [23].

Metabolites of bacteria from the gut microbiome can be associated with human diseases. For example, arginine can be transformed into glutamate and then deaminated to gamma-aminobutyric acid, which as a neurotransmitter. Alterations in the expression of receptors of this neurotransmitter are linked to the development of anxiety and depression [24]. Another example is lysine, which can be metabolized with the formation of cadaverine, which increased level can be associated with ulcerative colitis [25,26]. However, it is necessary to note that in several cases, the above mentioned pathological mechanisms are a consequence of metabolic disorders.

Physiological functions of the gut microbiome and its effect on the human health

The gut microbiome affects the physiological processes in the human body mainly through microbial metabolism. Microorganisms in the gastrointestinal tract can break down complex carbohydrates, proteins and some fats [1]. They are also capable of producing various enzymes that take part in metabolism [27]. The microbiome produces many metabolites that can enter blood and act throughout the whole body. Short-chain fatty acids, alcohols, ammonia, fatty acids, amines, sulfur compounds, phenols, indoles, glycerol derivatives, carbon dioxide and hydrogen are among such metabolites [1,27]. For example, the physiological functions of short-chain fatty acids are extremely important as they affect functioning of the epithelial cells of the colon [28]. Also, microorganisms take part in catabolism of amino acids and lipids [1].

In addition to the influence on metabolism, the gut microbiome is also important for formation of the human immunity, especially for the development and regulation of the immune system as the body develops [3,4]. The gut microbiome interacts with the immune system sending "signals" that facilitate the differentiation of the immune cells and immunity development. Moreover, it influences the antibody production, T-cell differentiation and enhancement of the phagocytic function of macrophages [29]. The gut microbiome also facilitates maintenance of the integrity of the intestinal epithelial cells [1,30].

Thus, the gut microbiome contains a large diversity of different bacterial genomes and can produce a wide range of metabolites. These metabolites and components of bacterial cells are important for the host organism as they are associated with the physiological development and maintenance of the innate and adaptive immunity.

Factors influencing the gut microbiome composition

The diversity of the human gut microbiome is influenced by various factors such as intake of antibiotics, age, stress and climatic conditions [23]. Factors are divided into dietary and non-dietary [1]. As for non-dietary factors, age is one of the factors affecting the gut microbiota composition. For example, *Clostridium* and *Bacteroides* dominate in the gastrointestinal tract of elderly people, while the number of *Bifidobacteria* is reduced [31]. An effect of dietary factors on the microbiota has been widely studied [1–10]. It is shown that a diet rich in simple carbohydrates leads to multiplication of *Proteobacteria* and *Firmicutes,* while a diet rich in fats leads to a decrease in the intestinal microbial diversity. Food rich in animal protein and saturated fats promotes the development of *Actinobacteria* and *Bacteroidetes* in the intestine [32,33].

Therefore, changes in nutrition affect the gut microbiome diversity.

Evolution of the human gut microbiome

During the whole human life, the human gut mirobiome demonstrates continuous dynamic changes that are manifested in constant evolution and adaptation [34]. In the first month after birth, the gut microbiome diversity in infants is very low. By the sixth month, the composition and number of cells of the gut microbiota significantly increase due to an increase in the variety of consumed food [31]. With the cessation of breastfeeding, the nutrition structure changes and consumption of carbohydrates by microorganisms increases. More short-chain fatty acids and vitamins metabolized by microorganisms appear in food [34]. In the course of time, the gut microbiome dominated by *Bifidobacteria* in infants transforms into the gut microbiome of adults with domination of *Firmicutes* and *Bacteroides* [35]. At the age of 2.5–3 years, rapid increase

in the bacterial diversity is significantly retarded and the gut microbiome composition gradually achieves the adult condition by the age of 7–12 years [34].

Acesulfame potassium

Acesulfame potassium is also known as acesulfame (E950). It is an acidic cyclic sulfonamide derivative, which is 200 times sweeter than sucrose (Figure 1). The acceptable daily intake of acesulfame is 15 mg/kg body weight/ day; 95% of this amount is fully excreted in urine after passing through the human digestive system [36]. A study of an effect of acesulfame potassium on the gut microbiota composition in mice showed that disorders of the metabolic pathways and intestinal microflora occurred after four weeks of its consumption. These disorders significantly differed depending on a gender. In male mice, the body weight significantly increased, the expression of the functional bacterial genes related to the carbohydrate and energy metabolism enhanced. In female mice, the body weight did not change significantly but several bacterial metabolites such as 2-oleoylglycerol, succinic acid and D-lactic acid were reduced. Moreover, the abundance of *Oxalobacteraceae*, *Clostridium*, *Lactobacillus* and *Ruminococcaceae* decreased, while the abundance of *Mucispirillum* increased [37].

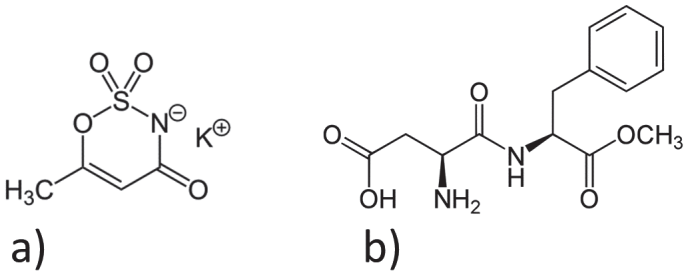

Figure 1. Structural formula of acesulfame potassium (a) and aspartame (b)

However, another study showed that acesulfame at doses equivalent to the human acceptable daily intake (ADI) did not significantly change the gut microbiome composition in mice. The abundance of *Clostridium IV, Clostridium IVXa*, *Bacteroides* and *Firmicutes* was the same in the experimental and control groups. [38]. Frankenfeld et al. [39] studied an effect of acesulfame potassium on the human gut flora. As a result, no significant differences were revealed between medians characterizing the number of bacteria in consumers and non-consumers of acesulfame. The ratio of *Bacteroidetes* to *Firmicutes* was also the same [1,39].

Aspartame

Aspartame (L-α-Aspartyl-L-phenylalanine methyl ester, E951) is 180 to 200 times sweeter than sucrose. Its acceptable daily intake (ADI) is 40 mg/kg body weight. The most part of aspartame is fully hydrolyzed in the intestine with formation of phenylalanine, methanol and aspartic acid [40]. Although many studies on aspartame safety for humans were carried out, little attention was given to an effect of aspartame intake on the human gut microbiome composition [1]. Palmnas et al. [41] reported about an effect of low aspartame doses (5–7 mg/kg body weight/ day) on metabolism and the gut microbiota in rats with and without diet induced obesity. For example, aspartame intake led to an increase in *Clostridium leptum* and *Enterobacteriaceae*, and an increase in the members of *Roseburia* was observed in rats with diet induced obesity. Compared to the control group, the gut microbiota composition in rats with obesity that consumed aspartame had the increased abundance of *Roseburia*, *Bifidobacterium*, *Clostridium leptum* and *Enterobacteriaceae*. Also, aspartame intake increased a level of circulated short-chain propionate and glucose, which may lead to hyperglycemia and insulin tolerance later on [1,41]. Suez et al. [38] analyzed an effect of aspartame on glucose metabolism and the gut microbiota. The glycemic response was significantly higher in the group consumed aspartame than in the control group ($p<0.001$). According to the authors' opinion, the revealed glucose tolerance can be associated with alterations in the gut microbiota composition, including a reduction of the abundance of *Clostridiales* and an increase in the abundance of *Bacteroides* [1,38].

Frankenfeld et al. [39] showed that although there was no significant difference in the ratio of *Bacteroidetes* to *Firmicutes* between the group consuming aspartame and the control group, the overall bacterial diversity differed between the groups. For example, aspartame intake was associated with an increase in *Actinobacteria*, *Deltaproteobacteria* and *Enterobacteriaceae* [1,39].

Saccharin

Saccharin (E954) is a derivative of naphthalene. It is about 240–300 times sweeter than sucrose and is one of the first artificial sweeteners used in various foods (Figure 2). It is slowly absorbed by the intestine and its acceptable daily intake is 5 mg/kg body weight/day, which is the lowest level among all artificial sweeteners [42].

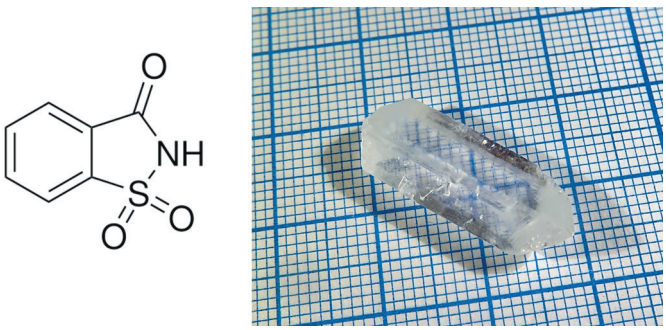

Figure 2. Structural formula of saccharin and a photo of its crystal grown in acetone

Suez et al. assessed an effect of saccharin on the blood glucose level and the gut microbiota composition in mice [38]. According to the authors' data, saccharin induced misbalance in the gut microbial community, including an increase in the abundance of *Clostridiales* and *Bacteroides* and a decrease in the abundance of lactobacilli and

Firmicutes. The same researchers reported that no disorders related to glucose tolerance were revealed in mice after saccharin consumption. However, transplantation of the gut microbiome from the *in vitro* cultures subjected to an exposure to saccharin to other mice led to impaired glucose homeostasis [38].

Sucralose

Sucralose (E955) is a chlorinated disaccharide. Its sweetening ability about 320–1000 times higher than that of sucrose (Figure 3). The acceptable daily intake is 15 mg/kg body weight/day. With that, the most part of ingested sucralose is excreted in faeces (65–95%) [36]. Uebanso et al. [43] reported that the number of *Clostridium IVXa* in faeces of mice given sucralose significantly reduced with an increase in the ratio of secondary/primary bile acids and a decrease in the level of luminal butyrate [43]. Bian et al. [44] studied an effect of sucralose on the gut microbiome in male mice. The gut microbiota composition altered significantly after sucralose intake during six months. After three months, the abundance of *Ruminococcus* increased and that of *Bacillales, Peptostreptococcaceae*, *Staphylococcus* and *Anaerostipes* decreased. After six months, an increase in the abundance of *Christensenellaceae*, *Clostridiaceae*, *Akkermansia*, *Roseburia* and *Turicibacter*, and a decrease in the abundance of *Erysipelotrichaceae*, *Dehalobacterium*, and *Streptococcus* were observed [44].

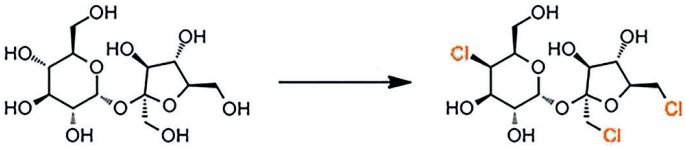

Figure 3. A simplified scheme of sucralose production from saccharose as a result of the five-stage reaction

Sodium cyclamate

Sodium cyclamate (E952) is used as a sweetener in more than 50 countries, and its sweetness is 30–40 times higher than that of sucrose (Figure 4).

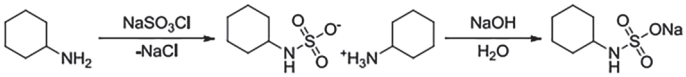

Figure 4. A scheme of sodium cyclamate production

However, the U. S. Food and Drug Administration (FDA) removed sodium cyclamate from the list of substances generally recognized as safe ("GRAS") in 1969 and fully prohibited it in 1970 [42]. It was found that sodium cyclamate can be metabolized to toxic cyclohexylamine under the action of the gut microbiota [1]. For example, the animal experiments showed that bladder cancer was found in rats fed with a mixture of cyclamate and saccharin [45]. However, correctness of these studies was subjected to question and the safety of sodium cyclamate was revised. In 1982, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established the acceptable daily intake of 11 mg/kg body weight/day for sodium cyclamate [1]. In the USA, the use of this substance is still prohibited. Subsequently, an effect of sodium cyclamate on the monkey gut microbiota was studied. Compared to the control group, total counts of coliforms and the presence of the microbial population including *Bifidobacterium*, *Clostridium*, *Enterobacteriaceae*, *Veillonella* and *Bacteroidaceae* did not have significant differences [46].

Neotame

Neotame (E961) is an artificial sweetener produced by reductive alkylation of aspartame. It is often used in combination with other sweeteners in sauces, fermented dairy drinks, lemon tea and soft drinks [1]. Although the structural formulas of neotame and aspartame are similar, neotame has higher sweetness, which is 7000–13000 times higher than that of sucrose (Figure 5).

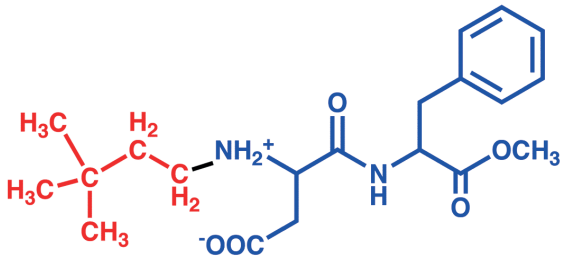

Figure 5. Structural formula of neotame. The blue color shows aspartame as part of neotame; this part of the molecule is responsible for sweet taste formation in this substance [49]

Both the FDA and European Food Safety Authority (EFSA) approved the use of neotame; with that, its acceptable daily intake (ADI) is 2 mg/kg body weight/day [47]. Neotame is quickly metabolized and is not accumulated in the body. Half of the consumed neotame does not enter blood and is excreted in faeces, while another half is excreted in urine as deesterified neotame. Up to now, there have been no reports about its toxicity for mice and other experimental animals [36].

Chi et al. [48] studied an effect of neotame on the gut microbiota in male mice. After four-week neotame consumption, a significant increase in *Bacteroidetes* and a significant decrease in *Firmicutes* were observed in the neotame-treated mice. Total microbial counts were significantly lower in the neotame-treated mice than in the control group [48].

Emulsifiers and their effect on the gut microbiome

Emulsifiers are substances having the surface activity that are capable of creating stable emulsions when mixing with other substances. They help improving the food structure and taste, extend product shelf life [1,50]. Some emulsifiers are present in food as a natural component, for example, surface active protein casein, while others are synthesized artificially, for example, substances such as carboxymethylcellulose and polysorbate 80. Over the last years, there has been a trend towards an increasing number of studies showing that food emulsifiers can af-

fect the gut microbiome, cause intestinal inflammation and facilitate the development of the metabolic syndrome [1,50,51].

Carboxymethylcellulose

Carboxymethylcellulose (CMC, E466) is an amorphous colorless substance, a weak acid. According to the classifier of food additives, it is assigned to the class of stabilizers; it imparts shape and viscosity to a product. Carboxymethylcellulose is a cellulose derivative obtained from cellulose upon its treatment with chloroacetic acid and alkali. It can be seen from the presented chemical structures (Figure 6) that carboxymethylcellulose has the higher water solubility than the initial raw materials due to the presence of the polar carboxymethyl group; however, the polymer structure remains to be unchanged. The chemical, food and pharmaceutical industries use the carboxymethylcellulose sodium salt (croscarmellose, E468), which aqueous solutions are viscous and have pseudoplasticity. The CMC sodium salt is an amorphous colorless substance with a molecular mass $(30–25)*10^3$, browning temperature 227 °C, carbonization temperature 252 °C. It is soluble in water, aqueous alkaline solutions, NH_3, NaCl and solvents for cellulose; it is not soluble in organic solvents, mineral oils. When solving in water, the CMC sodium salt forms viscous transparent solutions.

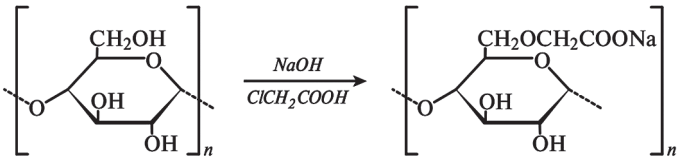

Figure 6. A scheme for production of the carboxymethylcellulose sodium salt

Toxicological studies showed that mice and rats treated with CMC did not have any significant side effects over 100 weeks [52]. Carboxymethylcellulose is a polysaccharide, which is hard to hydrolyze and digest by the enzymes of the human gastrointestinal tract; therefore, its fermentation usually depends on the gut microbiota [1]. Carboxymethylcellulose can be fermented by the gut microflora into short-chain organic acids, such as lactic acid, succinic acid, formic acid, acetic acid, butyric acid and propionic acid. Swidsinski et al. [53] studied an effect of CMC on mice with deactivated IL-10 gene (IL-10 gene suppresses production of all pro-inflammatory cytokines). Bacterial overgrowth was observed in CMC-treated mice. The bacterial concentration in the ileum was higher than 10^8 CFU/ml (colony forming units/ ml). It was found that leucocytes migrated into the intestinal lumen in four out of seven CMC-treated mice. CMC intake caused a reduction in *Eubacterium rectale* in the ileum and jejunum, and also increased *Bacteroides*. The authors assumed that CMC intake can lead to the bacterial overgrowth and cause inflammation of the intestine in susceptible mice [1,53].

Viennois et al. [54] studied an effect of CMC on intestinal inflammation and alterations of the mouse gut microbiota composition in colorectal cancer development. After CMC intake for 13 weeks, the mouse gut microbiome composition altered: the abundance of *Proteobacteria* and *Firmicutes* significantly decreased and the abundance of *Bacteroidetes* increased. With that, the levels of the marker of intestinal inflammation (lipocalin 2) were elevated. Several groups took part in the study and the frequency of tumor development also increased in a group treated only with CMC compared to the control group. The authors concluded that CMC intake promoted carcinogenic processes [1,54].

An effect of CMC on the gut microbial diversity was also studied with the use of *in vitro* models. For example, Chassaing et al. [55] studied an effect of CMC on the intestinal ecosystem using an artificial model that simulated the intestinal mucosa (M-Shime). The M-Shime model is an *in vitro* model consisting of several glass vessels with regulated pH, which simulate the stomach, small intestine and different parts of the colon. For better simulation of the human intestinal tract covered with mucus, the "mucous environment" was created in M-Shime using mucins, which allowed studying various microbiota types [55,56]. During simulation of the colon microflora treated with 1% of CMC in M-Shime for 13 days, *Bacteroidaceae* decreased, while *Enterobacteriaceae* and *Proteobacteria* increased. It was found that CMC treatment increased the flagellin gene expression. When microbiota treated with 1% of CMC was transplanted to C57BL/6 $Rag^{-/-}$ mice without microflora, alterations in the gut microbiota composition similar to those in the artificial system M-Shime were observed [55,56].

However, it is still necessary to study how CMC is related to alterations in the gut microbiota composition and the development of intestinal inflammation. One of the possible pathogenic mechanisms can be a damage of the intestinal mucosa by CMC, which can lead to mucosal inflammation [53]. Another potential mechanism can consist in CMC-induced increase in flagellin production, which, in turn, enhances the bacterial ability to penetrate into the mucus layer, and thereby, facilitates the overgrowth of gut bacteria and alters functional characteristics of the gut microbiota [50]. However, it is still necessary to investigate to what extent the above described pathogenic mechanisms can be extrapolated from artificial systems to the human body.

Polysorbate 80

In the USSR, polysorbates were synthesized for the first time in the All-Union Scientific Research Institute of Organic Intermediates and Dyes in 1958. Polysorbate 80 (Tween 80, P80, E433) is obtained by co-polymerization of sorbitol and its dehydrated monooleate with ethylene oxide. It is often used in the food industry as an emulsifier, solvent and stabilizer (Figure 7).

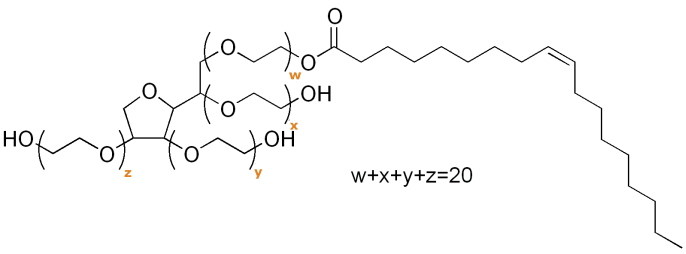

Figure 7. Structural formula of polysorbate 80

It can be seen from the presented formula that the polysorbate molecule actually consists of two parts: hydrophilic (left) and containing a long hydrophobic "tail" (right). Such structure from the hydrophilic and hydrophobic parts is typical for many emulsifiers. For polysorbate 80, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established the acceptable daily intake of 25 mg/kg body weight/day. Similar to carboxymethylcellulose (CMC), polysorbate 80 showed weak assimilation in the gastrointestinal tract and most part of it is excreted in faeces; however, it is known that it can increase serum levels of lipopolysaccharide *in vivo*, facilitate microbial invasion and lead to alterations in the gut microbiota composition [6]. Chassaing et al. [51] studied an effect of 1% polysorbate 80 solution on the gut microbiota, colitis development and metabolic syndrome in C57BL/6 mice and two lines of genetically modified mice (IL10 $^{-/-}$ and TLR5 $^{-/-}$). The 12-week study showed that intake of polysorbate 80 did not have a significant effect on the mouse microbial community; however, it was found that polysorbate 80 reduced the thickness of the intestinal mucosa, facilitated the contact between bacteria and epithelial cells and did cause changes in the gut microflora. Polysorbate 80 also led to an increase in the intestinal permeability and increased levels of lipopolysaccharide and flagellin. In addition, these authors studied effects depended on the amount of polysorbate 80. Mice treated with 0.1% solution of polysorbate 80 had low indicators of inflammation and obesity; while mice treated with 0.5% solution of polysorbate 80 showed mild hypoglycemia [1,51].

Singh et al. [57] studied an effect of polysorbate 80 on the mouse gut microflora and development of the intestinal inflammation and liver dysfunction [57]. Compared to the control group, the number of Gram-positive bacteria significantly increased in mice consumed polysorbate 80, which, according to the authors' opinion, promoted the development of non-alcoholic fatty liver disease due to an influence of the gut microflora on the enterohepatic circulation of bile acids. It was found that the abundance of *Bacteroides* decreased and the abundance of *Salmonella*, *Helicobacter*, *Clostridium* increased. With that, *Campylobacter jejuni*, *Salmonella* spp. and *Helicobacter* spp. are associated with the development of inflammation. Mice treated with polysorbate 80 showed the shortened colon, damage of epithelial cells, reduction in the faecal level of short chain fatty acids, such as butyrate, propionate and acetate, as well as a significant decrease in expression of proteoglycan mucin-2. Mice in a group received polysorbate 80 also demonstrated an increased level of the lipocalin-2 content in colon and faeces, an increase in intestinal permeability and in the flagellin content. These markers are associated with chronic intestinal inflammation. Also, intake of polysorbate 80 increased expression in the liver of alkaline phosphatase by 40%, alanine aminotransferase and aspartate aminotransferase by 50%. Moreover, lipid droplets and steatosis related to the increased activity of liver enzymes were observed in the liver, which indicated liver damage [1,57].

Viennois et al. [54] studied an effect of polysorbate 80 on microbiota and intestinal inflammation in mice. In their study, *Proteobacteria* and *Firmicutes* significantly decreased and *Bacteroidetes* increased in mice received polysorbate 80 [1,54].

Although both polysorbate 80 and CMC increase expression of pro-inflammatory flagellin, an increase induced by polysorbate 80 occurs more slowly than that induced by CMC. Compared to CMC, which promotes the overgrowth of bacteria in mice and, therefore, affects the development of inflammation, polysorbate 80 increases bacterial translocation through mucosa [58].

Therefore, both CMC and polysorbate 80 can affect microbiota in mice, increase its ability to penetrate the mucus layer, influence the signal pathways of cell proliferation and apoptosis, and lead to intestinal inflammation and disorder of metabolic homeostasis [1]. However, it is necessary to further investigate how these results obtained mainly on rodents can be extrapolated to humans.

Sodium sulfite

Sodium sulfite (E221) is a sodium salt of sulfurous acid and is used in foods to inhibit the microbial growth and stabilize color. In 1974, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established the acceptable daily intake of 0.7 mg/kg body weight for sodium sulfite. Sodium sulfite is readily soluble in water and is a strong reducer; therefore, it is quickly oxidized in the air. Irwin et al. [59] studied an effect of sodium sulfite in different concentrations on four bacterial species residing in the intestine: *Streptococcus salivarius, Lactobacillus casei, Lactobacillus rhamnosus* and *Lactobacillus plantarum*. Although the used media were suitable for the growth of all four species and the exposure time was short, sodium sulfite exerted the bacteriostatic and bactericidal effects against all four tested species at a concentration lower than the acceptable daily intake. Therefore, up to now, there are no data about an effect of sodium sulfite on the microbiome of the human gut, which is an environment more complex than those in *in vitro* experiments.

Nisin

Due to the high antibacterial activity, especially against Gram-positive bacteria, nisin (E234) is the only bacteriocin approved by JECFA as a food preserving agent.

Its acceptable daily intake is 2 mg/kg body weight/day. Lauková et al. [60] used rabbits to study changes in the gut microbiota after continuous nisin intake for 28 days. They observed a significant reduction in pseudomonads, clostridiae, coliforms and coagulase-positive staphylococci. Ronan et al. [61] studied an effect of nisin in two different matrices (starch dough and starch gel) on the mouse intestinal microbiota. They found that the relative abundance of *Bifidobacterium*, and Gram-positive bacteria belonged to the *Clostridium* cluster XIVa was significantly lower in two groups fed with a diet containing nisin than in the control group [61]. However, it is necessary to study further how these alterations in the microbial community can affect human health.

Potassium sorbate, sodium benzoate and sodium nitrite

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established that the acceptable daily intake for sodium benzoate (E211), sodium nitrite (E250) and potassium sorbate (E202) is 5, 0.07 and 25 mg/kg body weight, respectively [1]. Hrncirova et al. [62] determined in the *in vitro* experiments an effect of combinations of sodium benzoate, potassium sorbate and sodium nitrite on bacteria isolated from human faeces. It was found that microflora was very sensitive to the antimicrobial food additives. *Bacteroides coprocola* was most sensitive. This microorganism is associated with Crohn's disease and ulcerative colitis. *Clostridium tyrobutyricum* was also sensitive to sodium nitrite. *Lactobacillus paracasei* and *Bifidobacterium longum* were most sensitive to sodium benzoate. These microorganisms can enhance antitumor immunity *in vivo*. *Enterococcus faecalis* was also sensitive to sodium benzoate, while *Escherichia coli* was most sensitive to potassium sorbate [62]. In another study, Hrncirova et al. [63] used mice colonized with the human gut microbiome to investigate an effect of food preserving agents on the gut microbiota. In their investigation, the second generation of mice, similar to the first one, received sodium benzoate (4.8 mg/kg body weight/day), sodium nitrite (0.36 mg/kg body weight/day) and potassium sorbate (19 mg/kg body weight/day). Wild-type and Nod2-deficient C57BL/6 mice were included in this study. The experiment revealed a reduction of the overall microbial diversity, a decrease in *Clostridiales* and an increase in *Proteobacteria*. The authors noted that Nod2-deficient mice were particularly susceptible to gut microbiota disruption. They also noted that an impact of preserving additives on the human gut microbiota can cause dysbiosis even at low doses [1,62,63].

Titanium dioxide

Food-grade titanium dioxide (E171) is used as a colorant in many food products [43]. With that, 17–36% of titanium dioxide particles in food products have the nano size (<100 nm) [64]. JECFA did not establish an acceptable daily intake level for titanium dioxide in foods due to the absence of toxicity [1]. However, the studies on animals showed that titanium dioxide nanoparticles can affect the gut microbiota composition, which raises concerns about the potential health risks associated with oral exposure to titanium dioxide nanoparticles [1,65].

Essential oils

Essential oils are volatile oil-like compounds with typical strong odor and taste. They are insoluble in water, mainly colorless or slightly colored liquids. The majority of essential oils consist of light fractions; therefore, they are quickly evaporated and do not leave "fatty" stains on paper. Essential oils are complex mixtures of terpenes, terpenoides and other aromatic and aliphatic compounds extracted with organic solvents or by distillation from various spices and herbs [66]. Essential oils are widely used as flavoring agents, but they also have the antibacterial activity (Figure 8). Thapa [67] studied 21 essential oils and found that 19 of them possessed the antimicrobial activity against, at least, one of the following microorganisms: *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumonia, Bacillus subtilis* and *Staphylococcus aureus* [1,67].

It is known that essential oils can be added to feed for shrimps, broiler chickens, ducks and other animals [60,68,69]. Some essential oils are added into food as preserving agents and antioxidants, for example, thyme oil, cinnamon oil and clove oil [1].

Bento et al. [70] noted that the blend of cinnamon and thyme oils had a beneficial effect on the gut microbiome of monogastric animals and inhibited the growth of pathogens and opportunistic pathogens, for example, *Salmonella spp.* and *Escherichia coli* [70].

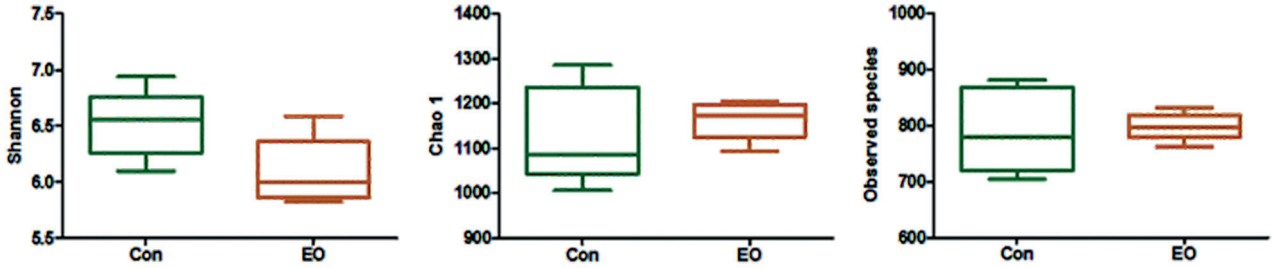

Figure 8. An effect of essential oils on colon microflora diversity in piglets
The values of Shannon and Chao1 indices, as well as the total counts of the studied bacterial species are given.
Con: the control group; EO: the experimental group [71].

Conclusion

In several cases, food additives exert a pronounced physiological effect. Theoretically, their influence on the body can have various features due to multiple classes of substances used as food additives and their subsequent metabolic transformation. With that, in case of a direct effect on the body, an action of food additives is well studied; however, it is studied insufficiently in case of indirect impact with participation of microbiome. Many studies demonstrate an importance of microbiome for the healthy functioning of the consumer's body. For example, it is known that the gut microbial composition affect immunity formation and production of different physiologically active substances.

As microbiome is an assembly of microorganisms, a researcher studying even one substance entering the body can encounter many metabolic pathways, in which various bacteria take part transforming the initial food additive into other substances. It is necessary to note that in several cases, genes responsible for encoding one or another metabolic enzyme can be on extra-chromosomal elements (for example, plasmids), which possibly would make prediction of metabolic pathways more difficult. Therefore, researchers can face a problem of explaining an effect on the human body of many minor metabolites even in investigation of one food additive. In this situation, machine learning can be used to process such volumes of data.

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FORTIFICATION OF FOOD WITH MICRONUTRIENTS: DEVELOPMENT OF METHODOLOGICAL AND REGULATORY FRAMEWORK IN THE RUSSIAN FEDERATION

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Abstract
The available scientific literature, domestic and international regulatory codes of normative documents concerning the fortification of various types of food products have been analyzed. The groups of food products of conventional and regular consumption included into the diets of all categories of consumers, recommended for fortification with essential micronutrients, have been determined: wheat and cereal flour (spelt wheat, buckwheat, oat, corn flour, etc.); pastry; milk and dairy products, including ice cream; non-alcoholic soft drinks; mineralized drinking water; fruit and vegetable juices; fat and oil products (vegetable oils, margarines, spreads, mayonnaise); confectionery and sweets (pastry, sugar, chocolate); cereals (breakfast cereals, muesli, ready-to-eat extruded cereals, instant pasta and cereals, mixtures for bakery, flour for sweet pastry); food concentrates (jelly, instant drinks, concentrates of sweet foods, instant food, instant cereal concentrates); table salt. The groups of food products assigned for certain categories of population are used as part of therapeutic diets for patients with various diseases (metabolic disorder syndrome, cardio-vascular system pathology with atherosclerotic vascular injury, type 2 diabetes mellitus, gastrointestinal tract diseases, non-alcoholic fatty liver disease, diabetic nephropathy, etc.), as well as assigned to reduce the risk of diseases developing, the nutrients are recommended for targeted fortification of certain types of food. Examples of micronutrients fortification of sausages and minced meat semi-finished products are given below. Requirements for fortification of mass consumption food products and for fortification of foods for special dietary uses are formulated in this article, the amount of fortifying components in the various groups of food products are justified, ensuring their efficiency for improving the micronutrient status and safety of its consumption. Based on the analysis of the available scientific literature, domestic and international regulatory framework of normative documents on fortification of various types of food products, recommendations have been developed for fortification of food with micronutrients.

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Introduction

In modern conditions the population of many countries, including Russia, suffers from multiple micronutrient deficiency, i. e. simultaneous deficiency of several vitamins (D, group B) and minerals (iodine, calcium, magnesium, iron, zinc, etc.) [1–4]. It is a cost-effective approach to increase micronutrient intake with the help of large-scale food fortification. In most economically developed countries (USA, Canada), as well as in some countries of the former USSR (Uzbekistan since 2005, Turkmenistan since 2006, Kazakhstan and Kyrgyzstan since 2009, Moldova since 2012), in many developing countries of Africa, Asia and Latin America, the problem of optimizing the vitamins supply of the population is solved by law-regulated fortification of food with vitamins B1, B2, B6, PP, folic acid and iron, added to the food of mass consumption: flour, pasta and bakery; vitamin D in the dairy food [5,6]. The flour was obligatory fortified with vitamins B1, B2 and PP by the decision of the USSR Council of People's Commissars since 1939, but later this practice was stopped [7]. Currently, the food products are fortified with micronutrient only voluntarily at the initiative of product manufacturer. Essential micronutrients, which include vitamins, minerals and polyunsaturated fatty acids (PUFA), are most often used as food fortifiers. The basic requirements for the fortification of food with vitamins and minerals were formulated in Unified Sanitary Epidemiological and Hygienic Requirements (i. e. SanPiN) 2.3.2.2804–10 "Hygienic requirements for safety and nutritional value of food" [8], which were included in SanPiN2.3.2.1078–01 [9] as Addendum and Amendment No 22, but this practice is currently suspended. The regulations for fortification in general concerned the mass consumption food products [10,11].

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The practice of creating the foods for special dietary uses (SFP) and the information presented in the Unified Register of the Customs Union show that biologically active substances and compounds with certain physiological effect are used for fortification of such products, in addition to essential micronutrients. This gives rise to a number of problems, including the choice of food products to be fortified, the levels of fortification that ensure the safety and efficiency of the fortified types of food products, the interaction of the fortifying elements or ingredients within the food product and their interaction with the body, etc. In this regard, the structuring of recommendations for the fortification of various groups of mass consumption food products and certain categories of foods for special dietary uses with essential nutrients on the basis of an analysis of the available scientific literature, domestic and international regulatory code of normative documents for fortification of various types of food products is now of particular relevance.

The purpose of the research is to determine the groups of food products of conventional and regular consumption to be fortified with essential micronutrients, included into the diets of all categories of population; definition of food groups intended for certain categories of population, which food products are used in clinical practice as part of therapeutic diets or are intended to reduce the risk of developing diseases; determination of micronutrients for their targeted application to the food and its fortification; justification of amount of fortifying elements/components application in various groups of food products, ensuring their efficiency for improving their micronutrient status and safety of consumption. The basic rules and principles of food fortification with essential nutrients are reduced to the following recommendations.

List of food products to be fortified and micronutrients recommended for food fortification

The following food product categories are subject to fortification with food active substances and/or biologically active substances, and/or probiotic microorganisms:

- food products for general population except for food products that are not technologically processed (fresh fruits, vegetables, meat, poultry, fish), spices, fermented drinks, as well as drinks containing more than 1.2% alcohol (except for low-alcohol tonic drinks where vitamins and minerals are added with different purpose);
- foods for special dietary uses for healthy children;
- foods for special dietary uses for pregnant and breast-feeding women;
- foods for special dietary uses for athletes' nutrition;
- foods for special dietary uses for nutrition of workers who are exposed in their work to harmful and extremely harmful conditions [12,13];
- foods for special dietary uses for dietary medical and preventive dietary nutrition, including baby food.

The food products for general population is fortified in accordance with recommended list of foods product groups and recommendations on the nutrients used for fortification of the appropriate food groups (Table 1).

Table 1. List of groups of mass consumption food products, recommended for fortification

Food products groups	Recommended nutrients for fortification
Wheat flour of the highest grade and of the first grade	vitamins: B1, B2, B6, PP, folic acid, A, D, B12, pantothenic acid; minerals: iron, calcium
Bakery and pastry	vitamins: B1, B2, B6, PP, folic acid, beta-carotene, A, D, B12, pantothenic acid; minerals: iron, calcium, iodine
Dairy products (dairy product, dairy compound product, product of milk processing, milk-containing product)	vitamins: C, A, E, D, K, group B, beta-carotene; minerals: iron, calcium, iodine; dietary fiber; polyunsaturated fatty acids; phospholipids; prebiotics; probiotic microorganisms; dihydroquercetin
Meat products	vitamins C, group B, Fe, Ca, iodine
Non-alcoholic soft drinks	vitamins: C, A, E, D, K, group B, beta-carotene and other carotenoids; minerals: iodine, iron, calcium; dihydroquercetin
Fruit (including berries) and vegetable juice products (juices, fruit and / or vegetable nectars, fruit and / or vegetable juice drinks)	vitamins: C, A, E, B1, B2, B6, PP, folic acid, beta-carotene; minerals: iodine, iron, calcium; organic acids; dietary fiber; polyunsaturated fatty acids; polyphenolic acids; prebiotics; phytosterols; flavonoids; phospholipids; dihydroquercetin
Cereals (breakfast cereals, ready-to-eat extruded cereals, instant pasta and cereals)	vitamins: C, A, E, D, group B, beta-carotene; minerals: iron, calcium, iodine
Fat and oil products (vegetable oils, margarines, spreads, mayonnaises, sauces)	vitamins: A, E, D, beta-carotene; dihydroquercetin
Food concentrates (jelly, instant drinks, instant food)	vitamins: C, A, E, D, K, group B, beta-carotene; minerals: iodine, iron, calcium, magnesium, potassium; dihydroquercetin
Pastry and confectionery	vitamins: C, A, E, beta-carotene, B1, B2, B6, PP, folic acid; minerals: iodine, iron, calcium, magnesium; dihydroquercetin (for chocolate and confectionery products only)
Fruit and berry concentrates with added sugar or other sweetening substances (jam, jam, confiture, jelly, popsicles, etc.)	vitamins: C, A, E, B1, B2, B6, PP, folic acid, beta-carotene; minerals: iodine, iron, calcium
Table salt	minerals: iodine, fluorine*

Note: *assigned for regions or areas with deficiency of this microelement

The criteria for classifying a food product as fortified with vitamins and/or minerals and/or other essential nutrients are given below in the Table 2.

Table 2. Criteria for classifying a food product as fortified with vitamins, minerals and/or other essential nutrients

Food group	Mass (volume) * of a food product, which must contain not less than 15% and not more than 50% of the normal physiological demand for this nutrient
Wheat flour of the highest grade and first grade	100 g
Bakery products from the highest grade and first grade of wheat flour and rye-wheat flour	150 g
Liquid dairy products, protein products made of grain, legumes, liquid grain food (soy milk)	200 ml
Dairy products and protein products from grain, leguminous crops (tofu), solid and pasty food	100 g
Minced meat products, cooked sausages	100 g
Fruit (including berries) and (or) vegetable juices, non-alcoholic soft drinks, incl. the drinks made from food concentrates	300 ml
Dry cereals (breakfast cereals, ready-to-eat extruded cereals, instant pasta and instant cereals)	50 g
Fat and oil products, sweet pastry, confectionery, hard rennet cheeses, canned food and vegetable, fruit and berry concentrates.	Per 100 kcal
Iodized edible table salt	1–2 g
Edible salt	5 g

Note: * average daily portion

The lists of food and biologically active substances used in the production of certain types of fortified food products are determined by the technical regulations of the Customs Union for food products of the corresponding homogeneous group.

Recommendations for food fortification

The food is fortified by adding one or more nutrients — i. e. additional food substance, biologically active substances or probiotic microorganisms — in accordance with the recommendations below:

- the food products for general population that do not contain nutrients or contain insufficient amount of nutrients, or which have lost those nutrients during the production (manufacturing) process, must be fortified with food substances (dietary fiber, prebiotics, etc.) and/or biologically active substances (vitamins, minerals, omega-3 PUFAs, etc.) and / or probiotic microorganisms;
- the added nutrients must be safe and stable during food storage;
- each nutrient must possess accurate physical and chemical characteristics that can be reliably determined using appropriate analytical methods;
- the beneficial properties of the introduced nutrients must be scientifically proved and substantiated;
- the amount of fortifying nutrients additionally introduced into the products must be calculated taking into account their natural content in the original product or raw materials used for its manufacture, as well as their losses during production and storage, in order to ensure the content of these nutrients at a level not below the regulated value for the entire shelf life of the fortified food product;
- the choice of types, forms, methods and stages of the introduction of fortifying nutrients shall be carried out taking into account their possible chemical interaction with each other and with the components of the fortified product and ensure maximum safety during production and storage;
- fortification of food products shall not worsen the consumer properties of these food products, shall not reduce the content and assimilability of other food substances contained in the food product; shall not significantly change the organoleptic properties of products and shall not reduce their shelf life;
- food fortification shall not affect safety of the food;
- the guaranteed content of nutrients in the fortified food products at the end of shelf life shall be indicated on the individual package of this food product.

The amount of each food substance or biologically active substance, used for fortification and guaranteed by the food manufacturer, must be brought to a level that meets the criteria for this food product. The source of the food substance or other distinctive features, and the maximum level of food and (or) biologically active substances in such products shall not exceed the safe upper level of such substances consumption from all possible sources (if any).

The content of vitamins and minerals included in the fortified food product of mass consumption shall be brought to a consumption level corresponding to 15–50 percent of the average daily requirement of adult person for vitamins and minerals per 100 g or 100 ml, or per serving of the fortified food products. The weights (volumes) of the averaged daily portions of fortified foods are shown in the Table 2.

For fortified high-calorie foods (where energy value per 100 g is 350 kcal or more) the content of the fortifying nutrient shall range between 15% and 50% of the recommended daily intake per 100 kcal (1 serving of the product).

The content of probiotic microorganisms in the fortified food products shall ensure the level of their consumption in a daily portion of such products that meets the established requirements and be at least 10^6 (for bifidobacteria) and 10^7 (for lactobacilli) colony-forming units (microbial cells) in 1 gram or 1 ml of these kinds of food.

The vitamins and minerals shall be used in the production of fortified food products only in the forms which are approved in accordance with the established procedure.

When fortifying salt with food iodine, the level of fortification element content shall be (0.04 ± 0.015) mg/g. When fortifying salt with edible fluorine, the permissible level of its content shall not exceed 0.025%.

Foods for special dietary uses include:

- food products for baby nutrition incl. food products for children of early age, preschool and school age;
- food products for pregnant and breast-feeding women;
- food products for athletes' nutrition;
- food products for preventive dietary nutrition, incl. baby nutrition, as well as food for workers exposed in their jobs to harmful and extremely harmful working conditions;
- food products of dietary medical nutrition for children and adults.

Adequate and upper permissible values of daily consumption of basic food and biologically active substances as part of foods for special dietary uses for persons older than 18 years are established in the corresponding regulation in the Unified Sanitary and Epidemiological and Hygienic Requirements for Goods Subject to Sanitary and Epidemiological Surveillance (Control) (hereinafter referred to as USE & HT) [14].

To produce fortified foods for special dietary uses for adult population, the vitamins and minerals shall be used in the officially approved forms in accordance with the established procedure.

In addition to vitamins, macroelements and microelements, polyunsaturated fatty acids (PUFA), it is possible to add dietary fiber, prebiotics, probiotic microorganisms, ingredients with known physical and chemical characteristics and safe for human health. These elements shall be added in amount recommended for daily consumption. It is necessary to provide a scientifically grounded and confirmed effect on one or several physiological functions, metabolic processes in the human body. The criteria for the selection of fortifying nutrients, their types, forms and doses are safety and proven (in terms of evidence-based medicine) efficacy.

The amount of fortifying nutrients in a daily portion of recommended foods for special dietary uses shall be at least 15% of an adequate level of consumption and shall not exceed the upper permissible level of these elements consumption [14].

Regulations for fortification of mass consumption food products, of foods for special dietary uses, incl. baby nutrition, as well as a list of regulatory documents on the food fortification, are presented below in the Table 3.

Table 3. Regulations for fortification of food products

Food product categories	Regulatory document for food fortification	Amount of the fortifying nutrient	List and forms of vitamins and / or minerals
Food products for general population	TR CU021/2011 [15], TR CU022/2011 [16], ESET [14] Appendix 5, TR CU023/2011 [17] (Article 5, clauses 14, 15)	not less than 15% and not more than 50% of the recommended daily intake of a particular food component in 100 ml or 100 g, or a single serving. For juice products, portion — 300 ml	In accordance with [14] Appendix 8
Foods for special dietary uses			
for pregnant women, breast-feeding women	TR CU027/2012 [18], TR CU022/2011 [16], Unified Sanitary Epidemiological and Hygienic Requirements [14], MP 2.3.1.2432–08 [19]	In accordance with [14] (Chapter 2, section 1, clause 11), for nutrients not specified in clause 11, at least 15% of the recommended daily intake of a specific component and not exceeding the upper permissible level of its intake in daily portion of the finished food product Appendix 5 [14].	In accordance with [14] Appendix 9
for dietary therapeutic and dietary preventive nutrition	TR CU027/2012 [18], TR CU022/2011 [16], Unified Sanitary Epidemiological and Hygienic Requirements [14]	not less than 15% of the recommended daily intake of a specific component and not exceeding the upper permissible level of its intake in a daily portion of a finished food product Appendix 5 [14].	In accordance with [14] Appendix 11
for children over three years old	TR CU021/2011 [15], TR CU033/2013 [20], TR CU023/2011 [17], Unified Sanitary Epidemiological and Hygienic Requirements [14], Sanitary Rules and Regulations 2.3.2.1078–01 [9]	In accordance with TR CU021/2011 [15], (Art. 7, item 12), TR CU033/2013 [19], (Appendix 13), TR CU023/2011 [17] (Art. 5, p. 14, 15, 28), Unified Sanitary Epidemiological and Hygienic Requirements [14] clause 13, [15] Appendix 20	In accordance with TR CU021/2011 [15], Appendix 9
for dietary preventive nutrition and dietary therapeutic nutrition of little children	TR CU021/2011 [15], TR CU027/2012 [18], TR CU033/2013 [19], TR CU023/2011 [17] Unified Sanitary Epidemiological and Hygienic Requirements [14]	Unified Sanitary Epidemiological and Hygienic Requirements [14] clause 16; TR CU027/2012 [18], (Appendix 3), TR CU033/2013 [19] (Appendix 14), TR CU023/2011 [17] (Article 5, paragraphs 14, 15, 28)	In accordance with TR CU021/2011 [15], Appendix 9; Unified Sanitary Epidemiological and Hygienic Requirements [14] Appendix 9
for baby nutrition, dietary prophylactic nutrition and dietary therapeutic nutrition	TR CU021/2011 [15], TR CU027/2012 [18] Unified Sanitary Epidemiological and Hygienic Requirements [14]	Unified Sanitary Epidemiological and Hygienic Requirements [14] p. 14	In accordance with TR CU021/2011 [15], Appendix 9; [14] Appendix11
for athletes' nutrition	TR CU021/2011 [15], TR CU027/2012 [18], Unified Sanitary Epidemiological and Hygienic Requirements [14]	Not less than 15% of the adequate level of daily consumption of a particular component and not exceeding the upper permissible level of its consumption in a daily portion of the finished food product Appendix 5 [14] (for saturated fatty acids with medium long chain (C8-C14), D-Ribose, sodium, caffeine, creatine — only separate items)	In accordance with [14] Appendix11, [21.22]

Recommendations on food fortification for healthy children

Requirements for the quality and safety of food products for healthy children, processes of this food production, its labeling, storage, transportation and sale are regulated in the prescribed procedure [14, 15].

In accordance with the established procedure, the daily dose of vitamins and minerals included in the composition of foods for special dietary uses for children from 1.5 to 3 years shall not exceed 50% of the daily physiological need for these substances or elements [19].

The daily dose of vitamins and minerals in the composition of foods for special dietary uses for children from 3 to 18 years old shall not exceed (in % of the daily physiological need for these substances, established for children from 3 to 18 years old, determined in accordance with the established procedure: for vitamins A, D, minerals (selenium, copper, zinc, iodine, iron) — 100%, for water-soluble vitamins and other fat-soluble vitamins and other minerals — 200%.

The forms of vitamins and minerals in the production of food products for baby food are determined by the requirements of TR CU021/2011 (Appendix 9) [15]. The forms of vitamins and minerals in the production of foods for special dietary uses for children from 1.5 to 3 years old are regulated by the law established procedure [14, Appendix 9]. The forms of vitamins and minerals in the production of foods for special dietary uses for children over 3 years old are regulated by the established procedure [14, Appendix 7].

Recommendations on food fortification for athletes' nutrition

In the production of foods for special dietary uses for the nutrition of athletes, it is allowed to use the forms of vitamins, vitamin-like and mineral substances determined by the relevant regulatory document [14, Appendix 11]. The types of foods for special dietary uses for the nutrition of athletes, recommended for fortification with essential nutrients, are shown in Table 4.

Recommendations for fortification of food products assigned for dietary preventive nutrition and dietary medical nutrition

The foods for special dietary uses intended for dietary preventive nutrition, including for the nutrition of workers engaged in jobs where they are exposed to harmful and extremely harmful working conditions, as well as dietary medical food, including baby food, shall be fortified on the basis of medical and biological requirements for the chemical composition and properties of the finished food product, taking into account the physiological needs of the body, working conditions or alimentary pathologies.

Foods for special dietary uses for dietary therapeutic nutrition and preventive dietary nutrition, which can be also used as part of standard diets, shall have proven therapeutic and/or) preventive properties, confirmed by the results of studies of their clinical efficacy based on the principles of evidence-based medicine and in accordance with the current regulations of the Russian Federation.

The food products for the nutrition of workers, employed in jobs with harmful and extremely harmful conditions, shall be fortified in accordance with the relevant Orders of the Ministry of Health and Social Development of the Russian Federation [12,13].

The list of types of foods for special dietary uses of dietary (therapeutic and prophylactic) nutrition, recommended for fortification with micronutrients, is given below in the Table 5.

Groups of foods for special dietary uses, including fortified food products, assigned for nutrition of people engaged in enterprises and institutions with harmful and extremely harmful working conditions, are determined by the Order of the Ministry of Health of the Russian Federation [12]. The main fortifying ingredients include soluble dietary fiber (in particular: pectin), antioxidants and vitamins.

The food products are preferably to fortify not with individual vitamins only, but a complete set of B vitamins [23] in combination with other vitamins. When adding a set of micronutrients to fortified products, it is more convenient to use ready-made vitamin mixtures,

Table 4. Types of athletes' foods for special dietary uses, recommended for nutrient fortification

Types of foods for special dietary uses, purpose	Recommended nutrients for fortification
high-protein foods for athletes' nutrition control of muscle ratio, control of fat mass	vitamins: C, E, A, group B, beta-carotene; minerals: potassium, magnesium, chromium, zinc, copper, selenium, iodine; PUFAs of omega-3 family
protein-carbohydrate food products and carbohydrate-protein food products restoration of the body's energy resources and an increase in absolute and relative parameters of muscle body mass	vitamins C, E, A, D, group B, beta-carotene; minerals: calcium, phosphorus, magnesium, potassium, iron, chromium, zinc, selenium, copper, iodine, manganese, molybdenum; PUFA of the omega-3 family; soluble dietary fiber: pectin, carrageenan, gums, carboxymethyl cellulose
carbohydrate-mineral drinks maintaining the body's water-electrolyte balance, preventing dehydration, maintaining the balance of fluid and minerals in an athlete's body	vitamins of B group; minerals: calcium, potassium, magnesium, sodium in the form of electrolytes (water-soluble salts of organic and inorganic acids: calcium chloride, calcium phosphate, sodium citrate, potassium chloride, magnesium phosphate)

Table 5. Types of food products for dietary uses of dietary (therapeutic and prophylactic) nutrition, recommended for fortification with nutrients

Types of foods for special dietary uses, purpose	Recommended Nutrients for Fortification
foods for special dietary uses with hypolipidemic and hypoglycemic effect, assigned for patients with metabolic disorder syndrome	vitamins C, E, A, group B, beta-carotene; minerals: potassium, magnesium, calcium, chromium, zinc, selenium; PUFA of omega-3 family; soluble dietary fiber; biologically active substances with an antioxidant effect: flavonoids (flavonols and their glycosides — quercetin, kaempferol, rutin; flavones — luteolin, apigenin; flavonones — naringenin, hesperidin; dihydroflavonols, proanthocyanidins, catechins)
foods for special dietary uses for patients with cardiopathology caused by atherosclerotic vascular injury (ischemic heart disease and hypertension)	vitamins B1, B2, B6, B12, folic acid, C, E, A, D, beta-carotene; minerals: calcium, phosphorus, magnesium, potassium, iron, chromium, zinc, selenium, copper, iodine, manganese, molybdenum; PUFA of omega-3 family; soluble dietary fiber: pectin, carrageenan, gums, carboxymethyl cellulose; polyphenols; plant sterols; organic acids; rutin; coenzyme Q10 (ubiquinone)
foods for special dietary uses for patients with type 2 diabetes mellitus	vitamins D, A, E, C, beta-carotene; minerals: calcium, phosphorus, chromium, zinc, selenium, iodine, manganese, vanadium; PUFA of omega-3 family; soluble dietary fiber: pectin, beta-glucans, gums
foods for special dietary uses for patients with diseases of the gastrointestinal tract (syndrome irritable bowel with constipation)	vitamins: B6; PUFA of omega-3 family; inulin; curcumin
foods for special dietary uses for patients with non-alcoholic fatty liver disease	vitamins: D, C, E, group B minerals: potassium, magnesium, calcium, chromium, zinc, selenium; PUFA of the omega-3 family; phospholipids; soluble dietary fiber: pectin, gums; coenzyme Q10; alpha lipoic acid
foods for special dietary uses for osteoporosis prevention	vitamins: D, K2, C, E, group B minerals: calcium, magnesium, phosphorus, zinc
foods for special dietary uses for patients with diabetic nephropathy	vitamins: C, E, A, group B, beta-carotene; PUFA of omega-3 family; flavonoids; catechins; curcumin

mineral or vitamin-mineral mixtures (premixes) made on the basis of a carrier substance (often it is this very food product that is being fortified) in accordance with GOST R58040–2017 "Complexes of vitamin mineral. General technical conditions". The use of premixes increases the accuracy of dosage of micronutrients added to the food product, ensures their uniform distribution in the food product being fortified, and also allows production control of the micronutrient content through the determination of amount of several vitamins and / or minerals included in the premix [24].

Deviation limits for fortifying nutrients in the fortified foods

The maximum permissible deviations of the nutritional value parameters (content of vitamins and minerals) of fortified food products, indicated on the food label on its packaging, from the actual nutritional value parameters shall be as follows: for vitamins C, B1, B2, B6, pantothenic acid, niacin and mineral substances (sodium, magnesium, calcium, phosphorus, iron, zinc) ± 20%, for vitamins A, B12, D, E, folic acid, biotin and iodine — ± 30%, iodine in iodized salt ± 38%. At the same time, the actual values of vitamins and minerals mass fractions shall comply with the requirements specified in the regulatory and technical documents or standards of the organizations, which standards are used for production, analyzing and labelling of these products.

In the production of fortified food products for general population and fortified foods for special dietary uses, it is allowed to increase the content of vitamins in relation to the declared values, but not more than 70% for vitamin C and not more than 50% for other vitamins, due to a natural decrease of vitamins amount in fortified foods during their storage during the shelf life. Calculations show that excessive amount of micronutrients in fortified foods at the level of 25% still does not achieve the maximum permissible level of their consumption and remains safe [25].

Labeling of the fortified food

The labeling of fortified food products for general population and fortified foods for special dietary uses must comply with the requirements of the technical regulations of the Customs Union TR CU022/2011 [16], TR CU021/2011 [15] and other technical regulations of the Customs Union for certain types of food products.

On the food packaging the word "fortified" must be written in the name of fortified food products or in the immediate vicinity. In addition the following shall be written:

- the names of the fortifying nutrients included in the composition of the food, their guaranteed content at the end of the shelf life of the food product in 100 g or 100 ml, or in one serving of the product, as well as the content expressed as a percentage of the average daily requirement for these nutrients;
- the content of a biologically active substance (mg, mcg, g), contained in the fortified food products for general population or fortified foods for special dietary uses and its percentage from the average daily (physiological) requirement for this element or substance in accordance with the established procedure [16,19]; if the daily requirement has not been established, it is necessary to write the amount of biologically active substance in the composition of fortified food products for general population or foods for special dietary uses and its ratio to the adequate level of consumption [14, Appendix 5];
- the number of probiotic microorganisms (CFU/g (ml)) included in the fortified food products for general population, or fortified foods for special dietary uses.

In the labeling of fortified food products for general population and fortified foods for special dietary uses, it is possible to use the principles of color indication, given in the relevant methodological recommendations [26].

The labeling of fortified food products for general population and fortified foods for special dietary uses may contain information on features and efficiency of the food product and (or) nutrient, characterizing its (their) nutritional and/or energy value, or information about the expected beneficial effect on the human body in case of this food systematic as part of food rations or as part of therapeutic diets in accordance with the regulatory documents [16, 27].

Packaging, storage, transportation and sale of fortified food products

The packaging of fortified food products for general population and fortified foods for special dietary uses must comply with the requirements of the Technical Regulations of the Customs Union TR CU005/2011 [28] and TR CU021/2011 [15].

When storing products, the temperature and humidity conditions and the expiration date set by the manufacturer must be complied with. The fortified food products for general population and fortified foods for special dietary uses shall be transported in accordance with the requirements of TR CU021/2011 [15].

Form of confirmation of compliance of fortified food products to the established requirements

Forms of confirmation of compliance of fortified mass consumption food products and fortified foods for special dietary uses are specified in TR CU021/2011 [15].

Verification of conformity of enriched food products for general population is carried out in the form of declaration, confirmation of conformity of fortified foods for special dietary uses is carried out in the form of state registration.

Meat food fortification

Analysis of the available literature proved that dairy products, bakery and pastry, and various drinks are most often fortified with micronutrients. Much less often meat products are fortified, despite the fact that boiled sausages and minced meat semi-finished products get a significant share in the diet of the population in our country. Nevertheless, at present there is a certain range of these food products, including meat-based products, assigned to compensate for iodine deficiency, chopped culinary products fortified with vitamins and minerals [29].

It was shown that vitamins B1, B2, PP and C added to minced meat are well preserved during the preparation and heat treatment of minced meat products (cutlets, schnitzel, beef steaks). Preservation level achieved 66–70% for vitamin B1, 85% for B2, 76% for PP, 65–70% for C [30]. Studies and tests run on rats with vitamins B1, B2 and PP deficiency, showed that the vitamins introduced into cutlets are effectively digested by the body of animals, ensuring the normal functioning of the dependent enzymes. Long-term (for 2.5 months) inclusion of vitamins-fortified cutlets in the diet of vitamin-deficient rats as the only source of vitamins B1, B2 and PP fully ensured the growth of animals, the activity of biochemical processes dependent on these vitamins and a normal state of internal organs (according to histological and histochemical parameters) [30].

When fortifying cooked sausages, the main losses of thiamine and ascorbic acid occur at the stage of minced meat cooking and amount to 32 and 38%. Heat treatment of the food product has less effect on the stability of these vitamins (20 and 10%). The losses of riboflavin and niacin at these stages are practically the same, accounting for vitamin B2–18 and 14%, and for vitamin PP — 21 and 19%. To increase the stability of vitamins, it is advisable to use vacuum cutting [31]. As a result, to ensure the content of vitamins in the finished product from 30 to 50% of the daily requirement of the human body for the fortification of cooked sausages with vitamins shall be added to the recipe, g / 100 kg of raw material — thiamine — 1.5, riboflavin — 1.0, nicotinamide — 15, ascorbic acid — 75 [31]. The introduction of a vitamin premix into the recipe of cooked sausage not only increased the content of vitamins B1, B2, PP, C in 100 g to a level covering the phisiological need for them by 30–55%, but also reduced the amount of sodium nitrite [32].

The All-Russian Scientific Research Institute of the Meat Industry named after V. M. Gorbatov (currently the Federal Scientific Center for Food Systems named after V. M. Gorbatov) has developed vitamin-mineral mixtures for sausages for baby nutrition, as well as recipes that are used in the production of cooked sausages for kids. In accordance with GOST 31498–2012 "Cooked sausage products for baby nutrition"" [33] sausages are produced fully

cooked and / or cooked pasteurized in the following assortment: sausages: "Kids sausages — Vita", "For diabetic kids", "Timka", "Lyubushka", "Gymnasium", sausages: "Zdorovye", "Skazka-vita", "For diabetic kids". In accordance with GOST R54753–2011 "Boiled ham in a casing for baby nutrition" [34], a ham fortified with vitamins B1, B2, PP, Fe, Ca, Zn and iodine is produced in the following assortment: "Kids sausages — Vita", "Klassnaya Vita", "Shkolnaya-vita", "Skazka-vita'. Canned sausages packed in cans or sterilized bags are fortified with vitamins B1, B2 and PP in an amount that provides 25–30% of the child's physiological needs [35].

Undoubtedly promising is the technology of canned beef products for enteral nutrition, fortified with biotin, vitamins B1, B2, B12, D, Ca: P = 1.5: 2, intended for people in the postoperative period with maxillofacial injury or burn injuries, who suffer from impaired swallowing and chewing [36].

In Belarus the special-purpose canned meat products have been developed for the nutrition of pregnant women and breast-feeding mothers "Vitaminized pate with pork and liver", "Fortified beef and pork puree", the recipe of which includes vitamins and minerals mixtures (vitamins A, D3, E, B1, B2, B6, B12, nicotinamide, folic acid, vitamin C, calcium, lactulose) and (iodine, selenium, folic acid, vitamin E) in a dose that covers the needs of a pregnant and breast-feeding woman by 20–25% [37]. Their clinical efficacy has been confirmed [37].

Conclusion

It is known that the efficiency of food fortification depends on initial degree of the population's consuming the micronutrients, the correct choice of the fortified product, i. e. its share in the nutrition, dietary habits, coverage of the population with the fortified food supply, effective quality control and levels of fortification, regular monitoring and evaluation of consumption of fortified foods [38,39]. The approaches presented in the article facilitate to a certain extent the choice of both the food product assigned for fortification, and the set of fortifying components.

Despite the heterogeneity of the analyzed studies (differences in fortified foods, composition of micronutrients, dosage, age of participants, duration of fortified foods consumption, differences in diet habits, initial consumption of micronutrients), it was found that consumption of foods in the diet fortified with several micronutrients led to an increase in serum concentration of these micronutrients; led to decrease in anemia occurrence by 34%, the development of bronchocele by 74%; probability of neural tube defects by 41% [40].

According to other meta-analysis data, despite the high heterogeneity and small sample size, compared with placebo or control sample, the consumption of foods fortified with several micronutrients can reduce the occurrence of anemia by 32%, iron deficiency anemia by 72%, iron deficiency by 56%, vitamin A deficiency by 58%, B vitamins deficiency by 64%, vitamin B_6 deficiency by 91%, vitamin B_{12} deficiency by 58% [41]. At the same time, it is emphasized that none of the included studies reported on disease incidence, adverse effects, mortality from all or any specific causes.

Results of randomized clinical trials involving people, who consume dairy products fortified with phytosterols and PUFAs of omega-3 family, showed the improved biological markers of cardio-metabolic risk (i. e. lowering of low density lipoprotein cholesterol and triglycerides in blood plasma) [42].

Unfortunately, the majority of the population is still not sufficiently aware of the possibilities and role of fortified foods in health maintaining, which issue requires wide educational activity among the consumers. The population's conscious choice of fortified food products will serve as an incentive to increase the production of such food products and expansion of their assortment.

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TECHNOLOGY OF ENZYMATIC-ACID HYDROLYSIS OF BONE RAW MATERIALS IN PRODUCTION OF GELATIN

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Keywords: *gelatin, enzymes, production, hydrolysis, quality indicators*

Abstract

Bone gelatin is an important and irreplaceable item widely used in the food industry and pharmaceutical production; it is also widely used in tissue engineering and other spheres. Due to widespread use of gelatin it is necessary to search for new safe and effective technologies for bone gelatin production. This research represents the results of enzymatic-acid hydrolysis of raw material in the process of gelatin production. The article presents the results of hydrolysis analyzes, the results of the main quality parameters of the obtained gelatin samples; and the major technological scheme for gelatin production is proposed here. As result of developed technology of enzymatic-acid hydrolysis of bone raw material with the ratio of raw material mass to the volume of solvent (HCl 1M and pepsin with an enzymatic activity of 40 units) as 1:9, duration of exposure: 180 minutes (3 hours), at the stage of demineralization, liming and de-ashing, we obtained samples of gelatin at yield rate of 12.1% from the initial mass of raw materials, which is 6.9% higher in comparison with the lowest yield of gelatin according to the proposed schemes. It is shown that the samples have a high protein mass fraction 91.4%, and a low fat mass fraction 0.4%, the obtained results indicate the high technological qualities of the obtained gelatin sample, this is also confirmed by high strength of gel according to Bloom scale, which value varies within the range of 290 ± 0.7 units.

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Introduction

Gelatin is a water-soluble protein with a molecular weight of 20 to 250 kDa. The main raw material for its production is the skin and bones of farm animals [1, 2]. This type of protein has found wide and significant application in various industries. In the modern market of food products and food components gelatin is a popular product, while a great demand for this product is observed in the market for medical and cosmetic products. According to the data of Grand View Research, in 2018 the gelatin market volume amounted to 2.91 billion USD [3]. The gelatin market will grow at rate of 8% per annum, and by 2025 the volume of market will reach 5 billion USD in monetary terms [4,5]. In the territory of the Russian Federation the production of gelatin still hasn't grown; so due to this situation a big import dependence on this type of raw material is observed. First of all, this underachievement is caused by complicated multistage processes in the production of gelatin, where large amount of acids, alkali and water are used. The application of a large amount of chemical substances leads to voluminous formation of wastewater. If this wastewater is not properly treated and neutralized, the untreated wastewater from gelatin production can cause serious environmental problems [6,7,8]. The second problem in gelatin production by the classical scheme is the long duration of certain technological stages of the production process, for example: demineralization, liming and de-ashing of bone raw materials can take up to 30–40 days, and therefore there is a need for vast industrial areas for continuous technological process of gelatin production [9,10].

In the classical scheme of gelatin production, demineralization, liming and de-ashing are the main preparatory technological stages before the extraction of gelatin. These processes take quite a long time, for example, demineralization lasts from 7 to 14 days, liming takes from 3 to 8 weeks, de-ashing takes from 1 to 2 days, while each of these preparatory technological stages requires a lot of chemical reagents, such as HCl and Ca $(OH)_2$. Modern highly efficient production cannot afford these long-term processes in its technological line. Therefore there is an urgent need for new technological solutions that will help optimize this stage of production [11,12,13].

One of the optimal solutions to accelerate the process of demineralization of fat-free bone raw materials is to expose the materials to hydrolysis with enzymes or enzyme preparations.

Bone gelatin is an important and indispensable product of food and pharmaceutical industries, but it is also widely used in tissue engineering and other spheres. Due to the widespread use of gelatin, it is necessary to search for new safe and efficient technologies for production of bone gelatin [14,15,16].

There is a modern, environmentally friendly and safer method that can be applied at certain stages of the technological production of gelatin — that is hydrolysis [17,18]. During hydrolysis proteolytic enzymes are widely used; they are increasingly used in enzymatic hydrolysis in pro-

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duction of gelatin from collagenous (gelatinous) structures of fish waste and animal skins. Enzymatic hydrolysis of raw materials with high collagen content makes it possible to obtain gelatin in a shorter time with less waste [19,20].

When using bone raw materials in the production of gelatin, enzymatic hydrolysis with pepsin is ineffective, since the bone matrix is not hydrolyzed by enzymes due to dense mineral intermolecular adhesion and interlacing, while the collagen structure inside the bone matrix is susceptible to the action of the enzyme pepsin to a high degree [21,22]. For enzymatic-acid hydrolysis of bone raw materials, it is necessary to seek a balance between acidic and enzymatic effects on the surface and internal structure of the bone matrix — on the mineral calcium phosphate, of which the bone matrix consists.

Thus, the objectives of the study were to determine the effect of the duration of hydrolysis according to the developed schemes on the yield of gelatin after extraction; to determine the main indicators of the quality of gelatin samples, which had the highest yield from the mass of the original bone raw material; to develop a major technological scheme for the production of gelatin from bone raw materials.

The aim of the work was to develop a technology for enzymatic-acid hydrolysis of bone raw materials in production of gelatin.

Objects and methods

To run the experimental part of the work, preliminarily defatted bovine bone raw material was used. The raw material was finely crushed to a size of 3 ± 0.5 mm. For experiment we used tubular bones of cows obtained from a farm located in the Kemerovo region — Kuzbass. For the experiment 3 kilograms of tubular bovine bones were crushed in the laboratory chain crusher. Further, the obtained bone raw material was subjected to hydrolysis by enzyme. Pepsin of microbial origin with an enzymatic activity of 300,000 units was used. The characteristics of the used enzyme are presented below in the Table 1.

Table 1. Characteristics of the applied enzyme

Parameter	Characteristics
Composition	Pepsin based on Rhyzomucor miehei (CAS:9001–92–7)
Origin	Microbial
Assumed decomposition	Phe [1] + Val, Gln [4] + His, Glu [13] + Ala, Ala [14] + Leu, Leu [15] + Tyr, Tyr [16] + Leu, Gly [23] + Phe, Phe [24] + Phe и Phe [25]
Appearance, color	White powder
Activity in 1 g, unit	Not less than 300 000
Activation temperature, °C	30 ± 2
Manufacturer	"Meito Sangyo Co., Ltd.", Japan

The enzyme pepsin is activated by interaction with hydrochloric acid (HCl) and during interaction pepsin shows high proteolytic properties, while HCl promotes the destruction of the bone matrix. Hydrolysis with microbial enzyme pepsin and hydrochloric acid (HCl 1M) was performed according to the schemes presented below in the Table 2.

Table 2. Schemes of preliminary treatment of bone raw materials

Scheme, No.	Enzyme concentration, unit	Bone raw material to solvent ratio, (weight / volume)	Duration of exposure, min.	pH, unit	Temperature of exposure, °C
1	30	1m HCl 1:7	60	1.5–2.0	27.0 ± 2 °C
			120		
			180		
			240		
		1m HCl 1:8	60		
			120		
			180		
			240		
		1m HCl 1:9	60		
			120		
			180		
			240		
		1m HCl 1:10	60		
			120		
			180		
			240		
2	35	1m HCl 1:7	60	1.5–2.0	27.0 ± 2 °C
			120		
			180		
			240		
		1m HCl 1:8	60		
			120		
			180		
			240		
		1m HCl 1:9	60		
			120		
			180		
			240		
		1m HCl 1:10	60		
			120		
			180		
			240		
3	40	1m HCl 1:7	60	1.5–2.0	27.0 ± 2 °C
			120		
			180		
			240		
		1m HCl 1:8	60		
			120		
			180		
			240		
		1m HCl 1:9	60		
			120		
			180		
			240		
		1m HCl 1:10	60		
			120		
			180		
			240		
4	45	1m HCl 1:7	60	1.5–2.0	27.0 ± 2 °C
			120		
			180		
			240		
		1m HCl 1:8	60		
			120		
			180		
			240		
		1m HCl 1:9	60		
			120		
			180		
			240		
		1m HCl 1:10	60		
			120		
			180		
			240		

The crushed bone raw material was submerged into solution of hydrochloric acid (HCl 1M), where the microbial enzyme was added to according to the submitted schemes. The raw material was exposed to hydrolysis at a temperature of 27 ± 2 °C for 60 to 240 minutes. pH of the medium varied within the range of 1.5–2.0 units. For uniform treatment of bone raw materials with a solution during the entire duration of the experiment, a magnetic laboratory stirrer "MM-5" (Russia) was used. The bone raw material was stirred at 100 rpm at the temperature up to 27 ± 2 °C. After hydrolysis, the bone raw material was subjected to centrifugation, which facilitated the separation of mineral sediment from ossein. The bone raw material was centrifuged on a high-speed centrifuge "Avanti J-26S — Beckman" (Beckman Coulter, USA). The technical characteristics of the centrifuge are presented below in the Table 3.

Table 3. Technical characteristics of the high-speed centrifuge "Avanti J-26S — Beckman"

Speed range for angle rotors, rpm	100–26 000
Maximum speed for bucket rotors, rpm	13 000
Maximum acceleration for angle rotors (x g)	81 800
Maximum acceleration for bucket rotors (x g)	26 500
Maximum volume for angle rotors, ml	6 000 ml (6 x 1 000 ml)
Maximum volume for bucket rotors, ml	4 000 ml (4 x 1 000 ml)
Volume of the tested sample in one capsule	1,5 ml — 1 000 ml
Time range	Up to 180 min., Hold mode
Engine's type	valve-inductor brushless motor
Number of acceleration / deceleration modes	2 acceleration / 3 deceleration
Friction reduction system	Yes
Temperature range	From –10 °C to +40 °C (in increments of °C)
Temperature control	± 2 °C of the setpoint
Heat dissipation (kW)	2
Noise level, dB	<57
Width x depth x height (cm)	71.0 × 86.0 × 86.0
Weight, kg)	290
Type of installation	Floor installation

After that the obtained ossein was washed with demineralized water. After removal of the mineral residue, the ossein was transferred to the stage of gelatin extraction. In order to save resources and ensure the environmental friendliness of production, gelatin was extracted with water at a temperature of 60 °C. The obtained gelatin broths were dried by a laboratory drying sprayer, model "Mini Spray Dryer B-290" (Buchi, Sweden) at a temperature of 95 °C, the feed rate of the broths into the spray chamber amounted to 3.0–3.2 ml/min. The mass fraction of protein was determined on a thermal digestor "FOSS Tecator Digestor 2520" (produced by Foss Tecator, Sweden). The strength of the gel according to Blum scale was determined on a texture analyzer "Structurometer ST-2" completed with an indenter "Bloom". The technical characteristics of the texture analyzer are presented below in the Table 4. The materials were prepared for analysis according to the following procedure: 7.5 g of gelatin was placed in a glass of cold water (105 ml of water) and this mixture was kept for 180 min at a temperature not over 22 °C. Then the swollen gelatin was heated in a water bath to a temperature of 60 °C and stirred for 15 min until complete dissolution. The prepared gelatin solution (with concentration of 6.67%) was poured into a special calibrated vessel and kept for 17 hours at a temperature of 10 ± 0.1 °C.

Table 4. Technical characteristics obtained by the texture analyzer "Structurometer ST-2"

Indenter speed (indenter penetration rate or rate of medium deformation), mm / s	0 … 4,5
Indenter stroke range, mm	0 … 220 ± 1
Reduced relative load measurement error,%, no more	1
Discreteness of setting the loading speed (increase of load), g / s	0,1
Device weight, kg	30
Frequency of the power voltage, Hz	50/60
Overall dimensions, mm	280 × 440 × 680

Experimental researches were run in the Department of Food Technology of Animal Origin and the Research Institute of Biotechnology, Kemerovo State University.

The experimental results were processed using the software *Statistica 10.0* (StatSoft Inc., 2007, USA).

Results and discussion

The yield of gelatin from bone raw materials is influenced by many production factors. The main of which are the method of processing bone raw materials and the method of its demineralization in order to obtain ossein [23,24,25]. In this regard, at the first stage of research we analyzed the influence of enzymatic-acid hydrolysis duration according to the proposed schemes (Table 2) on yield of gelatin during subsequent extraction of gelatin.

To determine this influence, after the extraction of gelatin from gelatin broths, the obtained samples were spray dried on a laboratory spray drier of model "Mini Spray Dryer B-290" (Buchi, Sweden). The influence of hydrolysis duration on gelatin yield during its extraction are shown below in the Figure 1.

The yield of gelatin after extraction ranged depending on the scheme of preliminary processing. According to the data presented in Figure 1, it can be seen that the minimum yield of gelatin among all the presented hydrolysis schemes, is 5.2%, the maximum is 12.1%, which is 6.9% higher in comparison with the least yield. At the same time, each of the schemes proves that pepsin efficiently dissolves collagen in the bone, breaking down the peptide bonds and providing a higher yield of gelatin, depending on enzymatic activity of applied enzyme and the volume of solvent in ratio to the mass of the initial raw material.

Figure 2 shows the results of the comparative yield of gelatin depending on the scheme of hydrolysis.

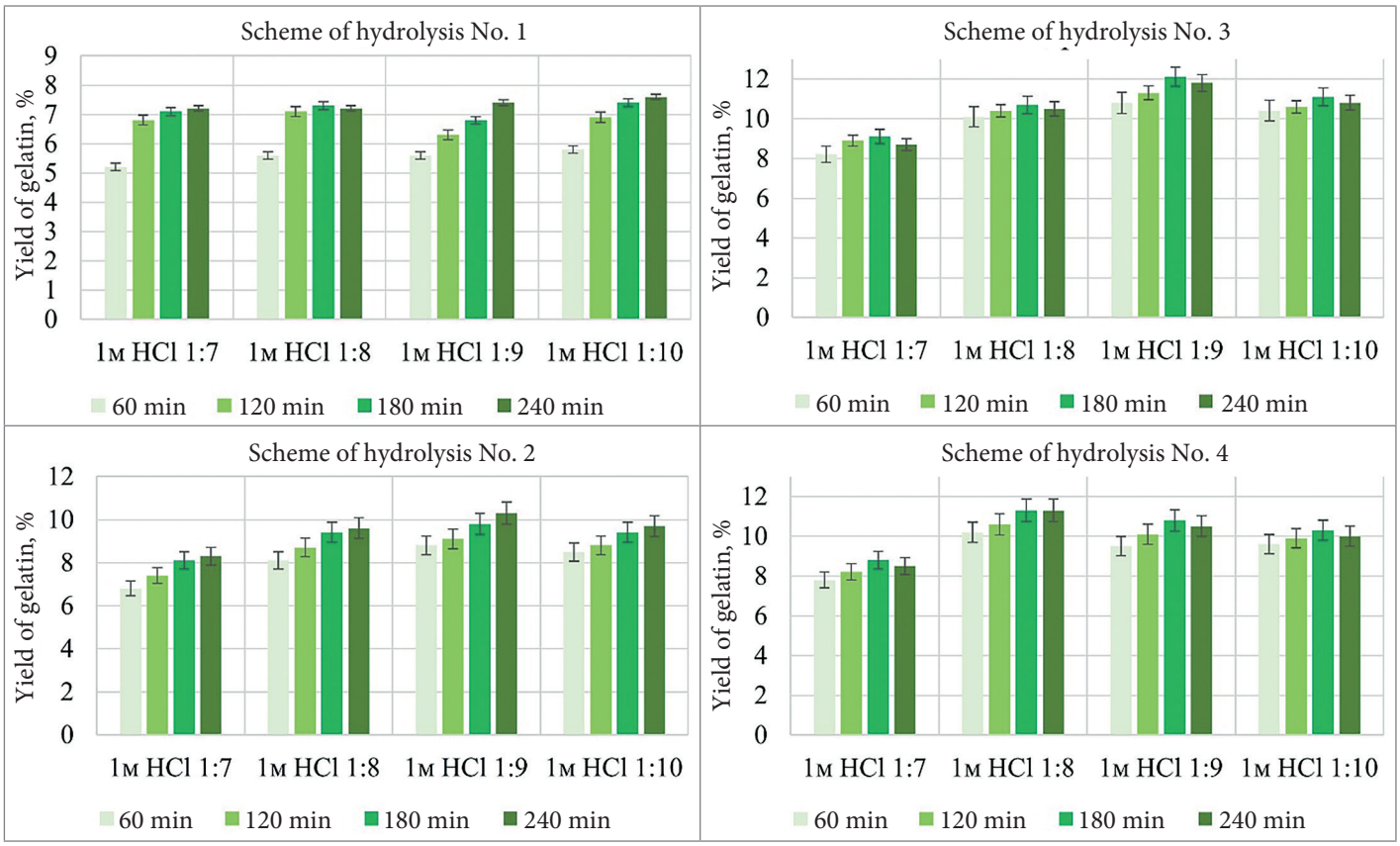

Figure 1. Influence of hydrolysis duration on yield of gelatin during its extraction

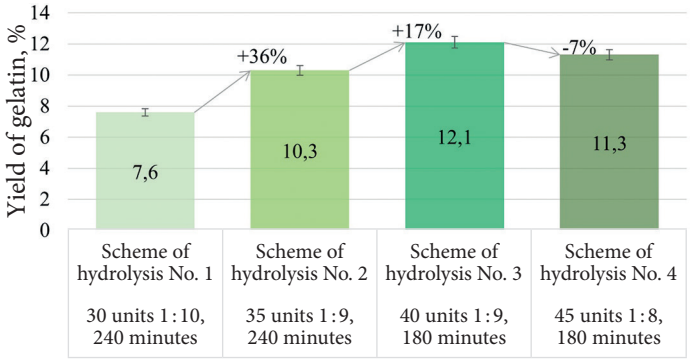

Figure 2. Comparative yield of gelatin

Basing on assessment of influence of hydrolysis duration on yield of gelatin during extraction, it can be concluded that the highest yield of gelatin is achieved during hydrolysis in the scheme 3 with a ratio of raw material to the volume of solvent 1:9 for 180 minutes (3 hours). It can also be noted that hydrolysis in the scheme 4 with a ratio of raw material to the volume of solvent 1:8 for 120 minutes also has a high yield of gelatin after its extraction; the yield amounts to 11.3% of the initial raw bone material.

Further, the main indicators of gelatin samples quality were determined, which had the highest yield from the mass of the initial raw bone material. The results are presented below in the Table 5.

If to compare the results of quality indicators of the obtained gelatin samples, with the standardized indicators GOST 11293–2017 "Gelatin. Specifications" for gelatin, the proposed hydrolysis technology allows obtaining gelatin of high quality. Some results of analysis of quality indicators of the obtained gelatin samples surpass the data obtained by other researchers. Thus, the mass fraction of protein in the obtained samples is 3–5% higher in average, the mass fraction of fat is also higher 0.2% in average [26,27,28,29].

Table 5. Main indicators of the obtained gelatin quality

Parameter	Sample obtained by hydrolysis scheme No. 3	Sample obtained by hydrolysis scheme No. 4	Normative value by GOST 11293-20171
Mass fraction of protein, %	91.4 ± 0.2	90.7 ± 0.1	—
Mass fraction of fat, %	0.4 ± 0.06	0.6 ± 0.03	—
Mass fraction of moisture, %	7.8 ± 0.2	8.1 ± 0.1	Not more than 16,0
Mass fraction of ash, %	0.4 ± 0.03	0.6 ± 0.04	Not more than 2,0
Gel strength by Bloom, unit	290 ± 0,7	275 ± 0.5	from 100 to 300

The obtained data confirm the applicability of enzymatic-acid hydrolysis of bone raw materials in order to obtain a high-quality gelatin, which makes it possible to develop new resource-saving methods for gelatin production and to launch a new branch of the industry in whole [30, 31].

At the final stage of scientific research, a schematic diagram of technology of gelatin production by enzymatic-acid hydrolysis of bone raw materials was developed according to the scheme No. 3. The developed scheme is shown below in the Figure 3.

[1] GOST 11293–2017 "Gelatin. Specifications". Moscow: Standartinform, 2020. — 35 p.

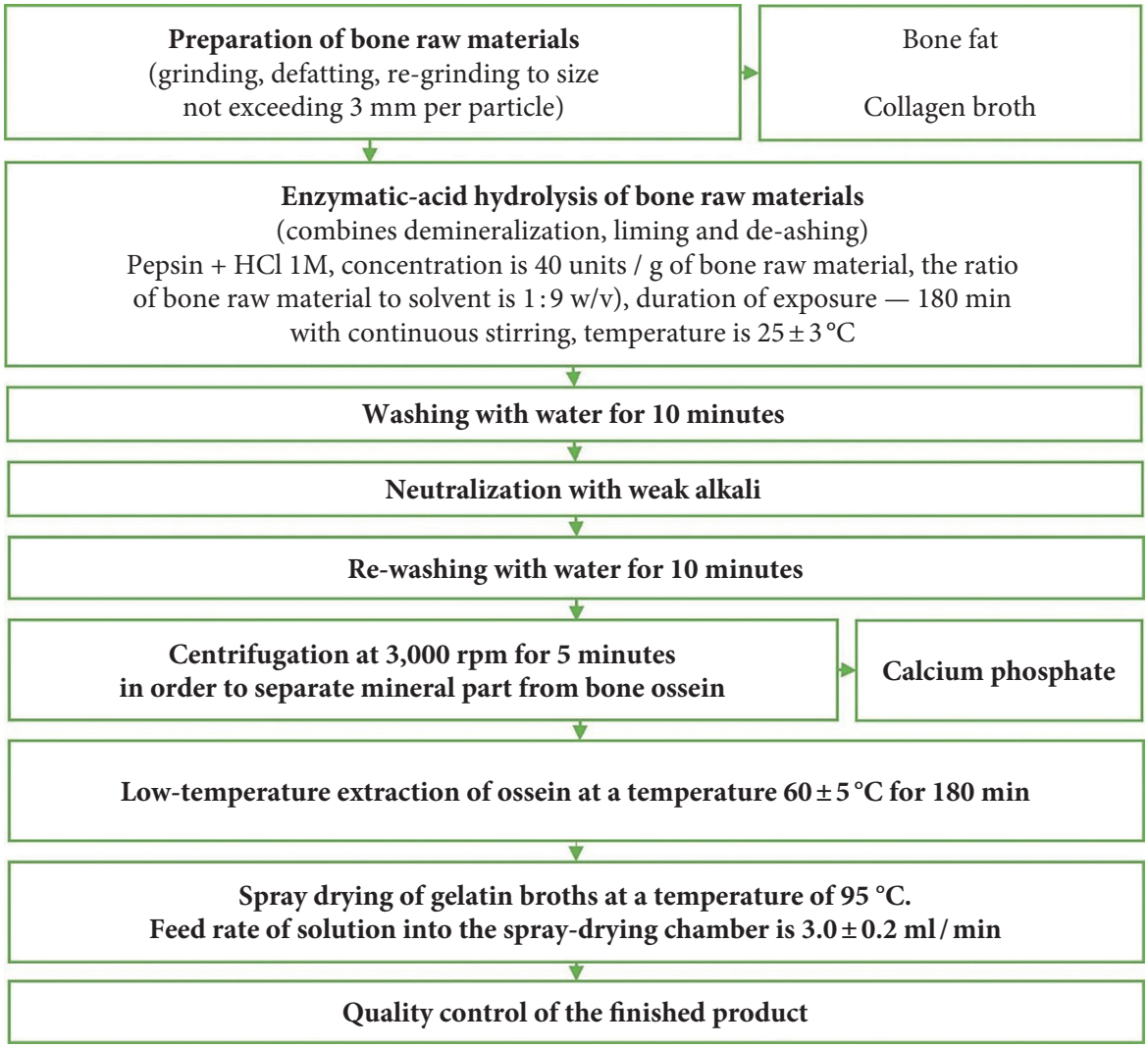

Figure 3. Basic technological scheme for production of gelatin from bone raw materials

On the basis of the performed studies, it is proposed to apply enzymatic-acid hydrolysis of bone raw materials at stages of demineralization, liming and de-ashing during the production of edible gelatin. The proposed technology is capable to shorten the technological process of gelatin production and to achieve sufficiently high yield of the final product in reference to the initial raw material.

Conclusion

The developed technology of enzymatic-acid hydrolysis of bone raw material with ratio of raw material mass to the volume of solvent 1:9 (HCl 1M and pepsin with enzymatic activity of 40 unit) for 180 minutes (3 hours) at stage of demineralization, liming and de-ashing allowed obtaining gelatin samples with a yield of 12.1% of the initial mass of raw materials, which is 6.9% higher than the lowest yield of gelatin in the proposed schemes. According to results of analysis of main indicators of quality, the obtained gelatin meets or exceeds the standards of GOST 11293–2017 as the obtained samples feature high mass fraction of protein — 91.4%, and low mass fraction of fat — 0.4%. The obtained results indicate high technological qualities of obtained sample of gelatin. This is also confirmed by the high strength of gel according to Bloom scale, which value varied within the range of 290 ± 0.7 units.

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THERMAL CONDUCTIVITY FACTOR FOR BEEF OF NOR AND DFD GRADES AT THE SUBCRYOSCOPIC TEMPERATURES

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Keywords: *thermophysical properties, superchilled, calculation formulas, temperature, humidity, frozen water, meat*

Abstract
Thermal conductivity factor and specific isobaric heat capacity of food products are currently the most important parameters in the development of mathematical models for food freezing and thawing and in improving production technology. There is significant variance among the existing experimental data for the thermal conductivity factor in meat. Most of the modern calculated relationships are based on the nutritional approach, which favorably differs by the ability to calculate the thermophysical characteristics of any food products. However, the calculation error at the subcryoscopic temperatures may be 15% to 20%. The development of superchilling as a way of storing meat requires high accuracy of freezing time calculation, including vacuum-packed boneless meat. In the presented article, the authors investigated hydrogen index, cryoscopic temperature, frozen moisture proportion and thermal conductivity factor for beef M. longissimus dorsi samples of NOR and DFD grades. It was found that DFD beef is characterized by 10% to 12% higher values of thermal conductivity factor in comparison with NOR grade. Using the method of regression analysis, the authors developed empirical relationships for calculating the thermal conductivity factor of meat depending on its temperature and pH level. Unlike cryoscopic temperature and frozen moisture proportion, pH is easy to measure and may be easily used on a conveyor belt for more accurate assessment of meat thermophysical properties. With an increase in pH from 5.3 to 7, an increase in cryoscopic temperature is observed from minus 0.94 °C to minus 0.72 °C. It has been shown that one of the factors for the higher cryoscopic temperature and higher pH level of DFD beef is higher water-holding capacity with less strongly bound moisture.

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Introduction

Simulation and control of food refrigeration and heat treatment processes based on the calculation of temperature profiles inside the food product, as well as the determination and justification of points for thermal control are inexorably associated with the need to know the thermophysical properties. Currently, according to the results of studies [1–3], the accuracy of food technology processes simulation is determined primarily by the availability and reliability of the initial data on the thermophysical properties of food products, local heat transfer coefficients, intensity of chemical processes, rather than by the computing power and principles of the numerical models used.

Cooling, freezing and thawing processes are among the main methods of meat preserving [4,5]. In addition, superchilled meat is becoming more common [6]. Superchilling of meat means its surface freezing and subsequent storage at the temperatures 1 °C to 2 °C below cryoscopic temperature [7]. According to [8], storing pork at minus 2 °C instead of plus 3.5 °C has increased its shelf life from 2 to 16 weeks. The main problem in introducing this technology is to determine the required time of meat freezing, as well as the development of appropriate calculation methods for various linear dimensions of samples and their thermophysical properties [7].

With the introduction of numerical simulation methods for the processes of refrigeration and heat treatment in food industry [9,10], the development of simple and accurate semi-empirical relationships describing the thermophysical properties of meat is of particular importance, including the thermal conductivity factor, which, together with the specific isobaric heat capacity, significantly varies at negative temperatures.

Currently, two methods for determining the thermophysical properties of food products are the most common: calculation based on the nutrient composition [11–13] and the use of experimental data [14–18].

The method for calculating the thermophysical characteristics of products based on the nutrient composition is associated with a higher error, which can be 15% to 20%, but it may be used for any product. In the ASHRAE handbook [11], data on the composition of products and cryoscopic temperature were obtained empirically (USDA), while the presented thermophysical properties of products were determined by a calculation method based on the thermophysical properties of their nutrients according to the model proposed in [12]. Researchers [12] were among the first

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to obtain and arrange the thermophysical characteristics of individual components in food products (proteins, fats, carbohydrates) in a wide temperature range and developed the corresponding calculation models. In [13], an extensive collection of empirical data from various researches on the nutritional composition of food products and polynomial relationships for calculating their thermophysical properties in the temperature range of minus 40 °C to plus 40 °C are presented. Experimental data [14] on the thermal conductivity of beef meat, fat and bones are of special interest.

Experimental studies of the thermal conductivity factor in meat products indicate a significant anisotropy and the effect of its refrigeration and grinding modes on the properties of the product [19,20]. A study of the ionizing radiation (dose 12 kGy) effect on the thermophysical properties of chilled beef and pork [21] showed an increase in the thermal conductivity factor by 15% to 20%, a decrease in the specific isobaric heat capacity by 10% to 12%, and a decrease in water activity by 3%. As a possible explanation, the author notes a decrease in the moisture content and water-holding capacity of meat [22].

Considering the product as a multicomponent dispersed medium, three main components may be distinguished for meat, which determine its thermal conductivity factor: muscle fibers (and fat), ice crystals and unfrozen moisture [23]. Attempting to improve approaches for calculating the thermophysical properties of moisture-containing products at subcryoscopic temperatures, the authors [24] noted the need to take into account the structure and size of ice crystals [25, 26], as well as to increase the accuracy of the product cryoscopic temperature and the frozen moisture proportion calculations [27].

Research on the thermophysical properties of NOR and DFD beef [28,29] indicated a strong correlation between cryoscopic temperature and meat pH level. With an increase in the pH of beef from 5.5 to 6.9, the cryoscopic temperature increases from minus 1.5 °C to minus 0.9 °C [28]. At the temperatures close to cryoscopic temperature, the amount of frozen moisture in meat of different grades may vary by more than 30% [29,30,31].

Thus, the aim of the presented work is the experimental research and development of a practical approach to the calculation of the thermal conductivity factor in meat of different grades.

Materials and methods

Measurement of the thermal conductivity factor in beef with the Linseis TNV-100 device equipped with the HotPoint Kapton-foil-sensors (Linseis Messgeraete GmbH, Germany). The metrological characteristics of the device are presented in Table 1. The stationary temperature mode required for testing was ensured by placing the sensor and the test sample in thermostat with a specified temperature (in the range of plus 20 °C to minus 35 °C with a step of 5 °C) and holding the sample for at least an hour at a constant temperature recorded by the sensor.

Table 1. Metrological characteristics of the HotPoint sensor by Linseis

Temperature measurement range, °C	minus 100 to plus 200
Thermal conductivity measurement range, W/m · K	0.02 to 30.0
Maximum absolute error when measuring thermal conductivity, %	±7.0

Thermal conductivity measurement using the HotPoint sensor is carried out by the method of a non-stationary heat source in the form of a plane. The measuring principle is as follows: the sensor is placed between the two halves of the test sample. During the measurement, a DC current (10 mA, 30 mW) flows through the film resistor of the sensor, causing the temperature to rise. The generated heat is scattered in the sample on both sides of the sensor. The temperature deviation from the initial temperature of the sample measured by the sensor makes it possible to calculate the thermal conductivity of the material under study according to the relationship:

$$\lambda \approx \frac{Q}{4\pi \cdot r \cdot \Delta T_s} \quad (1)$$

where:

Q is the heat flow, W;

r is the effective radius of the sensor, m;

ΔT_s is the deviation of the sample temperature from the initial temperature measured by the sensor, °C.

The studied beef samples for measuring the thermal conductivity factor were cut in the form of two plates with linear dimensions of at least 40 mm × 40 mm × 10 mm.

The HotPoint sensor was calibrated using reference materials: polymethyl methacrylate (PMMA) (thermal conductivity factor of 0.194 W/m · K), BK7 optical glass (thermal conductivity factor of 1.118 W/m · K), and titanium (thermal conductivity factor of 22 W/m · K). Control measurements of the thermal conductivity in ice and distilled water showed the deviation of the results from the reference values not exceeding 2.5%.

The pH value of beef was measured using the PCE-228 pH meter (PCE Deutschland GmbH, Germany) characterized by a measurement error not exceeding ± 0.5% at 20 °C.

The moisture content in the samples was measured on the AND ML-50 moisture analyzer by AND Japan, with a sample weight of 5 grams and an error in moisture content measuring not exceeding 0.2%.

The cryoscopic temperature of the samples was measured using the OSKR-1 cryoscope osmometer (KIVI, Russia). This device is equipped with a mechanical initiator of the crystallization process. In the temperature range of 0 °C to minus 0.93 °C, it provides an error of the cryoscopic temperature measurement not exceeding ± 0.002 °C. At the temperatures below minus 0.93 °C, the cryoscopic temperature measurement error is ± 0.010 °C.

The amount of frozen moisture in the samples depending on the temperature was measured on the DSC204 F1

Phoenix® differential scanning calorimeter (NETZSCH-Gerätebau GmbH, Germany). A detailed description of the methodology for carrying out these measurements and the settings of the device are described in [32]. The error in measuring the enthalpy of phase transitions according to the manufacturer's data does not exceed 3.0%.

For research, the authors used beef samples of NOR (6 samples) and DFD (6 samples) grades. Samples of beef *M. longissimus dorsi* were obtained at the slaughters of meat processing plants in the Moscow region. For each sample, at least 2 measurements of the thermal conductivity factor, moisture content, cryoscopic temperature and frozen moisture proportion were carried out.

Results and discussion

According to the studies, the average values of the moisture content in beef of NOR and DFD grades were extremely close and amounted to $W_{NOR} = 0.762 \pm 0.165$ and $W_{DFD} = 0.763 \pm 0.011$, respectively, with a confidence level of $p = 0.95$. Comparative results of the studies on cryoscopic temperature of samples depending on the pH values are presented in Figure 1.

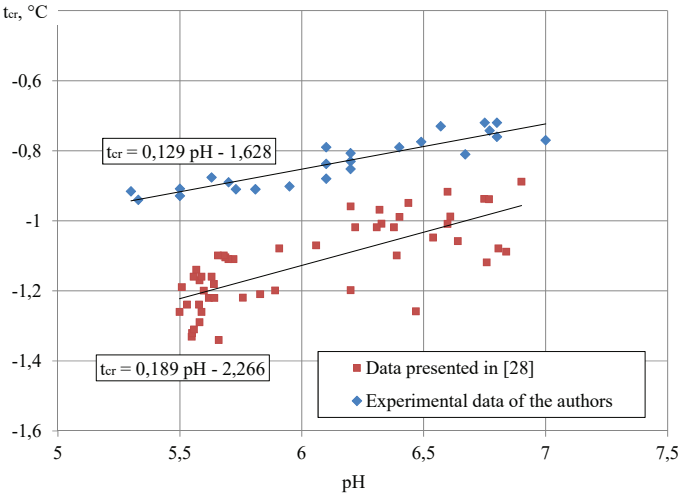

Figure 1. Dependence of the cryoscopic temperatur of *M. longissimus dorsi* on the pH value

The obtained results are in good agreement with the experimental data obtained by Farouk M. M. et al. in [28]. With an increase in pH from 5.3 to 7, an increase in cryoscopic temperature from minus 0.94 °C to minus 0.72 °C is observed. At the same time, the cryoscopic temperature of the beef samples studied in this work was on average 0.27 °C higher than that of Farouk M. M. et al. in [28]. This deviation may be due to both the physical characteristics of specific cattle breeds and the conditions for their feeding and maintenance, which requires additional research beyond the scope of this article. Calculation of the cryoscopic temperature of beef depending on the pH values may be carried out according to the following relationship:

$$t_{cr} \approx 0.129 \cdot pH - 1.628 \qquad (2)$$

Figure 2 shows the dependence of the frozen moisture proportion in the NOR and DFD beef samples obtained with a differential scanning calorimeter, the results obtained by calculation using formula (3), as well as the data presented in [18]. While the moisture content in NOR and DFD samples is equal, the amount of frozen moisture in DFD samples is 1.5% higher. This confirms the authors' hypothesis [28] that one of the reasons for the higher cryoscopic temperature and pH value of DFD beef is better water-holding capacity with less strongly bound moisture (by 16%).

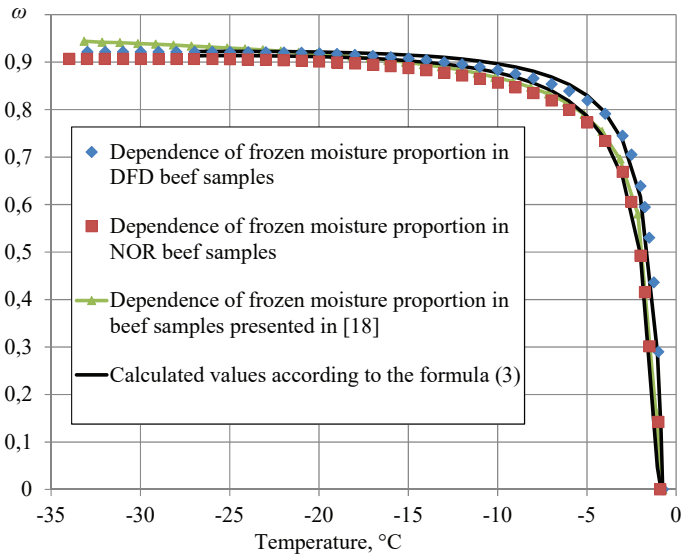

Figure 2. Experimentally determined amount of frozen moisture proportion in meat samples of different grades depending on temperature

The results of the measurements are in satisfactory agreement with the data of V. P. Latyshev [18]. Earlier experiments [30] showed that at temperatures below minus 35 °C, the amount of frozen moisture in meat remains almost unchanged. Taking this into account, a formula was developed for calculating the amount of frozen moisture (3). Its main advantage is the high accuracy of the frozen moisture proportion calculation at the temperatures close to cryoscopic temperature, regardless of the moisture content in the products.

$$\omega(t,w) = \omega_{fr}(w) \cdot \frac{1 - \frac{t_{cr}}{t} - \frac{t_{cr}}{t_{fr}^2}(t - t_{cr})}{\left[1 - \frac{t_{cr}}{t_{fr}}\right]^2} \qquad (3)$$

where:

ω_{fr} is the fraction of freezing water, which depends on the nature of the food product, its moisture content and water-holding capacity,

t_{fr} is the freezing point determined by differential scanning calorimetry on the DSC204 F1 Phoenix® instrument.

The freezing point for most types of meat is minus 33 °C to minus 35 °C.

The results of experimental studies on thermal conductivity λ for beef samples of NOR and DFD grades, in comparison with the data [14, 18], determined by the method [24], as well as obtained by calculation according to formulas (4) and (5) are presented in Figure 3. DFD beef is

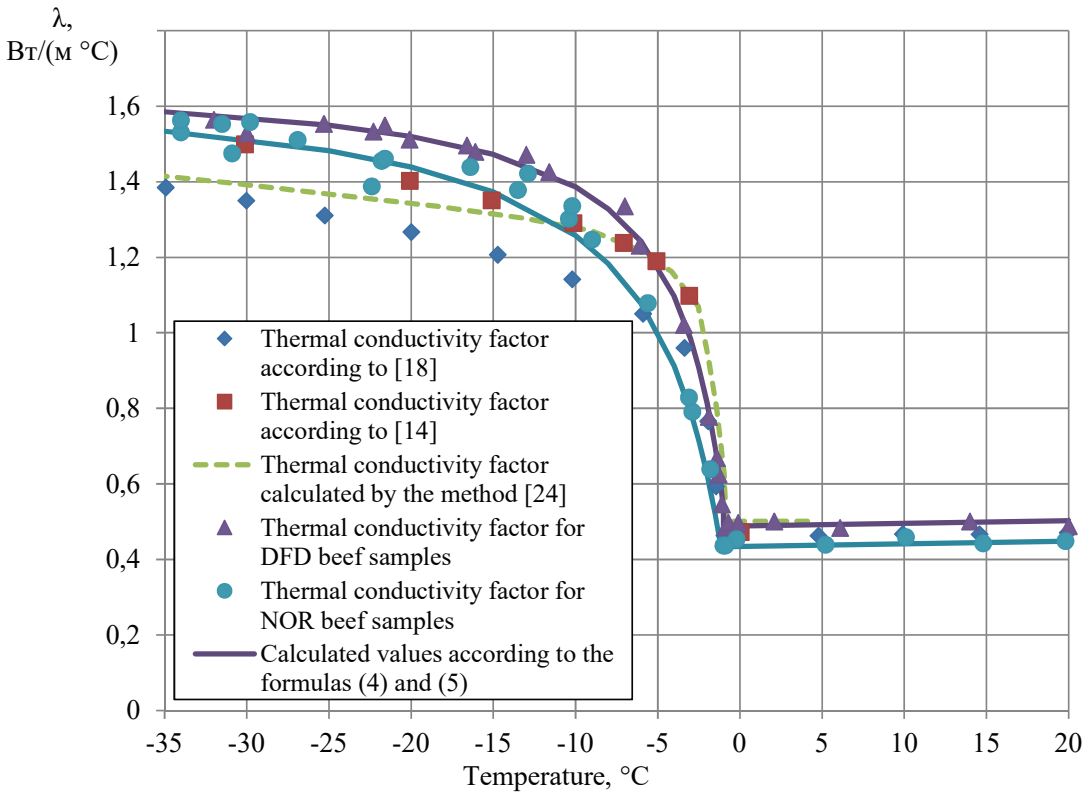

Figure 3. Thermal conductivity factor λ for beef of different grades

characterized by 10% to 12% higher values of thermal conductivity factor in comparison with NOR beef. Deviations in the thermal conductivity factors of beef obtained in this study, as well as in the works [14, 18], may be not only due to different properties of beef samples, but also due to the modes of meat freezing used by the authors. Low chilling rates promote the growth of larger ice crystals and lead to higher thermal conductivity of the samples [26].

For calculating the thermal conductivity factor of beef meat depending on temperature and pH, the authors obtained the following calculated relationship based on the method of regression analysis in the temperature range above cryoscopic temperature:

$$\lambda(t, pH) = 0.26 + 0.0007 \cdot t + 0.0317 \cdot pH \qquad (4)$$

To describe the thermal conductivity factor in the temperature range below cryoscopic temperature, the authors initially attempted to develop a semi-analytical relationship taking into account relationships (2) and (3), but the coefficient of the experimental data determination was very low and did not exceed 0.7. As a result, preference was given to the development of an empirical relationship and the regression analysis:

$$\lambda(t, pH) = \frac{t}{0.595 \cdot t + 0.435 \cdot pH - 4.398} \qquad (5)$$

The proposed relationship describes the data collected by the authors on the thermal conductivity of beef at temperatures below cryoscopic temperature with determination coefficient $R^2 = 0.95$. The choice of pH as one of the variables is primarily based on the simplicity of its measurements, in contrast to the cryoscopic temperature. These relationships are aimed primarily at the development of rapid numerical methods for determining the required duration of freezing for vacuum-packed boneless meat and improving conveyor technologies for meat superchilling.

Conclusion

With the introduction of superchilling technology for vacuum-packed boneless meat, the development of mathematical models for the process of its freezing, as well as obtaining relationships to describe its thermophysical properties, become more relevant than ever. The pH value is easy to measure and, unlike cryoscopic temperature, may be easily used in conveyor production for a more accurate assessment of meat thermophysical properties.

With an increase in pH from 5.3 to 7, an increase in cryoscopic temperature is observed from minus 0.94 °C to minus 0.72 °C. Frozen moisture proportion analysis was confirmed by other authors showing that one of the reasons for the higher cryoscopic temperature and pH of DFD beef is higher water holding capacity with less strongly bound moisture.

For meat of different grades, there is a difference in the values of thermal conductivity by 10% to 12%. The authors have developed empirical relationships for calculating the thermal conductivity factor of meat depending on temperature and pH value.

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