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BIOTRANSFORMATION OF HORSE MEAT PROTEINS BY PROBIOTIC MICROORGANISMS TO REDUCE ALLERGENICITY

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Abstract

Proteins may cause food allergies, the prevalence of which is growing worldwide. The intestinal microbiota takes an active part in stimulating and maintaining the functions of the body's immune system by producing humoral factors: enzymes, cytokines, mediators involved in the development of the immune response. The study of microbial consortium effect, which has high enzymatic activity, on reducing the reactivity of the body's immune response is of interest. The aim of the work was to study the biotransformation of horse meat proteins based on the use of probiotic consortium to obtain hypoallergenic meat raw materials. The object of the research was horse meat cooled for 24 hours at a temperature of 2 to 4 °C. The control: untreated meat samples; test 1: horse meat treated with a starter culture of one strain of Lactobacillus paracasei k-406; test 2: horse meat treated with a combined starter culture of four strains of Lactobacillus (Lactobacillus curvatus LCR-111–1, Lactobacillus plantarum 8RAZ, Lactobacillus fermentum 44/1 and Lactobacillus paracasei k-406). The microbial consortium was selected taking into account its biological compatibility and biotechnological potential, in particular, proteolytic activity. The degree of hydrolysis, technological parameters, fiber microstructure and the level of sensitization were determined. High proteolytic activity of the microbial consortium used for horse meat treatment was noted compared to the sample treated with a single-strain culture. Thus, the degree of hydrolysis after three days increased 4 times compared to the original raw material, which contributed to an increase in the hydrophilicity of the meat system by 11.8% and a decrease in the shear strength by 13.1% compared to the control (unfermented horse meat). Probiotic consortium caused a proteolytic modification of horse meat proteins, resulting in a decrease in the level of antigenic epitopes, which contributed to a decrease in the sensitizing activity of meat raw materials. This indicates the prospects for using such raw materials in the production of hypoallergenic meat products.

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Introduction

The dynamic growth of patients with food allergies all over the world indicates an urgent need to develop products that provide adequate nutrition and quality of life for people with a specific reaction to allergens. The relevance of this problem is confirmed by the Technical Regulations of the Customs Union TR CU 022/2011[1]. In Article 4 "Requirements for the labeling of food products", the manufacturer is obliged to indicate in the composition of food products the ingredients, biologically active additives, the use of which may cause allergic reactions or is contraindicated in certain types of diseases. The most common food allergens include nuts (almonds, cashews, peanuts), milk and dairy products, fish, shellfish, eggs and others. There are a number of proteins that may cause a strong allergic reaction in some people. The most well-known include casein from cow's milk, egg albumin, tropomyosin and parvalbumin from fish. Clinical manifestations of allergic reactions in humans may be expressed as gastrointestinal disorders (vomiting, diarrhea, upset stomach), rash, itching in different parts of the body, allergic rhinitis (runny nose) and even asthma attacks (anaphylaxis) [1].

Serum albumin and gamma globulin are the most prevalent and studied proteins and represent the two main potential allergens of animal meat. Serum albumin is one of the most common blood proteins; its concentration in plasma is 35 to 55 mg/ml. These are proteins with a globular spatial conformation formed by several domains [2,3,4]. Allergenic proteins contain specific segments known as epitopes. They are recognized by the immune system and trigger an allergic reaction. Epitopes may be linear or conformational. Linear (peptide) epitopes are sequences of amino acid residues in the allergenic protein that are recognized by IgE antibodies. They may be relatively short (5 to 20 amino acids) and may be present in different parts of the

[1] TR CU022/2011 Technical Regulations of the Customs Union "Food products regarding their labeling". (as amended as of April 22, 2024)" Retrieved from https://docs.cntd.ru/document/902320347. Accessed February 20, 2025 (In Russian)

allergenic protein. Conformational epitopes are formed by the three-dimensional structure of the protein, which is formed after the spatial folding of individual amino acid sequences. They may be recognized by IgE antibodies only in their three-dimensional conformation. These epitopes are usually more specific than linear ones. Epitopes on the surface of allergenic proteins are quite diverse, depending on the type of allergen, its chemical nature and spatial structure. In addition, epitopes may change depending on the storage and processing conditions of the allergen. This may affect the ability of the immune system to recognize and respond to them [5,6,7].

Food allergies most often affect children, starting from an early age. According to experts, this process is associated with problems in the formation of intestinal microflora under the influence of various factors. Due to the active growth of the urbanization process, the number of cases of milk allergy is also growing, among which there are cases of beef allergy, which is apparently associated with the antigenic affinity of bovine proteins [5]. In the absence of specific methods of therapy, one of the approaches to the prevention and treatment of such patients is the exclusion of the allergen-containing products from the diet [8,9]. Since the growing body of children requires satisfying all physiological needs, especially with regard to proteins of animal origin, deep hydrolysates of proteins from cow's milk or other dairy animal's milk are used for artificial feeding [10,11].

To date, the food industry does not produce hypoallergenic products for mass consumption. Various types of technological processing of food raw materials such as thermal treatment, hydrostatic pressure, microwave, ultrasound, chemical modification and others are not so effective. The principle of hypoallergenicity is based on the destruction of the conformational structure of the protein, on the surface of which there are IgE-antigenic epitopes capable of interacting with antibodies [12,13]. The most promising method used by many authors is enzymatic hydrolysis of proteins, resulting in short-chain peptides and amino acids formation, that lose their conformational active center of interaction with Ig E. According to a number of authors, with a molecular weight below 3.5 to 10 kDa, allergenicity becomes minimal [14–16]. However, during enzymatic hydrolysis, potential epitopes that are inside protein globules or those that are newly formed during interaction with components of food raw materials may appear [17–19].

The COVID-19 pandemic has drawn the attention of the entire population to the problems of immunoprophylaxis and products that help boost immunity. The role of probiotic consortia in the prevention of a number of diseases was discussed back in 2015 at the FEMS (Federation of European Microbiological Societies) congress [20]. Healthy intestinal microflora is one of the most important factors in human well-being. In this regard, the production of probiotic food products based on the most significant representatives of the human gastrointestinal microflora remains relevant [21].

Intestinal microflora is an integral part of the body and is involved in various life support processes. The protective function is manifested in maintaining internal homeostasis due to the release of bacteriocins and the destruction of bacterial toxins [22,23]. Microbiota takes part in digestion processes, synthesizing a number of enzymes involved in the breakdown of polysaccharides, proteins, fatty acids and other compounds involved in the regulation of metabolism. Microflora plays a major role in the synthesis of essential micronutrients: vitamins, amino acids (arginine, glutamine) [24]. As for the immunomodulatory activity of intestinal microbiota, it takes part in the formation and development of the intestinal lymphoid system, promotes the synthesis of sIgA, stimulates the synthesis of cytokines and immunomediators [25]. Thus, intestinal microflora plays an important role in the formation of sensitization to food allergens, since the maturation and development of the body's immune system occurs to a significant extent under the influence of various microorganisms inhabiting the intestine [26].

As for the mechanism of allergic diseases, according to a number of authors, the immunoregulatory effect of the intestinal microbiota is associated with its influence on the production and activity of regulatory T-cells. Intestinal biocenosis disorders are associated with an increase in the immune response from Th2 cells [27]. Th2 cells produce IL-4, IL-5 and IL-13, which contribute to the development of allergic inflammation. The effect of the intestinal microbiota shifts this response towards the development of Th1 cells, which contributes to immune tolerance and maintains the balance of Th1/Th2 cells [27]. Intestinal microbiota is also one of the environmental factors that contribute to the maturation of T-cells [28]. Finally, the intestinal microflora plays a significant role in the development and maintenance of the intestinal barrier function, the damage of which is believed to lead to allergic sensitization of the host organism [29].

Thus, the intestinal microbiota takes an active part in stimulating and maintaining the functions of the immune system by producing humoral factors: enzymes, cytokines, mediators involved in the development of the immune response. Given the above, it was interesting to search for and select a probiotic consortium with high proteolytic activity, capable of reducing the level of residual antigenicity of meat proteins and under the action of which hydrolysis products, metabolites, cytokines and other biologically active substances are formed that could be used to reduce the reactivity of the immune system. The aim of the work was to study the processes of biotransformation of horse meat proteins using a probiotic consortium to obtain hypoallergenic meat raw materials.

Material and methods

Experimental setup

Experiments were conducted in the laboratories of the department "Animal Products Technology. Commodity Science", Biotechnology Center of the East Siberian State

University of Technology and Management. Horse meat was chosen as the object of the study, as it is a traditional raw material in the places of their breeding and has a high nutritional value due to the increased content of iron, vitamins B and E. Expanded and productive horse breeding determines the feasibility of using horse meat for the production of mass-market products.

Meat was selected from animals aged 24 months, raised in the agricultural production cooperative "Ulekchin" (Ulekchin village, Zakamensky district, Republic of Buryatia). For the study, samples were isolated from the semitendinosus muscle of the hip horse cut cooled for 24 hours at a temperature of 2 to 4 °C. For the experiment, pieces of horse meat of 100 to 150 g were cut out; fat and connective tissue were separated. The control: untreated meat samples; test 1: horse meat treated with a starter culture of one strain of *Lactobacillus paracasei k-406*; test 2: horse meat treated with a combined starter culture of four strains of *Lactobacillus (Lactobacillus curvatus LCR-111–1, Lactobacillus plantarum 8RAZ, Lactobacillus fermentum 44/1* and *Lactobacillus paracasei k-406)*. The bacterial strains were obtained from the National Bioresource Center "All-Russian Collection of Industrial Microorganisms" of the National Research Center "Kurchatov Institute". The microbial consortium was selected taking into account its biological compatibility and biotechnological potential [30].

To conduct the experiment, the horse meat was injected with starters (2 units of activity per 100 kg) using syringe followed by massaging to evenly distribute the preparation inside the muscle tissue and aging for 2 to 3 hours at a temperature of 18 to 20 °C. Then, the sample was salted with 15% brine in an amount of 20% of the sample weight, massaged, packed in film and stored at a temperature of 2 to 4 °C for 3 days. The physicochemical properties, technological parameters, microstructure and sensitization level were studied using the methods given below.

Methods for determining physicochemical properties and technological parameters

The degree of hydrolysis was determined by the Formula 1:

$$DoH = (N_a / N_t) \times 100\%, \quad (1)$$

where N_a is the content of amino nitrogen, %; N_t is the content of total nitrogen, %.

The protein content was determined by the following formula:

$$P = N_t \times 6.25 \times 10\ (\%), \quad (2)$$

where N_a and N_t were determined by the Kjeldahl[2] method using KDN-812 semi-automatic installation (Xian Yima Optoelec CO. LTD, China).

Water-holding capacity (WHC) of meat raw materials was determined using the Grau-Hamm method modified by Volovinskaya-Kelman, based on the release of moisture by the test sample during light pressing (weight of 1 kg for 10 min), sorption of the released water by filter paper and determination of the amount of separated moisture by the size of the area of the spot on the filter paper.

To determine the shear strength, samples were cut out from boiled muscle tissue along the fibers using probe No. 5 (10 mm diameter) and the force required to destroy the sample by cutting was measured using Warner-Bratzler device (Russia).

Methods of morphology evaluation

For microstructural studies, horse meat samples (1.5×1.0×0.5) were fixed in 10% neutral formalin for 48 hours. After washing the samples from excess formalin, standard alcohol processing was performed through a battery of ethyl alcohol with increasing concentration (40 to 100°). Subsequently, after holding the biological material in a paraffin/chloroform mixture for 12 hours, processing was continued with three changes of melted paraffin every 60 minutes. Upon completion, melted paraffin was poured into molds with samples (1×1.5) with the addition of beeswax to improve the quality of microsections. MS-2 sledge microtome was used to make 6 to 7 μm thick sections. Sections were stained with hematoxylin and eosin according to Ehrlich, and with picrofuchsin according to the Van Gieson method to identify collagen fiber nuclei [31]. Measurements of muscle tissue structures and other cellular inclusions in horse meat samples were performed using Micromed 3 microscope and Toup Cam 5/1 video eyepiece (Optics & Electronics Co., Ltd., China) at a magnification of 100×.

Methods of biological research

The sensitizing activity of the samples was determined using a delayed type hypersensitivity (DTH) model on 21 white outbred mice weighing 22 to 24 g[3]. Three groups of seven animals each were formed: Group 1 — intact (without the administration of extracts); Group 2 — control (unfermented horse meat extract); Group 3 — test (horse meat extract fermented by the microbial consortium). The extracts were prepared from horse meat samples by homogenization in 0.15 M NaCl with water ratio of 1:10.

Sensitization with the antigen was carried out once at a tailset in amount of 60 μl of the extract and Freund's complete adjuvant (FCA) emulsion (ratio 1:1). The challenging dose of the antigen in amount of 40 μl was administered after 5 days into the pad of the mouse's hind paw (test paw). The other hind paw was a control (control paw). 24 hours after testing, the edema value was measured using MK-0-25 engineering micrometer. The difference in the thickness of both paws characterizes the edema value, which can be used to evaluate the intensity of the DTH reaction.

[2] GOST 25011-2017. Meat and meat products. Protein determination methods. Retrieved from https://docs.cntd.ru/document/1200146783. Accessed February 20, 2025 (In Russian)

[3] Guidelines for conducting preclinical studies of medicinal products. Part one. Chapter 2. Moscow: Grif and K, 2012. — P. 57–58. (In Russian)

After the animals were removed from the experiment, the hind paws were cut off at the ankle joint and weighed. Local (popliteal) lymph nodes were also isolated, weighed on an analytical scale (Shinko Denshi HT224RCE, Japan), and the cellularity was counted in a Goryaev chamber. For this, each lymph node was homogenized in a glass homogenizer using 3 ml of 0.9% NaCl. Then the number of leukocytes was counted by diluting the previous sample 20 times with a 3% solution of acetic acid, colored with methylene blue.

Leukocyte count was performed in 100 large squares of the Goryaev chamber using the Formula 3:

$$X = \frac{A \times 250 \times 20}{100}, \quad (3)$$

where:

X is the number of leukocytes in 1 µl of sample;

A is the number of leukocytes in 100 large squares of the chamber.

Reaction indices (RI) were determined using the Formula 4:

$$RI = \frac{Value_{test} - Value_{control}}{Value_{control}} \times 100\%, \quad (4)$$

where:

$Value_{control}$ is the value of the control paw (or lymph node);

$Value_{test}$ is the value of the test paw (or lymph node).

Intact animals were sensitized with FCA emulsion and sterile 0.15 M NaCl solution according to the same scheme as in the groups receiving horse meat extracts.

The analysis of the sensitizing properties of the extracts was also carried out on 6 albino guinea pigs divided into 2 groups: Group 1 — control (non-fermented horse meat) and Group 2 — test (fermented horse meat). The animals were sensitized at the pads of two hind paws once with the corresponding horse meat extract mixed with FCA in amount of 0.5 ml (ratio 1:1). After 20 days, the animals were intradermally injected with a challenging dose of the corresponding extract (0.05 ml) at the shaved area of the back. To control the reactivity of the skin, 0.05 ml of sterile 0.15 M NaCl was injected intradermally at the opposite shaved area of the back. The response was assessed after 1, 24 and 48 hours by the appearance of erythema and its size (diameter)[4].

The mice and guinea pigs used in the experiment were obtained from the Buryat Republican Scientific and Industrial Veterinary Laboratory. Animal experiments were conducted in the vivarium of the Federal State Budgetary Educational Institution of Higher Education "East Siberian State University of Technology and Management".

The experiments were carried out in compliance with the principles of humanity in accordance with international moral and ethical standards and the requirements of the European Convention ETS N123[5] and Directive 2010/63/EU[6]. Animal care and maintenance were carried out in accordance with GOST 33216-2014[7] and Order of the Ministry of Health of the Russian Federation No. 199-N[8].

During the preparation for the experiment, the mice and guinea pigs underwent 10 days of quarantine and adaptation to the vivarium environment. During the experiment, the general condition of the animals, their food and water consumption were monitored daily. The experimental animals were divided into groups of 7 individuals for mice and 3 individuals for guinea pigs each in separate cages of the appropriate size, and were on the same (standard) diet with free access to feed and water. The light regime in the vivarium was provided by changing "day/night" illumination every 12 hours. The air temperature was 20 to 25 °C, the relative humidity was 60 to 70%.

Mice were removed from the experiment by cervical dislocation, and guinea pigs by chloroform vapors.

The study protocol was approved at a meeting of the ethics committee.

Statistical data processing

The obtained experimental data were processed using the calculation of mean values (M), standard error of the mean (m) and parametric test (Student's t-test) using Statistica software and Microsoft Excel. To analyze the physicochemical properties of horse meat during storage, the degree of the studied parameter change was calculated using Friedman test, which allows estimating the variability of the parameter over time. Changes were considered significant at $p \leq 0.05$.

The results of morphometric and biological studies are presented as medians (Me), upper and lower quartiles (Q1-Q3) and processed using the nonparametric Mann-Whitney test. The results were considered reliable when the significance level of differences was reached ($p \leq 0.05$).

Results and discussion

The process of meat aging includes a number of biochemical processes that may affect the technological and physicochemical properties of the raw material. The degree of hydrolysis and technological characteristics of horse meat were studied during the processing of meat raw materials with a single-strain starter (test 1) and a microbial consortium (test 2) compared to untreated meat (control). The data are presented in Table 1.

[4] Guidelines for conducting preclinical studies of medicinal products. Part one. Chapter 2. Moscow: Grif and K, 2012. — P. 58–59. (In Russian)

[5] Council of Europe — European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123). Strasbourg, 18.III.1986

[6] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Retrieved from https://eur-lex.europa.eu/eli/dir/2010/63/oj/eng/pdf. Accessed February 20, 2025

[7] GOST 33216–2014 Guidelines for accommodation and care of animals. Species-specific provisions for laboratory rodents and rabbits Retrieved from https://docs.cntd.ru/document/1200127506. Accessed February 20, 2025 (In Russian)

[8] Order of the Ministry of Health of the Russian Federation dated 01.04.2016 No. 199-N "On approval of the rules of good laboratory practice". Moscow — P. 9 (In Russian)

Table 1. Physicochemical changes in horse meat during processing with microorganisms

Samples under study	Storage, days			
	0	1	2	3
Degree of hydrolysis, %				
Control	5.54 ± 0.11[a]	5.74 ± 0.27[c]	6.31 ± 0.21[bc]	8.67 ± 0.34[bc]
Test 1	5.59 ± 0.12[a]	6.32 ± 0.26[b]	8.55 ± 0.25[bd]	21.75 ± 0.36[bd]
Test 2	5.63 ± 0.23[a]	7.75 ± 0.14[bd]	10.73 ± 0.32[bd]	22.87 ± 0.28[bd]
Water-holding capacity, %				
Control	70.83 ± 1.6	70.9 ± 1.8[c]	72.4 ± 2.3[c]	73.9 ± 2.7[c]
Test 1	71.24 ± 2.2[a]	76.2 ± 1.9	77.3 ± 2.1[bd]	80.9 ± 2.2[bd]
Test 2	72.17 ± 2.1[a]	78.2 ± 2.2[d]	79.9 ± 1.9[bd]	82.6 ± 1.8[bd]
Shear strength, 10^2 N/m^2				
Control	4.85 ± 0.14	4.95 ± 0.11	4.38 ± 0.12[c]	4.29 ± 0.15[c]
Test 1	4.81 ± 0.11[a]	4.55 ± 0.18	4.21 ± 0.17	3.83 ± 0.13[bd]
Test 2	4.89 ± 0.16[a]	4.43 ± 0.16	3.92 ± 0.14[bd]	3.65 ± 0.19[bd]

Note: a and b indicate that within one row, the difference in mean values is statistically significant ($p \leq 0.05$); c and d indicate that within one column, the difference in mean values is statistically significant ($p \leq 0.05$) according to Friedman test.

Analysis of the data in Table 1 showed that in the process of protein proteolysis, the degree of hydrolysis after two days increased in the control by 13.9%, in Test 1 by 53%, in Test 2 by almost 2 times compared to the initial value (Day 0). Aging for 3 days enhanced proteolytic processes, which is confirmed by an increase in the degree of hydrolysis in the control by 56.5%, in Test 1 by 3.9 times, in Test 2 by 4.1 times ($p \leq 0.05$).

A literature review showed that the treatment of protein-containing raw materials with microorganism strains promotes the development of proteolytic processes in the raw materials; it was shown that due to the effect of enzymes produced by *Aspergillus oryx season*, the degree of hydrolysis within 72 hours increased to a level of 20 to 30% of the total amount of nitrogen [32]. The authors [33] noted the proteolytic activity of propionic acid bacteria, which contributes to an increase in the amount of amine nitrogen in beef treated with microorganisms, indicating hydrolysis of meat proteins.

The hydrophilicity of the meat system plays an important role in the formation of quality characteristics of the finished meat product; a reliable increase in the degree of hydrophilicity of fermented horse meat was revealed. On Day 3 in Test 1, the increase in the value of WHC compared to the control was 10.1%, in Test 2 it was 11.8% ($p \leq 0.05$). It is possible that the synergistic effect of probiotic microorganisms in the consortium contributes to the destruction and disorientation of protein molecules and the emergence of additional hydrophilic groups. An increase in the level of WHC value was found when processing beef with propionic acid bacteria [33].

The texture of horse meat, characterized by shear strength, improved after aging with a starter culture. On Day 3 of horse meat aging, a decrease in the level of shear strength was observed in Test 1 by 8.8%, in Test 2 by 13.1% compared to the control ($p \leq 0.05$). Proteolytic processes contributed to the loosening of the muscle fiber structure (lysis), which led to a decrease in the level of horse meat rigidity.

Thus, the obtained data indicate that the microbial consortium enhances proteolytic processes in horse meat, which causes an improvement in the technological and microstructural characteristics of meat.

Proteolytic changes in muscle tissue proteins lead to the destruction of fibers, which is seen by microstructural methods. Table 2 presents the results of morphometric studies. Figures 1 to 3 show photo images with the microstructure of the studied samples.

As can be seen from Figure 7A, it was found that in the control samples of horse meat not exposed to probiotic microorganisms, the surface of the muscle fibers is uneven and transverse striation in some fibers of the control group is weakly expressed.

During the morphometric study, the thickness of the fibers in the control samples of chilled horse meat averaged 48.0 ± 5.0 μm. The fibers in the control samples are located quite densely with insignificant connective tissue layers between them. During microscopy of the control samples, the thickness of the endomysium averaged 10.5 ± 5.0 μm.

Table 2. Microstructural dimensions of the studied horse meat samples

Horse meat samples	Parameter	Diameter, μm		Thickness of connective tissue layers, μm	
		Muscle fibers	Fiber nuclei	Endomysium	Perimysium
Control	M ± m	48.0 ± 15.0[a]	4.8 ± 3.0	10.5 ± 5.0	30.5 ± 7.0
	min-max	30.5–60.5	2.5–8.5	5.0–20.0	24.5–40.0
Test 1	M ± m	80.0 ± 15.0[b]	4.0 ± 0.5	9.5 ± 2.5	25.0 ± 5.0
	min-max	65.5–95.5	3.5–4.5	7.5–12.5	22.5–35.0
Test 2	M ± m	100.0 ± 20.0[b]	5.5 ± 1.0	7.0 ± 1.5	20.5 ± 5.0
	min-max	76.5–110.0	4.5–6.5	5.5–8.5	18.0–25.0

Note: a and b indicate that within one column, the difference in mean values is statistically significant ($p \leq 0.05$) according to Student's t-test.

At the same time, the connective tissue layers between the fiber bundles (perimysium) of horse meat are more clearly visible in a longitudinal section (Figure 7A) and vary from 24.5 to 40.0 μm. As can be seen from Figure 7B, a dense row of small muscle ridges is located between the transverse fibers of horse meat.

Among the structural components in chilled horse meat, when stained according to Van Gieson, the nuclei of muscle fibers are distinguishable, which have an oval, elongated and rod-shaped form.

Histological examination of horse meat samples treated with the monoculture revealed that the sections obtained were stained pink-red and the muscle fibers were mostly folded when cut longitudinally (Figure 8A). The surface of the muscle fibers was uneven and consisted of alternating thick, swollen and loosened muscle fibers. Microscopy of sections in Test 1 showed transverse striation on most fibers in longitudinal sections (Figure 8A). In transverse sections, the muscle fibers were dense, swollen and the connective tissue layers were thinner in contrast to the control samples (Figure 8B). Among the structural elements in Test 1, the nuclei of muscle fibers attract special attention. When staining the preparations according to Van Gieson, nuclei were not detected in contrast to the control samples. Only the use of hematoxylin and eosin allowed to find that the nuclei of muscle fibers treated with the monoculture mainly had an elongated, rod-shaped form with an average thickness of 4.0 ± 0.5 μm. At the same time, the transverse striation on the surface of the fibers in Test 1 is expressed better than in the control samples, and finely looped structures are visible on some muscle fibers. In Test 1, all layers of loose connective tissue in both the endomysium and perimysium became denser and more compressed (Table 2, Figure 8B).

Microstructural analysis of horse meat samples in Test 2 treated with the microbial consortium allowed to establish the disintegration of muscle fibers into separate segments (Figure 9A). At the same time, collagen fibers significantly swelled and increased in size. The sarcolemma of the fibers was swollen and had no visible damages. As a result, collagen fibers were glued together with a decrease in the connective tissue layers between the fibers and a decrease in the width of the perimysium between the fiber bundles (Figure 9B). The results of morphometric measurements of the structural components in these samples are presented

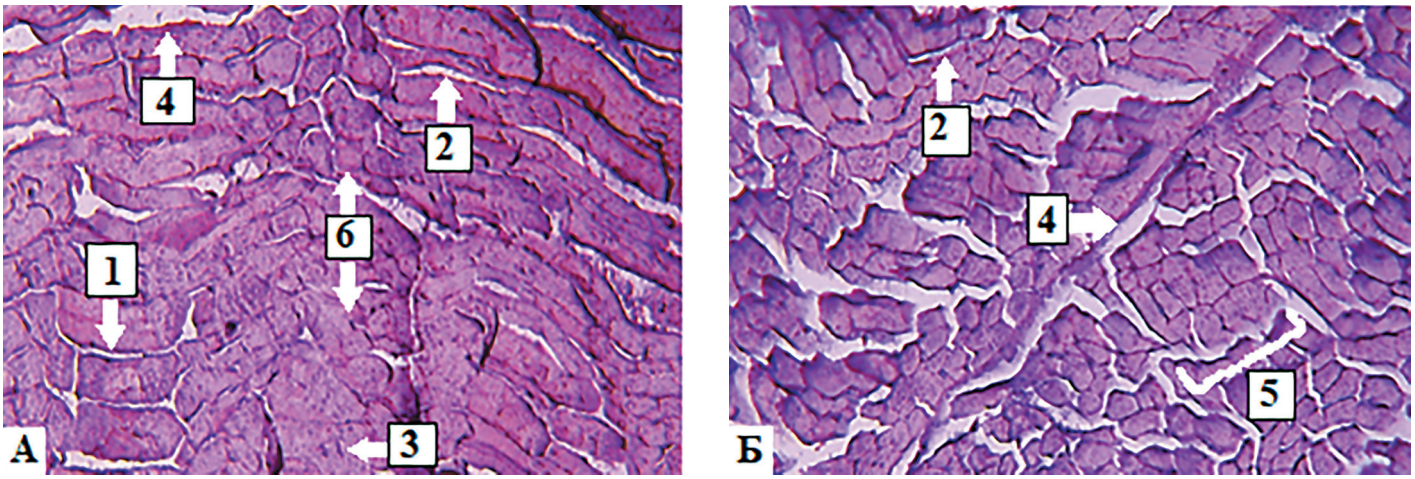

Figure 7. Micrographs of control horse meat samples: A, longitudinal section; B, transverse section: 1, muscle fibers; 2, endomysium; 3, fiber nuclei; 4, perimysium; 5, fiber bundles; 6, muscle ridges. Staining with hematoxylin and eosin (PL magnification 10×/0.25)

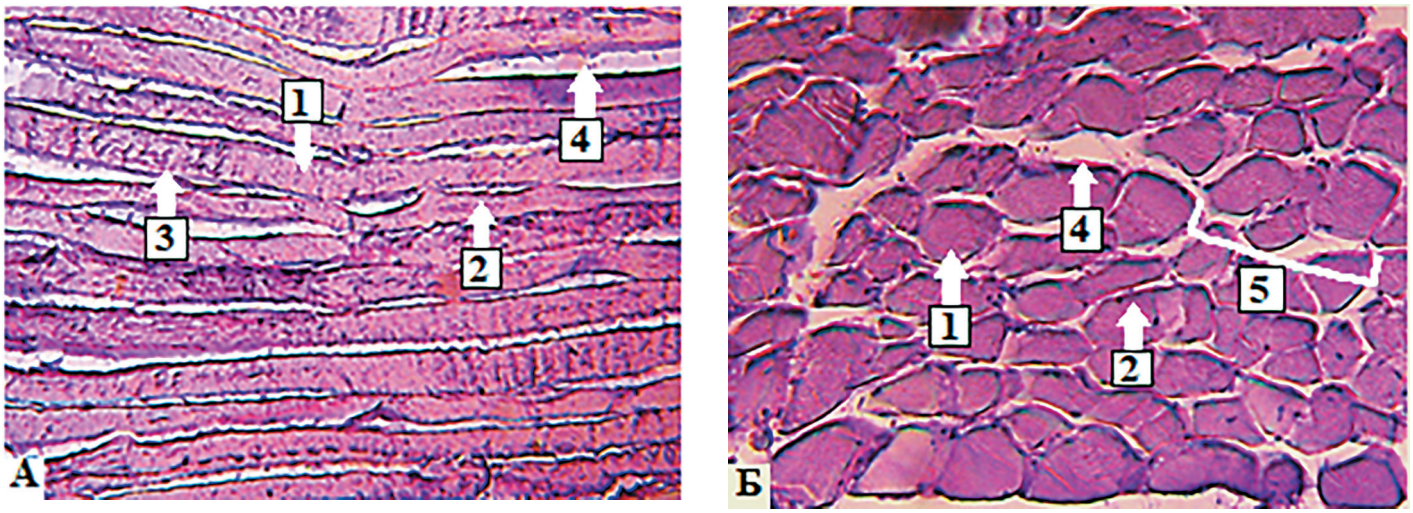

Figure 8. Micrographs of horse meat samples treated with the monoculture; A, longitudinal section; B, transverse section: 1, muscle fibers; 2, endomysium; 3, finely looped fiber structures; 4, perimysium; 5, fiber bundles. Staining with hematoxylin and eosin (PL magnification 10×/0.25)

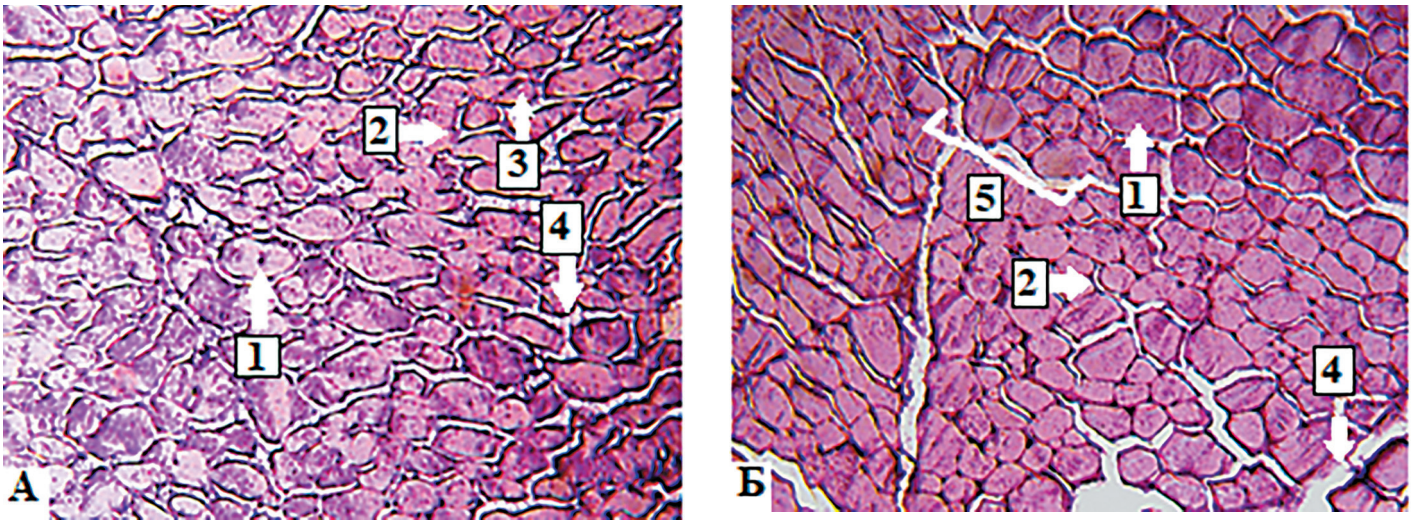

Figure 9. Histological preparations of horse meat samples treated with the microbial consortium; A, longitudinal section; B, transverse section: 1, muscle fibers; 2, endomysium; 3, thickened nuclei; 4, perimysium; 5, fiber bundles. Slight reduction of endomysium and perimysium. Staining with hematoxylin and eosin (PL magnification 10×/0.25)

in Table 2. Microscopy of sections of meat samples treated with the microbial consortium showed that most muscle fibers were completely free of transverse striation, but it was weakly visible in some fibers (Figure 9A). Treatment of horse meat with the microbial consortium affected the shape and visibility of the nuclei. Just as in Test 1, muscle cell nuclei were not detected in horse meat fibers treated with the microbial consortium using Van Gieson staining. Using another stain, slightly thickened, elongated nuclei became visible along the fibers under microscopy, in contrast to the control samples.

The conducted studies revealed that the treatment of horse meat samples with the monoculture stimulated the swelling of collagen fibers and the loosening of muscle tissue with the subsequent formation of an intense pink-red color. Microstructural analysis of horse meat samples in Test 2 treated with the microbial consortium revealed that the collagen fibers significantly swelled, increased in size, and revealed reliable differences in fiber thickness compared to the control. At the same time, the connective tissue layers between individual fibers and bundles of muscle fibers became thinner and smaller compared to those in the control samples and in Test 1 treated with the monoculture.

Based on the conducted studies, more effective hydrolysis of horse muscle tissue was revealed when using the four-strain starter. In this regard, further studies to determine the level of sensitization were carried out by comparing untreated horse meat (control) and horse meat treated with the microbial consortium (test).

Allergenicity of products may be assessed by studying the reaction of delayed type hypersensitivity (DTH) in experimental animals. Table 3 presents the parameters of the delayed type hypersensitivity reaction in white mice when administered extracts of unfermented and fermented horse meat.

When administered the extract of unfermented horse meat (control), a positive 100% reaction was noted for reaction indices 1, 2 and 4. The degree of the DTH reaction for the corresponding indices (1 to 4) was 2.2, 6.3, 7.7, and 13.4 times greater than in the intact group of animals, respectively. Administration of the extract of horse meat exposed to the consortium (test) caused a positive 100% reaction only when calculating RI-2. At the same time, in this group, the degree of the DTH reaction was lower than in the control by 1.9, 3.1, 3.1, and 2.1 times, respectively (Table 3).

Table 3. Parameters of the DTH reaction in white outbred mice when administered horse meat extracts

No.	Group name	Parameter	Reaction index			
			1 — by paw volume	2 — by paw weight	3 — by lymph node weight	4 — by lymph node cellularity
1	Intact	Me	1.49[a]	2.24[a]	5.62[a]	3.07[a]
		Q1–Q3	1.19–1.79	1.88–2.60	5.11–6.13	2.10–4.04
2	Control — extract of unfermented horse meat	reaction	+	+	+	+
		degree of reaction	100%	100%	80%	100%
		Me	3.32[b]	14.03[b]	43.21[b]	41.21[b]
		Q1–Q3	2.93–3.71	11.28–16.78	38.32–48.10	35.92–46.50
3	Test — extract of horse meat treated with the microbial consortium	reaction	+	+	+	+
		degree of reaction	60%	100%	80%	80%
		Me	1.77	4.58[c]	14.13[c]	19.92[c]
		Q1–Q3	1.33–2.21	3.76–5.40	9.80–18.46	14.06–25.78

Note: data with [b] superscript differ significantly from data with [a] superscript, data with [c] superscript differ significantly from data with [a, b] superscripts within one column ($p \leq 0.05$) according to Mann-Whitney test.

A similar pattern was established when carrying out the DTH reaction on guinea pigs (Table 4).

It was noted that after 1 hour, no pronounced reaction was observed (slight swelling and redness, the size of the erythema is difficult to determine), and after 24 and 48 hours, pink erythema was observed around the injection site. There was a reliable decrease in the severity of the DTH reaction in the test group (2) compared to the control group (1). After 24 and 48 hours, the reaction value was 1.6 and 1.5 times lower, respectively. Thus, the data obtained on two species of animals indicate a decrease in the sensitizing activity of meat raw materials exposed to the probiotic consortium. A decrease in the sensitizing properties of meat proteins is caused by the destruction of antigenic epitopes due to the proteolytic activity of the probiotic consortium.

According to the authors, one of the promising methods in the development of hypoallergenic products of animal origin is the modification of antigenic epitopes in protein molecules, which reduces their allergenicity. The degree of allergenicity reduction depends on the type of proteases used. Non-specific proteases or protease mixtures reduce protein allergenicity more effectively [6]. This approach is used by many authors to reduce the allergenicity of cow's and mare's milk, soy and egg proteins [34–36]. Considering the role of intestinal microbiota in the development of an allergic reaction of the host organism, we attempted to use the proteolytic activity of probiotic microorganisms to reduce the allergenicity of raw meat. A decrease in the sensitizing properties of horse meat due to the proteolytic activity of the microbial consortium was shown in two species of experimental animals. The role of metabolites, cytokines and other compounds produced during hydrolysis and having an immunomodulatory effect remains unclear, which requires further research.

Conclusion

As a result of horse meat treatment with the single-strain culture and the microbial consortium, data were obtained indicating high proteolytic activity of the consortium compared to the control and the sample processed with the monoculture. It was noted that the degree of hydrolysis of horse meat proteins after 3 days increased 4 times compared to the original raw material, which caused an increase in the hydrophilicity of the meat system by 11.86% and a decrease in shear strength by 13.1% compared to the control (unfermented horse meat). It is possible that the microbial consortium has a more pronounced proteolytic effect on the destruction and disorientation of protein molecules due to the synergistic effect, which contributes to the emergence of additional hydrophilic groups.

Analysis of the molecular weight distribution of proteins in the studied samples confirmed a deeper hydrolysis of horse meat proteins in the test samples compared to the control. When comparing test samples, deeper proteolytic processes were noted in the sample treated with the microbial consortium, compared to the sample treated with the monoculture. The content of proteins with a molecular weight of 65 to 170 kDa in the test sample with the monoculture decreased by 10.2 rel.%, and in the test sample with the microbial consortium this parameter decreased by 2 times. Therefore, the amounts of proteins with a molecular weight of 15 to 26 kDa increased in Test 1 by 17 rel. %, and in Test 2 by 1.88 times. The data obtained indicate a deeper destruction of protein molecules under the influence of the consortium of lactic acid microorganisms producing proteolytic enzymes.

Microstructural analysis showed that treatment of horse meat samples with the monoculture stimulated swelling of collagen fibers and loosening of muscle tissue with subsequent formation of intense pink-red color. Microstructural analysis of horse meat samples in Test 2 treated with the microbial consortium allowed to establish that collagen fibers significantly swelled, increased in size and reliable differences in fiber thickness were revealed compared to the control. At the same time, connective tissue layers between individual fibers and bundles of muscle fibers in samples treated with the microbial consortium became thinner and smaller than in the control and in samples exposed to the monoculture.

It was noted that when using unfermented horse meat extract (control), a positive 100% delayed type hypersensitivity reaction in experimental animals was observed. The administration of the fermented horse meat extract (test) caused a positive 100% reaction only when calculating the DTH reaction index by the weight of the paws. Compared with the control, a decrease in the reaction indices was noted: by paw volume by 1.9 times; by paw weight by 3.1 times; by lymph node weight by 3.1 times and by lymph node cellularity by 2.1 times. Similar results were obtained in an experimental model of delayed type hypersensitivity (skin test) in guinea pigs. Thus, the treatment of meat raw material with probiotic microorganisms caused a decrease in its sensitizing activity. Therefore, this biotechnological technique indicates the prospects for using such raw materials in the production of hypoallergenic meat products for mass consumption.

Table 4. Parameters of the DTH reaction in albino guinea pigs when administered horse meat extracts

No.	Group name	Parameter	Reaction results (d of erythema, mm) / time (hours)		
			1	24	48
1	Control — extract of unfermented horse meat	Me	No pronounced reaction	8.30[a]	6.45[a]
		Q1–Q3		7.4–9.2	5.65–7.25
2	Test — extract of horse meat treated with the microbial consortium	Me	No pronounced reaction	5.15[b]	4.41[b]
		Q1–Q3		4.25–6.05	3.61–5.21

Note: data with [b] superscript differ significantly from data with [a] superscript within one column ($p \leq 0.05$) according to Mann-Whitney test.

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