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

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ENHANCING FUNCTIONAL FERMENTED DENDENG (INDONESIAN JERKY) WITH *MORINGA OLEIFERA*: NUTRITIONAL, MICROBIOLOGICAL, AND SENSORY ASPECTS

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Keywords: *fermented chicken dendeng, Lactobacillus plantarum, Moringa oleifera, Mailard reaction, functional food*

Abstract

Fermented chicken dendeng is a traditional dried meat product with improved nutritional and microbiological quality due to fermentation. This study evaluates the effects of Lactobacillus plantarum and Moringa oleifera supplementation on pH, titratable acidity (TA), bacterial count, and proximate composition. The pH values ranged from 4.09 to 4.62, with the lowest in the 6 % L. plantarum + 2.5 % M. oleifera treatment. Titratable acidity was the highest (1.47 %) in the 6 % L. plantarum group but decreased with M. oleifera addition. The highest bacterial count (6.535 log CFU/g) was observed in the 6 % L. plantarum + 5 % M. oleifera group, indicating probiotic activity. Proximate analysis shows that fermentation enhances quality. Protein content increased with M. oleifera, reaching 58.2 % in the 6 % L. plantarum + 5 % M. oleifera treatment. Fat content remained stable (8.6 % — 9.3 %), while ash content increased to 5.1 %. These findings suggest that fermented chicken dendeng with L. plantarum and optimal M. oleifera supplementation had improved microbial stability and protein retention. Further research is needed to assess storage stability and sensory attributes.

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Introduction

Recent studies have reinforced the potential of *Moringa oleifera* and *Lactobacillus plantarum* as functional ingredients in the development of fermented meat products. *Moringa oleifera*, commonly referred to as the "miracle tree," is rich in protein, essential vitamins (A, C, and E), minerals (iron, calcium, potassium), and a wide array of bioactive compounds including polyphenols, flavonoids, tannins, and saponins. These compounds are well-documented for their potent antioxidant, antimicrobial, and anti-inflammatory properties [1,2]. Such properties make Moringa an ideal candidate for use in functional food formulations aimed at enhancing both nutritional value and health benefits.

One of the most significant attributes of Moringa is its prebiotic potential. Polyphenolic compounds found in Moringa not only suppress pathogenic bacteria but also promote the proliferation of beneficial lactic acid bacteria (LAB) in the gastrointestinal tract and fermented food matrices [3,4]. These polyphenols undergo microbial biotransformation into short-chain fatty acids (SCFAs) and other metabolites that exhibit antioxidant and immunomodulatory functions, thus contributing to improved gut health and metabolic balance. Such metabolic synergy underscores the relevance of Moringa in the context of fermented food innovation.

In parallel, *Lactobacillus plantarum* is a widely used LAB in meat fermentation and has a well-established role in ensuring product safety and quality [5]. It lowers the pH of meat substrates by producing organic acids such as lactic, acetic, and succinic acids. These acids create an inhospitable environment for spoilage and pathogenic microorganisms. Furthermore, *L. plantarum* synthesizes

bacteriocins, antimicrobial peptides that inhibit undesirable microbes, thereby extending shelf life and enhancing microbiological safety [6,7]. Beyond its antimicrobial properties, *L. plantarum* exhibits proteolytic activity that results in the degradation of meat proteins into smaller peptides with potential bioactive functions. These peptides contribute to flavor development, improve digestibility, and offer health benefits such as antihypertensive and antioxidant effects [8,9]. The dual functionality of *L. plantarum* as both a preservative agent and a probiotic makes it a strategic addition to meat fermentation systems.

Animal model studies by [10] demonstrated that Moringa supplementation improves antioxidant enzyme activity, notably superoxide dismutase (SOD) and catalase (CAT), while also increasing populations of *Lactobacillus* in the gut. These findings highlight the dual prebiotic and antioxidant role of Moringa, reinforcing its value in functional meat product development. Elabd et al. [11] further confirmed that polyphenols from Moringa are metabolized by gut microbiota into compounds with significant biological activity, including anti-inflammatory and anti-carcinogenic effects. Abdallah et al. [12] reported that in fermented food systems, natural plant ingredients like Moringa not only enhance sensory attributes such as color, aroma, and texture but also improve microbial safety. The suppression of key foodborne pathogens, including *Listeria monocytogenes* and *Escherichia coli*, was observed in meat products enriched with plant-based additives. This reinforces the notion that functional ingredients can offer multi-dimensional benefits when integrated into traditional food matrices. In the article [13] identified *Moringa oleifera* as a prime candidate for use in the development of functional fermented meat products. The high protein content and pharmacologically active constituents of Moringa position it as an effective plant-based ingredient that can simultaneously address nutritional gaps and enhance product appeal [14]. These findings support the growing trend of incorporating Moringa into functional food design, particularly within culturally significant dishes.

In the Indonesian context, traditional fermented dendeng, a dried meat product, is well known for its unique flavor, firm texture, and extended shelf life. Traditionally made from beef, dendeng is processed through marination, fermentation, and drying, which contribute to its sensory appeal and microbial stability [15]. However, economic and health considerations have led to growing interest in replacing beef with more affordable and leaner meats such as chicken. Chicken meat is characterized by its lower fat and saturated fatty acid content, making it a healthier alternative to beef. It is also a good source of high-quality protein, essential amino acids, zinc, and B vitamins [16,17]. Nonetheless, chicken dendeng often lacks the texture, flavor depth, and microbial resilience of its beef counterpart, thus requiring functional enhancement through innovative ingredients and fermentation strategies.

Given the functional attributes of *Moringa oleifera* and the proven efficacy of *Lactobacillus plantarum*, this study aims to evaluate the nutritional composition, microbiological safety, and sensory qualities of fermented chicken dendeng supplemented with 2.5 %, 5 %, and 7.5 % Moringa leaf powder. By assessing their combined impact, the research seeks to identify the optimal inclusion level that maximizes product quality and health benefits. The findings are expected to contribute to the advancement of functional meat products and provide a sustainable solution for enhancing traditional fermented foods in Southeast Asia.

Objects and methods

Experimental Materials

Fresh chicken carcasses were purchased from a certified poultry market in Malang, East Java, Indonesia. The chickens were slaughtered under hygienic conditions on the day of purchase and transported in insulated cool boxes (4–6 °C) within 1 hour to the laboratory of the Faculty of Animal Science, Universitas Brawijaya. Only chicken breast meat (*pectoralis major*) was used after deboning and removal of visible fat and connective tissue. *Moringa oleifera Lam*. leaves were collected from a local organic farm in Ngadiluwih District, Kediri Regency. The leaves were manually harvested, sorted to remove damaged or yellowing foliage, and transported in paper bags to the laboratory for further processing into powder. Additional ingredients such as salt (Refina, PT Pangan Lestari, Indonesia), granulated sugar (Gulaku, PT Sugar Labinta, Indonesia), garlic (sourced fresh from Kediri traditional market), coriander powder (Rajawali, Surabaya, Indonesia), and traditional spice mix (homemade blend) were used in the formulation of fermented dendeng. The probiotic culture used was *Lactobacillus plantarum* FNCC 0027, obtained from the Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. The bacterial starter was activated in sterile MRS broth (Oxoid, UK) and incubated at 37 °C for 24 hours before application. All reagents and chemicals used for analysis, including lactic acid standards, NaOH, phenolphthalein, and others, were of analytical grade and sourced from Merck (Germany) unless otherwise specified.

Preparation of moringa leaf powder

Fresh *Moringa oleifera Lam*. leaves were harvested and thoroughly washed with clean water to remove dirt and debris. The leaves were then air-dried in the shade for 48 hours to retain their nutritional and phytochemical integrity. To achieve uniform drying, the leaves were further dehydrated using a Miyako DH-600 food dehydrator (Miyako Electronics, Indonesia) at 45–50 °C for 6 hours. After drying, the leaves were pulverized into a fine powder using a Philips HR2221/00 blender (Philips Electronics, Netherlands). The resulting Moringa leaf powder was stored in an airtight glass jar at room temperature (25–27 °C) in a dark, dry place until used in the formulation process.

Preparation of fermented chicken dendeng

Chicken breast meat (without skin and visible fat) was used as the main raw material. The meat was deboned and ground using a stainless steel meat grinder. The formulation of fermented chicken dendeng was divided into four treatment groups based on *Moringa oleifera* leaf powder inclusion levels: T0 = 0 % (control), T1 = 2.5 %, T2 = 5 %, and T3 = 7.5 %. All formulations contained the same base ingredients (in % w/w of total mixture) as detailed in Table 1.

Table 1. Formulation of fermented chicken dendeng with different *Moringa oleifera* inclusion levels

Ingredient	T0 (%)	T1 (%)	T2 (%)	T3 (%)
Chicken breast meat	90.0	87.5	85.0	82.5
Salt	1.5	1.5	1.5	1.5
Sugar	2.0	2.0	2.0	2.0
Garlic (fresh, mashed)	2.0	2.0	2.0	2.0
Coriander (powder)	1.5	1.5	1.5	1.5
Traditional spice mix	2.0	2.0	2.0	2.0
Lactobacillus plantarum culture	1.0	1.0	1.0	1.0
Moringa leaf powder	0.0	2.5	5.0	7.5

After thorough mixing, the meat mixture was fermented at ambient temperature (27–30 °C) for 24 hours. It was then shaped into thin slices (~0.5 cm thickness) and dehydrated using a Miyako DH-600 food dehydrator (Miyako Electronics, Indonesia) at 55 °C for approximately 8 hours. Moisture content was monitored until it reached < 20 %, measured using the oven drying method at 105 °C for 6 hours, following AOAC [18] standards. Once dried, the dendeng was vacuum-packed in sterile polyethylene pouches and stored at room temperature for subsequent analyses.

Experimental design

The study employed a Completely Randomized Design (CRD) consisting of four treatments (T0 = 0 % Moringa, T1 = 2.5 %, T2 = 5 %, and T3 = 7.5 % *Moringa oleifera* leaf powder) with six replications per treatment. In this design, experimental units (meat batches) were randomly assigned to each treatment group, ensuring that all samples had an equal chance of receiving any treatment. This random allocation minimizes bias, controls for uncontrolled variables, and allows for valid statistical comparisons among groups. CRD was chosen due to the homogeneous nature of the experimental material and environment, which met the assumptions of independence, normality, and equal variance required for ANOVA analysis.

Analysis methods

Nutritional composition analysis, proximate analysis

The nutritional composition of fermented chicken dendeng was analyzed using standardized methods as recommended by [19]. The moisture content was determined using the oven-drying method at 105 °C to constant weight, fat content by the Soxhlet extraction method using petroleum ether, and ash content by incineration at 550 °C in a muffle furnace (Memmert MU200, Germany). Crude protein was determined by the Kjeldahl method (AOAC 2001.11) using a Kjeldahl digestion unit (Buchi K-425, Switzerland) and distillation unit (Buchi B-324). Carbohydrate content was calculated by difference:

$$\text{Carbohydrate (\%)} = 100 - (\text{\% moisture} + \text{\% crude protein} + \text{\% crude fat} + \text{\% ash}).$$

All analyses were conducted in triplicate and results are reported on a wet basis.

pH Measurement

The pH of the fermented chicken *dendeng* samples was recorded immediately after the 24-hour fermentation process, prior to the drying stage. Each sample (T0, T1, T2, and T3) was collected in triplicate for analysis. Approximately 10 g of each sample was homogenized in 90 mL of distilled water using a laboratory homogenizer (IKA T18 Ultra-Turrax, Germany). The pH value was measured at room temperature (27–30 °C) using a calibrated digital pH meter (Hanna Instruments HI2211, Romania). Calibration was performed before each use with standard buffer solutions at pH 4.0 and 7.0, following [20].

Titratable acidity (TA) determination

Titratable acidity was assessed at the same time as the pH measurements, i.e., after the 24-hour fermentation period and before dehydration. A 10 mL aliquot of the homogenized sample was titrated with 0.1 N NaOH using phenolphthalein as the indicator until a stable pink color was observed. The titratable acidity was expressed as a percentage of lactic acid equivalent. All determinations were carried out in duplicate for each treatment group. This method was adapted from [21].

Microbiological analysis

Microbiological quality was assessed by evaluating the Total Plate Count (TPC) of fermented chicken dendeng samples after the 24-hour fermentation period. TPC analysis was conducted using Plate Count Agar (PCA; Merck, Germany). Approximately 10 g of sample was aseptically homogenized in 90 mL of sterile peptone water (0.1 %) using a stomacher (BagMixer 400, Interscience, France) for 2 minutes. Serial dilutions were prepared and 1 mL aliquots were plated using the pour plate method. Plates were incubated at 37 °C for 48 hours, and the results were expressed as colony forming units per gram (log CFU/g) [22]. Microbiological analysis was performed in duplicate. Microbiological analysis was carried out exclusively on fermented samples; the initial raw materials (fresh chicken meat and Moringa leaf powder) were handled hygienically but not analyzed for microbial load. The microorganisms identified in this study focused on mesophilic aerobic bacteria that contribute to the total microbial load. Specific pathogenic or spoilage organisms (e.g., *E. coli, Salmonella, Listeria*) were not assessed in this experiment.

Statistical analysis

All experimental data were subjected to statistical analysis using Analysis of Variance (ANOVA) to assess the effect of Moringa supplementation across treatments (T0, T1, T2, and T3). When a significant difference was detected ($p < 0.05$), Duncan's Multiple Range Test (DMRT) was employed as a post-hoc test to determine differences between group means. The results are presented as means ± standard deviation (SD). Statistical analyses were conducted using SPSS software version 26.0 (IBM Corp., USA).

Results and discussion

Proximate composition of fermented chicken dendeng

The proximate composition analysis of fermented chicken dendeng with different levels of *Moringa oleifera Lam*. supplementation (T0, T1, T2, and T3) showed significant variations in carbohydrate, protein, ash, and fat content ($p < 0.05$). The proximate composition of the Moringa leaf powder used in this study was also analyzed to better understand its contribution to the final product. Based on laboratory analysis using AOAC standard methods, Moringa leaf powder contained approximately 25.6 % protein, 6.1 % fat, 8.3 % ash, and 50.4 % carbohydrate (by difference). The proximate composition analysis of fermented chicken dendeng formulated with *Lactobacillus plantarum* and varying concentrations of *Moringa oleifera* Lam. is shown in Figure 1. These changes indicate the effect of Moringa leaf powder as a functional ingredient on the nutritional quality of the product.

The protein content of the samples increased with the addition of *Moringa oleifera* by about 2.5 % but varied slightly with further additions. The control group (6 % *L. plantarum*) had a protein content of 19.96 %, while the sample with 6 % *L. plantarum* + 2.5 % *Moringa oleifera* had the greatest protein content (24.36 %). But when the Moringa concentration went up to 5 % and 7.5 %, the protein content went down slightly to 22.60 % and 22.83 %, respectively.

Carbohydrates were the most abundant macronutrients in all samples, making up between 60.35 % and 67.43 % of the total. The control group (6 % *L. plantarum*) had the most carbohydrates (67.43 %), while the sample with 6 % *L. plantarum* and 2.5 % *Moringa oleifera* had the least (60.35 %). Adding Moringa seems to lower the amount of carbs, which could mean that Moringa boosts microbial activity, and microorganisms use carbohydrates more as an energy source for fermentation.

The fat percentage of all the samples stayed rather consistent, between 10.63 % and 11.40 %. The small differences seen could be due to the ability of *Moringa oleifera* to absorb or hold onto fat during fermentation. The changes were small, though, which means that adding Moringa did not have a big effect on how fat was broken down during fermentation.

The overall ash content slowly went up ($p > 0.05$) when the amount of *Moringa oleifera* increased. The control group had the lowest amount of ash (1.48 %), and the 7.5 % *Moringa oleifera* group had the highest (2.10 %). This is what we predicted because *Moringa oleifera* has a lot of minerals, such as calcium, potassium, and magnesium, which makes the ash level larger.

pH and titratable acidity

The pH and titratable acidity (TA) levels play crucial roles in fermentation, influencing microbial activity, product stability, and sensory attributes of fermented meat products. The graph shown in Figure 2 illustrates the

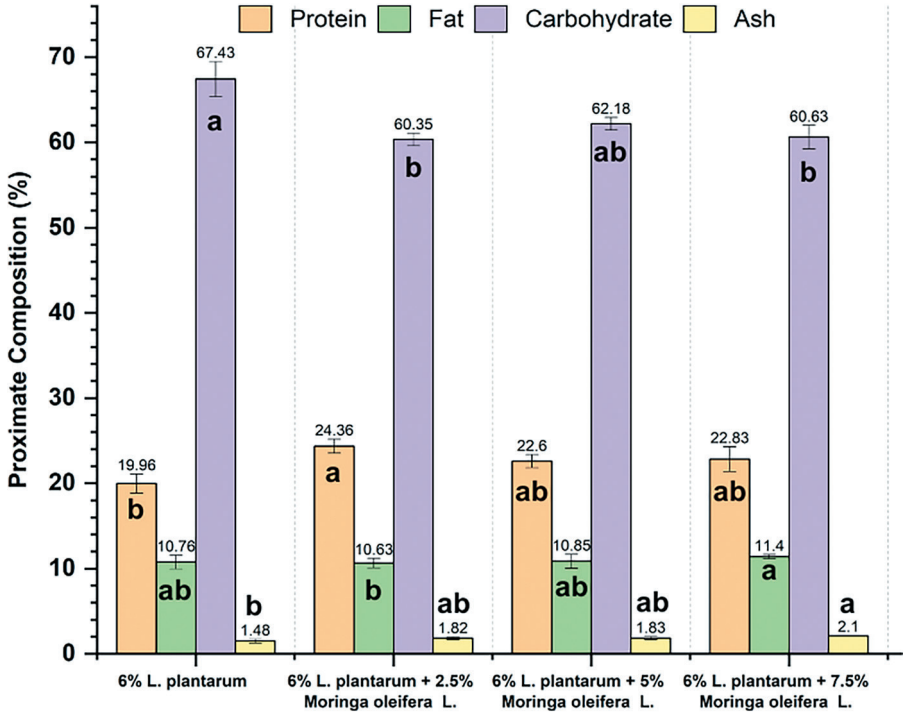

Figure 1. Proximate composition (%) of fermented chicken dendeng prepared with 6 % *Lactobacillus plantarum* and supplemented with varying concentrations of *Moringa oleifera* leaf powder. Error bars denote standard deviations ($n = 3$). Different superscript letters within the same nutrient category indicate statistically significant differences ($p < 0.05$) according to Duncan's multiple range test

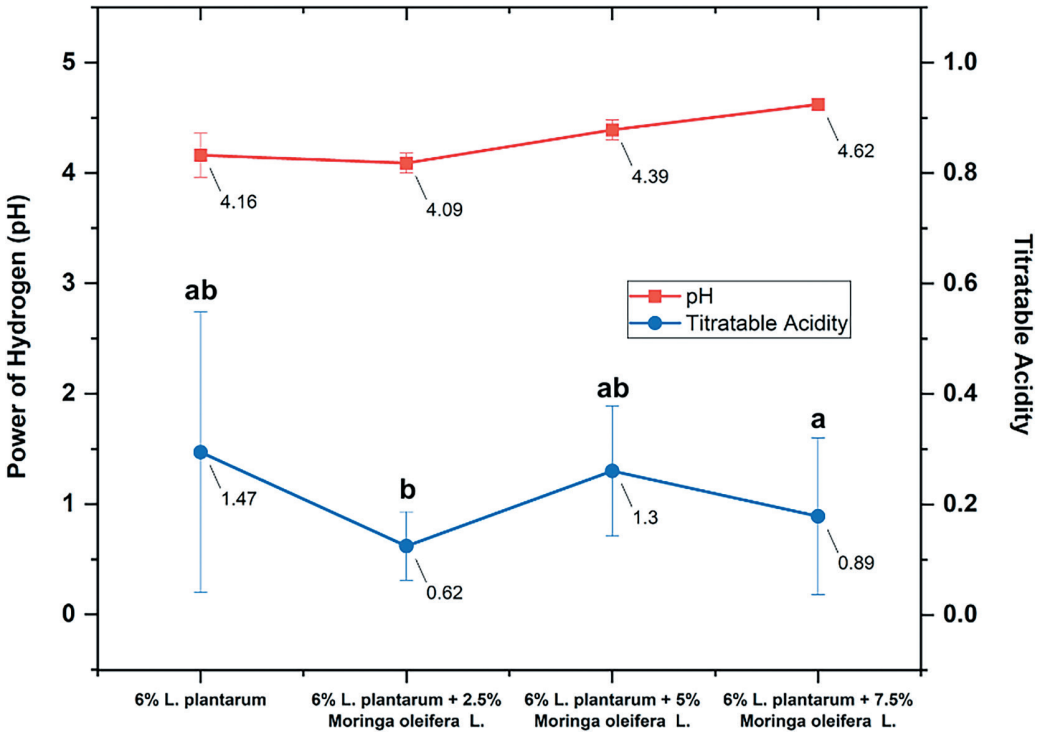

Figure 2. pH values (red line) and titratable acidity (blue line) of fermented chicken *dendeng* formulated with different levels of *Moringa oleifera* leaf powder (T0 = 0 %, T1 = 2.5 %, T2 = 5 %, T3 = 7.5 %). Values represent mean ± SD ($n = 3$). Titratable acidity values marked with different superscript letters (a, b, ab) differ significantly ($p < 0.05$) according to Duncan's multiple range test

variation in pH and TA values during the fermentation of chicken dendeng with *Lactobacillus plantarum* and different concentrations of *Moringa oleifera* (2.5 %, 5 %, and 7.5 %). The pH values varied from 4.09 to 4.62, and the titratable acidity (TA) values changed, with the lowest TA value (0.62) at 2.5 % Moringa and the highest (1.47) at 6 % *L. plantarum* alone. These findings suggest that Moringa supplementation influences the acidification dynamics of fermented dendeng, potentially modifying the microbial equilibrium and fermentation efficacy.

Total plate count

The one-way ANOVA test showed a statistically significant difference in bacterial counts among different Moringa inclusion levels ($p < 0.05$), indicating that Moringa significantly affects microbial proliferation as shown in Figure 3. The bacterial count analysis of fermented chicken dendeng under different formulations shows a significant impact of *Moringa oleifera* supplementation on microbial growth. The total plate count (log CFU/g) increased by adding 2.5 % and 5 % *Moringa oleifera*, reaching a peak at 6.535 log CFU/g and slightly declined at 7.5 % Moringa concentration (6.33 log CFU/g). The control group (6 % *L. plantarum* only) exhibited the lowest bacterial count (5.6175 log CFU/g), suggesting that Moringa contributes to bacterial proliferation in moderate concentrations.

Discussion

Protein content

The observed increase in protein content at 2.5 % Moringa supplementation in fermented chicken dendeng can be primarily attributed to the high protein content of *Moringa oleifera* leaves, which range from 25 % to 30 % on a dry matter basis. These leaves are also rich in essential amino acids such as lysine, methionine, and tryptophan, which are often limited in animal-based products. The contribution of these amino acids improves the overall amino acid balance and nutritional value of the final product, particularly benefiting populations with higher protein needs, such as children, athletes, and elderly individuals.

Moreover, Moringa leaves are known to contain up to 17 different fatty acids, with α-linolenic acid (44.57 %) as the dominant compound, followed by heneicosanoic (14.41 %), γ-linolenic (0.20 %), palmitic (0.17 %), and capric acid (0.07 %). These unsaturated fatty acids not only contribute to the nutritional profile but also interact with proteins during fermentation, potentially influencing their digestibility and functionality. This aligns with findings by Li et al. [23] who noted that lipid protein interactions, especially in systems with elevated polyunsaturated fatty acid content, can modify protein solubility and accessibility.

The role of *Lactobacillus plantarum* in enhancing protein quality is also crucial. This LAB produces proteolytic enzymes particularly serine proteases and metalloproteases that hydrolyze complex muscle proteins into peptides and free amino acids [24]. According to [7] supported by [25], such enzymatic activity improves the functional properties and digestibility of fermented products. These peptides may exhibit additional bioactivity, such as antioxidant or antihypertensive effects, further enhancing the health-promoting qualities of the product.

At higher Moringa concentrations (5–7.5 %), however, the protein content did not continue to increase and in some cases slightly decreased. This could be due to protein-lipid

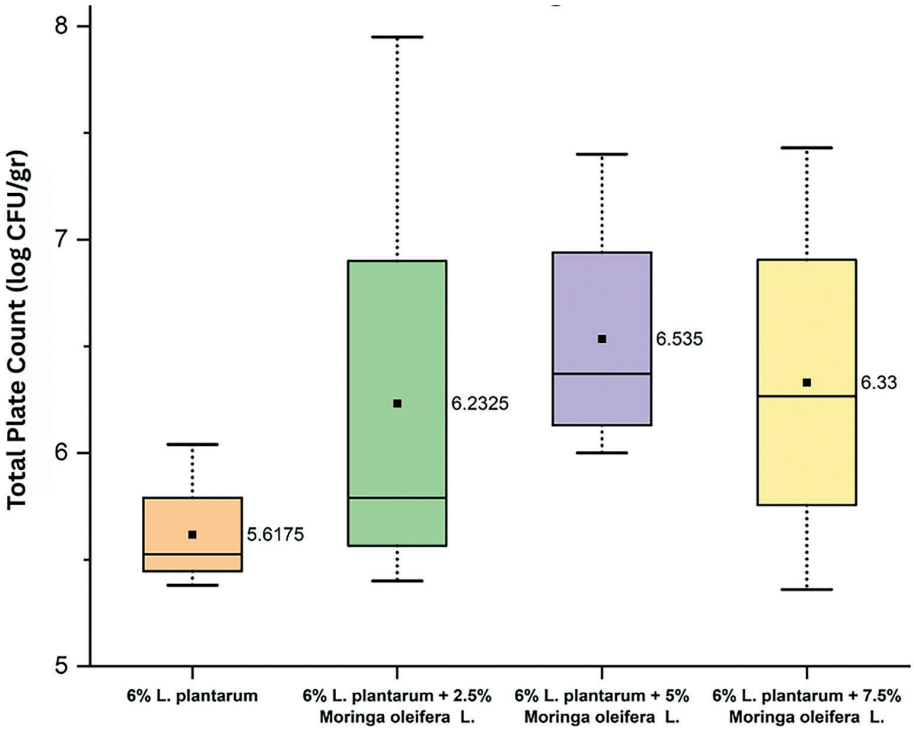

Figure 3. Box plots display distribution of total plate counts in T0 (control), T1 (2.5 %), T2 (5 %), and T3 (7.5 %) formulations. Central boxes represent interquartile range, horizontal lines indicate medians, and black squares indicate means (n=3 per group)

or protein-polyphenol interactions, which lead to reduced protein solubility or enzyme inhibition during fermentation. Polyphenols in Moringa, while beneficial as antioxidants, may also bind to proteins and proteases, forming complexes that are less digestible or bioavailable. Similar effects were reported by [25] in the context of polyphenol-protein binding in functional feed systems, where high phenolic concentrations affected enzymatic hydrolysis. Taken together, these findings suggest that a moderate inclusion of Moringa leaf powder (around 2.5 %) in fermented chicken dendeng achieves an optimal balance: enhancing protein content and digestibility through both direct nutritional input and microbial proteolysis, while avoiding the inhibitory effects observed at higher doses. This strategy supports the development of functional meat products enriched with plant-derived bioactives and improved nutritional profiles.

Effect of carbohydrates on Maillard reaction and color attributes

Carbohydrate reduction is associated with microbial consumption during fermentation, as well as Moringa's low digestible carbohydrate and high fiber content. *L. plantarum* changes carbs into organic acids, and Moringa may help this process along due to its polyphenols speeding up sugar breakdown and fermentation. *Lactobacillus plantarum* uses carbohydrates as a substrate and turns sugars into organic acids through fermentation. This metabolic process lowers the amount of carbohydrates remained in the final product. During fermentation, *L. plantarum* changes fermentable carbohydrates (sugars) into lactic acid and other organic acids. Moreover, *L. plantarum* strains exhibit enzymes, including tannase and gallate decarboxylase, that accelerate the degradation of complex polysaccharides and phenolic compounds during fermentation [22].

Moringa leaf powder, on the other hand, has a lot of fiber and protein but not a lot of carbohydrates that can be digested [26]. The overall carbohydrate intake goes down because Moringa takes the place of some of the ingredients that are high in carbohydrates. When sugars are dried, they react with amino acids in a Maillard process. This may lower the measured carbohydrate content displayed in Figure 4 even more. The gradual darkening of fermented chicken dendeng as the concentration of *Moringa oleifera* increases illustrates the intricate interplay of Maillard reaction products (MRPs), polyphenol oxidation, and fermentation metabolites. The control sample stayed light brown, which is a color that fermented meat products usually have. The samples got darker when more Moringa was added (T1-T3). At 2.5 % (T1), they were greenish-brown, and at 7.5 % (T3), they were almost black. This means that the Maillard reaction and oxidative browning of Moringa polyphenols are likely what caused the color change in these samples.

The Maillard reaction is a non-enzymatic browning process that happens when reducing sugars and amino acids are heated together. This makes melanoidins, which are the brown polymeric pigments that give thermally processed foods their color [27]. This reaction happens in three basic steps: (1) Schiff bases are generated between reducing sugars and amino acids; (2) Amadori products break down into reactive carbonyl compounds; and (3) these intermediates are turned into melanoidins through polymerization. The light brown color in P0 (0 %) reveals that the Maillard reaction happened at a steady rate.

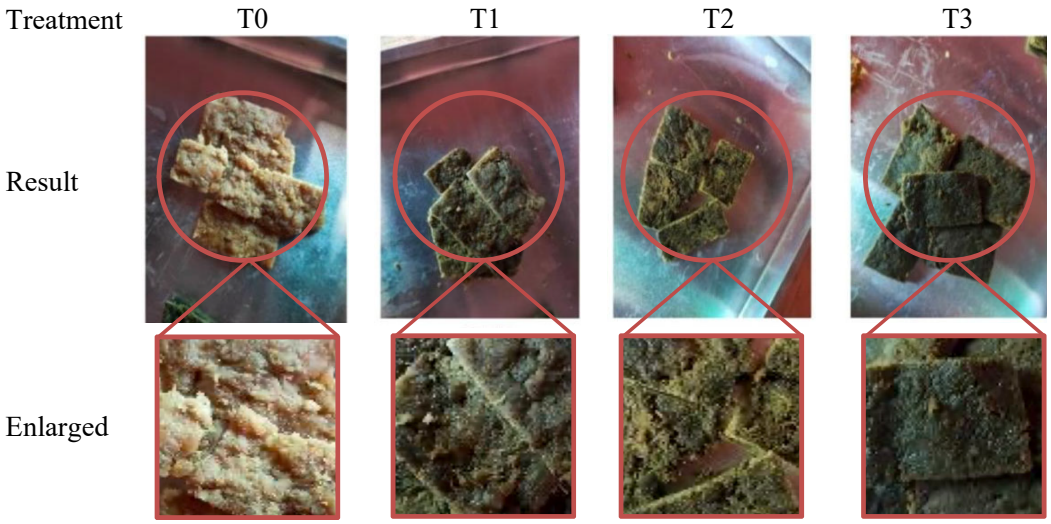

Figure 4. Representative images show T0 (light brown) to T3 (dark brown/greenish black), illustrating the effect of *Moringa oleifera* on product appearance. The darker color in T3 is attributed to enhanced Maillard reaction and polyphenol oxidation during heat treatment

The dark color in T1-T3, on the other hand, suggests that the process happened faster, presumably because of the bioactive chemicals in Moringa.

The increased browning observed at elevated Moringa concentrations can be ascribed to the interaction between Maillard intermediates and Moringa-derived polyphenols. Flavonoids, tannins, and alkaloids are all found in moringa. These compounds can act as secondary reactants and speed up the Maillard reaction, which leads to more melanoidin being made [28]. Polyphenol oxidation also contributes to darkening, as substances such as quercetin and chlorogenic acid undergo auto-oxidation, creating quinones and polymerized pigments that add to the final dark color. This impact is most clear in T3 (7.5 %), where the very black color may be due to too much polyphenol oxidation and pigment production from the Maillard reaction. Furthermore, the control of pH affects the effectiveness of the Maillard reaction. Lower pH levels inhibit Maillard reactions, but neutral to slightly alkaline conditions facilitate melanoidin formation. The minerals in Moringa, such as calcium, potassium, and magnesium, may have helped to stabilize the pH, making it easier for Maillard browning to happen in T2 (5 %) and T3 (7.5 %).

From a sensory standpoint, the noted hue alterations may influence consumer approval. The Maillard reaction creates roasted and umami flavors in moderation, but too much melanoidin can make food taste bitter and burnt, which is not what consumers want. The 2.5 % and 5 % Moringa formulations (T1, T2) may provide balanced sensory characteristics, whereas the 7.5 % formulation (T3) may lead to excessive darkening and bitterness, diminishing customer preference. Lund et al in [29] also said that melanoidins have antioxidant capabilities, which may help fermented dendeng work better by getting rid of free radicals and improving gut health. However, too much Maillard reaction can also cause the development of unwanted substances such as acrylamide, thus it is important to carefully optimize the levels of Moringa that are added.

The changes in color of fermented chicken dendeng with more Moringa are mostly caused by the Maillard reaction, polyphenol oxidation, and the by-products of fermentation. Moderate Moringa inclusion (2.5–5 %) may boost flavor complexity and antioxidant effects, while higher amounts (7.5 %) risk excessive darkening and possibly bitterness. Future research should enhance processing settings to equilibrate Maillard reaction regulation, sensory acceptability, and functional attributes in plant-enriched fermented meat products.

Fat content

The reduction in fat content observed in fermented chicken dendeng with *Moringa oleifera* supplementation and *Lactobacillus plantarum* fermentation can be explained by multiple, overlapping biochemical and structural mechanisms. Moringa leaves are rich in polyphenolic compounds, flavonoids, and tocopherols (vitamin E), all of which are potent natural antioxidants [30]. These compounds protect lipids from oxidation during fermentation and drying, thereby slowing the formation of secondary oxidation products such as malondialdehyde (MDA). This antioxidant protection preserves fat integrity while simultaneously preventing the accumulation of harmful oxidation by-products in the final product.

Moreover, *Lactobacillus plantarum* contributes to fat reduction through enzymatic lipolysis. This strain is known to secrete lipases capable of hydrolyzing triglycerides into free fatty acids and glycerol [31]. These liberated fatty acids are susceptible to further oxidation or may be metabolized into energy-rich compounds such as short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate. As demonstrated by [32], these SCFAs have been associated with enhanced lipid catabolism, improved gut health, and reduced fat accumulation in host organisms, particularly in high-fat diet models. Additionally, *L. plantarum* may also participate in the breakdown of structural phospholipids in muscle tissue, further decreasing the overall fat content while contributing to a softer texture and

enhanced flavor. This dual action of enzymatic hydrolysis and microbial biotransformation leads to the development of fermented meat products with a leaner profile and more favorable health implications. According to [33], such microbial processing not only reduces crude fat but also generates bioactive fatty acid derivatives that contribute to cardiovascular and metabolic health.

The dehydration process during dendeng production may also physically reduce fat levels. As water is removed under controlled drying conditions, lipid molecules can migrate to the surface or be partially lost through evaporation or drip loss, depending on the drying technique. Özer and Kılıç [34] highlighted that heat-induced structural changes in the meat matrix during drying facilitate lipid migration, further contributing to fat loss and a firmer texture. Interestingly, in [35] found that solid-state fermented okara accelerated lipid metabolism without significantly altering pH or SCFA levels, suggesting that the modulation of fat composition in fermented systems may also involve pathways unrelated to acidification. In the case of Moringa-supplemented dendeng, this implies that the observed fat reduction might arise from antioxidant activity, microbial hydrolysis, and structural transformation rather than a direct effect of fermentation acidity alone.

Taken together, the combined effect of Moringa's antioxidant properties, lipolytic activity of *L. plantarum*, and processing factors such as dehydration create a favorable environment for fat reduction. This positions fermented chicken dendeng as a functional meat product with improved lipid profile, particularly suited for health-conscious consumers or individuals managing fat intake. Furthermore, the development of short-chain fatty acids and bioactive lipid metabolites during fermentation enhances the potential probiotic and metabolic health benefits of the product.

Ash content

The greater amount of ash content is because Moringa leaves have a lot of minerals in them. They are high in calcium (for bone health), iron (for making hemoglobin), potassium (for balancing electrolytes), and magnesium (for enzyme function and muscle relaxation). The fermentation process produces lactic acid, which can enhance mineral bioavailability by forming soluble mineral complexes. The metabolic pathway for lactic acid fermentation involves the Embden-Meyerhof pathway (EM pathway), where pyruvate is reduced to lactate in the presence of NADH, influencing the pH of the medium and creating a favorable environment for mineral solubility. The acidification of the medium leads to the pH reduction, which can facilitate solubilization of bound minerals such as calcium, magnesium, and iron, making them more accessible for absorption in the gastrointestinal tract [36]. A higher ash content indicates that Moringa supplementation enhances the mineral profile of fermented chicken dendeng, making it a nutritionally superior product.

pH and titratable acidity

The lower pH in the control is in line with how LAB fermentation usually works. The minerals in moringa have a buffering action that keeps pH stable and changes TA. The changing TA values show that microbes are getting used to Moringa's polyphenols, which may stop acid production at first but then help LAB grow. Moderate amounts of Moringa (2.5 % and 5 %) encourage the growth of LAB since they have prebiotic properties. At 7.5 %, antimicrobial polyphenols (flavonoids and tannins) slow down development a little bit. More LAB counts make products safer and last longer, but too much polyphenols may throw off the balance of microbes. For the best fermentation efficiency, microbiological stability, and sensory quality, a 5 % addition seems to be the best.

L. plantarum breaks down carbohydrates, mostly glucose and other sugars that can be fermented, into organic acids such lactic acid, acetic acid, and succinic acid. This mechanism lowers the pH and raises the TA, which is important for keeping microbes alive and improving fermented product flavor [37]. The pH of the control sample dropped slightly, which is in line with normal LAB fermentation patterns. This is because acid production makes microbial competition stronger and makes food safer [17]. The inclusion of *Moringa oleifera* seems to stabilize or slightly raise pH levels, which could be due to Moringa's high mineral content (such calcium, potassium, and magnesium), which could neutralize some acidity and slow down acidification [38].

The changes in titratable acidity levels show that this buffering effect is even stronger. The dramatic drop at 2.5 % Moringa inclusion shows that acid generation is temporarily slowed down. This could be because polyphenolic chemicals in Moringa affect the metabolism of LAB. But when the amount of Moringa goes up to 5 % and 7.5 %, the TA levels go back up a little bit, which suggests that the microbes adapt and the LAB can keep digesting sugars even when there are bioactive compounds present. This result aligns with prior research on plant-derived antimicrobials, wherein polyphenols initially impede bacteria metabolism but subsequently facilitate adaptability.

From a food safety standpoint, the noted pH stabilization at elevated Moringa concentrations (pH 4.39 at 5 % Moringa and pH 4.62 at 7.5 %) may exert multiple effects. Higher pH levels may reduce excessive sourness, which can make the taste more appealing [39]. On the other hand, a higher final pH may make fermentation less effective at killing bacteria, which could mean longer fermentation times or extra ways to keep food safe from bacteria [40]. Adding *Moringa oleifera* changes the rate at which fermented chicken dendeng becomes acidic, which could alter the survival of microbes, the taste, and the stability of the shelf life. The minerals and antibacterial polyphenols in Moringa may change the way LAB metabolizes, which could raise the final pH and cause TA readings to change.

Microbial dynamics

These findings are consistent with prior research demonstrating that nutritional supplements and bioactive substances can influence microbial activity in fermented meals. Recent studies on the impact of Moringa on microbial communities have shown that moderate amounts can help some bacteria grow while stopping others from growing. A study by [2] demonstrated that the incorporation of Moringa leaf powder into fermented dairy products promoted the proliferation of advantageous *Lactobacillus* strains, indicating that its bioactive components may function as prebiotics.

The rise in bacterial count at 2.5% and 5% Moringa may be due to the prebiotic-like effect of Moringa, which gives LAB the nutrients they need to flourish, such as proteins, vitamins, and minerals. Previous research indicates that LAB flourish in nutrient-dense environments, especially when plant-derived bioactive chemicals that facilitate bacterial metabolism are present. Plant-derived bioactive chemicals, including polyphenols and flavonoids, exert a substantial impact on LAB metabolism by serving as carbon sources or cofactors. Studies have shown that LAB may biotransform phenolics found in plant substrates, creating compounds that are better at fighting oxidative stress [41]. Polyphenols can shield LAB from oxidative harm, enhancing their viability during fermentation operations [42]. The increased bacterial growth indicates that *Moringa oleifera* facilitates LAB proliferation, hence optimizing the fermentation process and possibly augmenting the probiotic potential of fermented dendeng. Nonetheless, at an elevated dose (7.5%), the bacterial count demonstrated a little reduction, potentially attributable to the antibacterial properties of Moringa polyphenols. Flavonoids, tannins, alkaloids, and saponins are all found in *Moringa oleifera*. These compounds can stop bacteria from growing and kill them. These bioactive substances might damage the membranes of bacterial cells or their metabolic processes, which would lower the number of bacteria overall. Flavonoids in Moringa have been related to these antibacterial effects because they can change how bacteria metabolize and damage the membranes that protect them.

From a food safety and shelf life point of view, the controlled rise in LAB count that comes with moderate Moringa addition is good. LAB is important for preventing spoilage microorganisms from growing by making organic acids, bacteriocins, and hydrogen peroxide [43]. This makes the environment unfavorable for pathogenic bacteria. Thus, the 5% *Moringa oleifera* formulation might be the best balance between promoting bacterial growth and killing bacteria, which would keep microbes stable and extend shelf life. A small decrease in LAB at 7.5% Moringa could also help keep the food from becoming too acidic, which would improve its taste by keeping the sourness and umami profile softer [44]. Adding *Moringa oleifera* to fermented chicken dendeng has a big effect on how bacteria develop. The best growth happens at a 5% concentration, and at greater percentages, it changes the way bacteria grow. These results show that Moringa could be a useful addition in fermented meat products because it can improve nutrition, keep microbes safe, and improve taste all at the same time.

Conclusion

Adding *Moringa oleifera* to chicken dendeng in the process of its fermentation has a big effect on its physical, chemical, and microbiological qualities, especially the pH, titratable acidity, and number of bacteria. Moreover, *Moringa oleifera* appears to help the growth of good LAB, which sped up the fermentation process and changed the color and texture through biochemical reactions such as the Maillard reaction. These findings suggest that *Moringa oleifera* can improve the functional and nutritional value of dendeng, making it a promising natural ingredient for future food innovations. However, further research is needed to optimize formulation, processing conditions, and sensory acceptance to maximize its benefits while maintaining desirable product characteristics.

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EXPERIMENTAL STUDY ON ANTIBIOTICS STABILITY TO DEGRADATION IN MEAT SAMPLES UPON SIMULATING CONTAMINATION

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Keywords: *antibiotics, veterinary drugs, animal husbandry, livestock, meat products*

Abstract
Residual concentrations of antibiotics in animal-derived foods represent a serious threat for human health facilitating formation and spreading of antimicrobial resistance. Despite of normative restrictions that regulate the content of antibiotics in foods, mechanisms of their degradation and transformation under an effect of various technological factors, such as freezing and heat treatment, have been studied insufficiently yet. The topicality of this issue is determined by the fact that residual amounts of antibiotics can enter the food chain, which leads to their unintended consumption by humans. This, in turn, can promote selection of resistant pathogenic microorganisms, which is one of the key problems of modern medicine. The aim of this study was a complex assessment of model matrices containing meat raw materials contaminated with antibiotics of different pharmacological groups: penicillins, tetracyclines, amphenicols, aminoglycosides and polypeptides. Contamination of samples (chicken breast, rabbit meat and chicken liver) was carried out in concentrations that corresponded and exceeded maximum permissible levels specified in the normative documentation. Confirmation of the contamination levels was performed by HPLC–MS/MS with deviations not higher than 5 %. In this study, a comprehensive assessment of an effect of storage conditions at a subzero temperature and heat treatment (a range from –18 °C to +72 °C) on the residual concentrations of antibiotics was carried out. The study included analysis of the stability of the molecular structure, degradation kinetics of active components and preservation of pharmacological properties of antibiotics. Freezing did not exert a significant effect on the concentration, while heat treatment led to various degrees of degradation of the tested compounds. The most heat labile were chloroamphenicol and tetracyclines (losses up to 90 %), whereas bacitracin and streptomycin showed the highest stability (losses ≤40 %). The results obtained corroborate partial destruction of antibiotics upon culinary treatment and emphasize a need for strict control of the residues in meat products.

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Introduction

The constant growth of the population and economic development have significantly increased human needs for animal-derived food sources. A rapid increase in urbanization, globalization and market-oriented demand has facilitated the use of antibiotics in industrial animal husbandry and poultry breeding. Most developing countries have ineffective surveillance systems for the antibiotics use in the systems of animal feedstuff production [1]. It is estimated that the global consumption of antibiotics by farm animals will be more than 200,235 tons (growth by 11.5 %) by 2030 [2]. The question of the use of forbidden antibiotics in animal husbandry remains to be an issue of discussion. Antimicrobials used in veterinary medicine potentially can also affect the human body when entered with foods. In the practice of animal breeding, the most frequently used antimicrobials are penicillins, macrolides, aminoglycosides, sulfanilamides and tetracyclines [1].

Ingestion of residual amounts of antibiotics with foods is considered a risk factor for human health. Individuals with increased sensitivity, who can have allergic reactions with different degrees of manifestation, are the most vulnerable group. With chronic ingestion of such compounds, an effect on the hemopoietic system accompanied with the development of certain hematological deviations cannot be excluded. Anaphylactic shock is quite a rare phenomenon but it is potentially dangerous complication that can occur upon high sensitivity to residual antibiotics.

Regarding the gastro-intestinal tract, the effect can be manifested by the disruption of the microbiota, development of diarrhea and other functional disorders. Potential toxicity for the liver and kidneys linked with the risk of formation of hepato- and nephropathies deserves a special

attention. A significant problem remains to be a contribution of residual antibiotics to formation of antimicrobial resistance, which reduces efficacy of therapy of infectious diseases.

There is evidence that upon long-term impact on the body, including the prenatal period, certain antibiotics can negatively influence the developmental processes, including possible teratogenic and mutagenic effects. In addition, an effect on the nervous system is considered, including a possibility of formation of cognitive disorders upon long-term exposure. The toxic effect on the bone marrow also presents a serious threat, which can be manifested as myelosuppression and other hematological disorders [3–5]. Moreover, these compounds disturb the fermentation processes in the food industry [6]. Also, there are reports that antibiotic residues can change sensory characteristics of foods [7].

Overuse or improper use of antibiotics in animal husbandry has led to emergence of the antibiotic resistance genes (ARG) in several microorganisms [2,8]. Resistant bacteria can be transmitted from animals to humans via zoonoses (infectious diseases transmitted from animals to humans), food chain or environmental sources such as contaminated water [9]. Antimicrobial resistance is a serious public health crisis in the developing societies influencing the general health state of humans and animals, rates of economic growth and social conditions [10]. According to the Centers for Disease Control and Prevention (CDC), about 2.8 million people fall ill with antimicrobial-resistant infectious diseases in the USA annually, which leads to at least 35.000 deaths [11].

In the conditions of modern challenges in the area of human and veterinary medicine, a task of the development of comprehensive and efficient approaches to reduction of using antimicrobials in key spheres — in public health, veterinary practice and animal husbandry — becomes more and more urgent. A solution to this problem is impossible without the interdisciplinary interaction that combines data from pharmacology, microbiology and epidemiology. An increase in antibiotic resistance enhances the urgency of introduction of new tools for control and monitoring of using antimicrobials. In parallel, attention to the issues of food safety and quality is strengthening. A range of strategic initiatives aimed at restriction of the unjustified use of antimicrobials in animal husbandry has been created at the international level. The corresponding recommendations have been developed by the Food and Agriculture Organization of the United Nations (FAO), World Organization for Animal Health (WOAH) and World Health Organization (WHO) [12]. For example, member countries of WOAH (no less than 53 %) stopped using the antimicrobials as growth promoters in farm animals. Recently, the USA has withdrawn the claims regarding growth promotion for antibiotics intended for farm animals [13]. An expanding data array about negative effects of bio- and technogenic contaminants determines a permanent extention of the regulatory list of controlled compounds. In this situation, food manufactures and authorities controlling food quality and safety should have methods for reliable, high-performance and sensitive control of contaminants that can potentially be present in used raw materials and cause negative consequences for consumers' health.

An influence of freezing and heat treatment on residues of ciprofloxacin and oxytetracycline was demonstrated by Shaltout F. A.E. et al. [14]. The researchers have found that heat treatment significantly reduced the content in poultry meat of oxytetracycline residues, while ciprofloxacin was more stable when using three types of heat processing — roasting, boiling and microwaving. However, the opposite picture was observed when samples frozen to a temperature of –20°C were stored for six and twelve months. In this case, oxytetracycline was more stable, its amounts reduced only by 2.05 % during frozen storage for six months and by 32.38 % during frozen storage for twelve months. At the same time, the ciprofloxacin content reduced by 62.63 and 100 % over the same periods, respectively [14].

The difference in the sensitivity to temperature among different groups of antibiotics was shown in [15]. As a result of the conducted research, it has been established that bacitracin begins to degrade at a temperature of 100 °C, whereas neomycin sulfate, amoxicillin and amoxicillin-clavulanate are stable at 100 °C and begin to degrade at a temperature of 121 °C, much like tetracycline. Similarly to [14], the stability of quinolones was demonstrated by the example of enrofloxacin — enrofloxacin practically did not degrade at a temperature of 121 °C as well as gentamicin, kanamycin, tiamulin, tilmicosin, tilvasolin and colistin. An important factor is also duration of heat treatment. For example, milk sterilization at a temperature of 120 °C for 20 min led to greater destruction of beta-lactams than sterilization at 140 °C but over 10 min. [16]. In general, it is worth noting that milk sterilization can reduce amounts of penicillins by 65 %, and cephalosporins up to 100 % [17].

Zorraquino et al. [18] carried out an experiment similar to [16] but with macrolides and lincomycin. According to the results, macrolides, similar to beta-lactams, degrade to the highest degree at a temperature of 120 °C over 20 min; however, macrolides showed a higher dispersion within the group — from 51 % for tylosin to 93 % for erythromycin. Lincomycin demonstrated stability at all temperature regimes — its losses were no more than 5 %. Macrolides and tetracyclines are also sensitive to the environmental pH — in acid environment with pH of 4–5 their amounts reduced in a significant degree compared to pH close to neutral [19].

It is necessary to note that the destruction of antibiotics at heat treatment does not always indicate that an antimicrobial agent was completely destroyed. As any other chemical compound, an antibiotic can enter into reactions with other components of a food product. As a result, new compounds are formed, which potentially can also be harmful for the body and can be a cause of the emergence of antibiotic resistance genes as was shown by Jiang H. et al. [20]. According to the results, erythromycin incorporated into turbot

fish meat before its heat treatment by different methods degraded practically fully. However, seven products of its degradation were detected. Their composition depended on a method of heat treatment and they theoretically can be as harmful as erythromycin per se [20].

Researches in this area demonstrated that effectiveness of deactivation of antibiotics depends on multiple factors including an antibiotic type, treatment conditions and characteristics of the processed product. However, systemic data about an influence of these factors on the antibiotics stability in animal-derived foods are still limited.

Therefore, a deep study of the mechanisms of antibiotics degradation under an influence of technological factors is necessary to develop efficient strategies to control and minimize the risk of their residues in foods. This requires the interdisciplinary approach including chemistry, microbiology, biotechnology and food engineering for the complex understanding of the processes that occur at all stages of production and processing of animal-derived foods.

The goal of the research is experimental assessment of the antibiotics stability in meat samples with simulated contamination.

This research aims at the study of degradation of antibacterial preparations in conditions that are maximally approximated to the real conditions of storage and heat treatment of meat products. The data obtained will help assess and form opinions on optimization of the technological impact with account for residual concentrations and propose methods for their reduction at stages of a production processes.

Objects and methods

Objects

Substantiation of the choice of matrices for contamination

The choice of studied matrices was determined by their chemical composition and potential effect on stability of antibiotics in the conditions of heat treatment. Poultry meat is widely represented in a diets and is actively used in processing, whereas the liver is distinguished by more complex biochemical structure including an increased content of enzymes, lipids and pigments. These features can significantly change pharmacokinetics and pharmacodynamics of antibiotics, which finely influence their stability to temperature impact.

The emphasis was made on antibiotics, for which maximum permissible levels (MPL) were established precisely for the liver, and also on antimicrobials that are found in this organ most often. This approach allowed for obtaining more representative data and better assessing real risks linked with contamination, which helps build effective control measures later on.

To create model samples, chilled raw materials were taken: chicken breast meat (*m. pectoralis superficialis*) without skin and tendons, meat from the shoulder part (of rabbits *regio scapularis*) and chilled chicken liver. These matrices can be considered typical representatives of meat raw materials that are most often used in the control of residual amounts of veterinary medicines. Sample preparation included homogenization up to the homogeneous fine-dispersed mass using a rotor homogenizer Ultra-Turrax T18 basic (IKA, Germany).

For matrix contamination, standard samples of antibiotics of the analytical purity were used: bacitracin zinc salt (TRC, Canada); penicillin G potassium salt (TRC, Canada); phenoxymethylpenicillin (penicillin V, Sigma-Aldrich, USA); tetracycline hydrochloride (Sigma-Aldrich, USA); chlortetracycline hydrochloride (Sigma-Aldrich, USA); doxycycline (Sigma-Aldrich, USA); oxytetracycline hydrochloride (EDQM, France); chloramphenicol (TRC, Canada); streptomycin sulfate (VETRANAL, Germany). All standard samples had purity no less than 98 % and were used without additional purification. Deionized water (resistivity ≥18.2 MΩ·cm) obtained using the water purification system Milli-Q Direct 8 (Millipore Merck KGaA, Germany) was used.

Methods

Preparation of initial solutions

Solutions of standard samples of antibiotics were prepared by step-by-step dilution of individual standards up to the required concentration:

- Solution 1 (100 μg/ml): 1 mg of the standard sample was dissolved in 1 ml of methanol and the volume was brought up to 10 ml with deionized water.
- Solution 2 (10 μg/ml): 1000 μl of solution 1 was transferred to the volumetric flask and the volume was brought up to 10 ml with deionized water.
- Solution 3 (1 μg/ml): 1000 μl of solution 2 was transferred to the volumetric flask and the volume was brought up to 10 ml with deionized water.
- Solution 4 (0.1 μg/ml) (only for chloramphenicol): 1000 μl of solution 3 was brought up to 10 ml with deionized water.
- Solution 5 (0.01 μg/ml) (only for chloramphenicol): 1000 μl of solution 4 was brought up to 10 ml with deionized water.

Prepared solutions were stored at +4 °C for not more than 7 days in hermetically sealed dark glass bottles.

Sample preparation

The homogenized samples (20.00 ± 0.30 g) were placed into 50 ml Falcon tubes. Five milliliters of the standard antibiotic solution were added to each sample and mixed using a vortex mixer (L–VM–MINI, Labgic, China) until obtaining the homogeneous mass. After addition of antibiotics, the samples were held at a temperature of 4 °C for 12 hours for uniform distribution of the analyte. The control samples without antibiotics were used as negative controls. All measurements were done in triplicate. An increment of contamination and detection threshold in a range of concentrations from 0.0001 to 10 mg/kg were determined for each matrix, depending on the group of antibiotics (Table 1).

Table 1. Increment of contamination of the model samples for target analytes of antibiotics

Contaminating analyte	Types of meat raw materials	Minimal threshold of contamination, mg/kg	Increment of incorporation to determine a detection threshold, mg/kg (No. of sample)
1. Bacitracin (as zinc bacitracin)	Poultry meat (chicken)	0.01	0.01 (1.1) 1.0 (1.2) 10.0 (1.3)
	Rabbit meat	0.10	0.1 (2.1) 1.0 (2.2) 10.0 (2.3)
	Chicken liver	0.10	0,1 (3.1) 1,0 (3.2) 10.0 (3.3)
2. Penicillin (penicillin potassium salt)	Poultry meat (chicken)	0.02	0.02 (4.1) 0.1 (4.2) 1.0 (4.3)
	Chicken liver	0.02	0.02 (5.1) 0.1 (5.2) 1.0 (5.3)
3. Phenoxymethylpenicillin (synonym: penicillin V)	Poultry meat (chicken)	0.020	0.020 (6.1) 0.1 (6.2) 1.0 (6.3)
	Chicken liver	0.020	0.020 (7.1) 0.1 (7.2) 1.0 (7.3)
4. Streptomycin/ Dihydrostreptomycin	Poultry meat (chicken)	0.2	0.2 (8.1) 1.0 (8.2) 10.0 (8.3)
	Chicken liver	0.2	0.2 (9.1) 1.0 (9.2) 10.0 (9.3)
5. Chloramphenicol	Poultry meat (chicken)	0.0001	0.0001 (11.1) 0.001 (11.2) 0.01 (11.3)
6. Tetracyclines tetracycline	Poultry meat (chicken)	0.01	0.01 (12.1) 1.0 (12.2) 10.0 (12.3)
oxytetracycline		0.01	0.01 (13.1) 1.0 (13.2) 10.0 (13.3)
chlortetracycline		0.01	0.01 (14.1) 1.0 (14.2) 10.0 (14.3)
doxycycline		0.01	0.01 (15.1) 1.0 (15.2) 10.0 (15.3)

As a result of the experiment on confirmation of the increment of contamination of the model samples, all declared concentrations of target antibiotics were analyzed in triplicate (X_1, X_2, X_3). Each sample represented an individually taken specimen additionally analyzed in the conditions of repeatability by two parallel detections (injections). Sample preparation was carried out on the day of production of model samples. Samples were analyzed after thorough mixing with sampling material from various parts of Falcon tubes, which ensured homogeneity of the analyte distribution in the matrix. Storage and freezing until the time of analysis were not used, which enabled minimizing possible losses of antibiotics upon extraction and excluding factors influencing reliability of results.

Heat treatment

The influence of storage and technological processing was assessed on the third series of samples. Freezing was carried out at a temperature of –18 ± 0.5 °C for 10 days in a chamber DF 168 IAP (Nordfrost, Germany) with constant registration of temperature with a logger Testo 174T (Testo SE & Co. KGaA, Germany). Heating was carried out in a drying chamber Binder ED 115 (BINDER GmbH, Germany) until reaching 72 °C in the sample center, controlling temperature with a thermometer SP-83 (Termopribor, Russia). After heating, samples were cooled to room temperature and analyzed on the day of preparation.

Chromatographic analysis (HPLC–MS/MS)

Analysis of antibiotics from different groups was conducted according the current national and international standards:

- GOST 33934-2016[1] — determination of zinc bacitracin by HPLC–MS/MS;
- GOST ISO 13493-2014[2] — determination of chloramphenicol by liquid chromatography;
- GOST 34533-2019[3] — determination of sulfonamides, nitroimidazoles, penicillins, amphenicols;
- GOST 31694-2012[4] — determination of the antibiotics of the tetracycline group;
- GOST 32798-2014[5] — determination of the aminoglycosides (streptomycin).

Antibiotics were quantified by high performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS; QTRAP 5500, AB Sciex Pte. Ltd., Singapore). Detection was carried out in the multiple reaction monitoring (MRM) mode using positive and negative ionization depending on a type of analyte. Calibration was carried out by the external standard in a range of concentrations of 0.001–10 mg/kg ($R^2 \geq 0.995$).

[1] GOST 33934-2016 "Meat and meat products. Determination of Zn-bacitracin using high performance liquid chromatography with mass spectrometry detector" Retrieved from https://docs.cntd.ru/document/1200144233 Accessed December 11, 2025 (In Russian)

[2] GOST ISO 13493-2014 "Meat and meat products. Method for determination of chloramphenicol (levomycetin) content using liquid chromatography" Retrieved from https://docs.cntd.ru/document/1200113004 Accessed December 11, 2025 (In Russian)

[3] GOST 34533-2019 "Food products, food raw materials. Method for determination of sulfonamides, nitroimidazoles, penicillins, amphenicols by high performance liquid chromatography-mass spectrometry" Retrieved from https://docs.cntd.ru/document/1200167054 Accessed December 11, 2025 (In Russian)

[4] GOST 31694-2012 "Food products, food raw materials. Method of determination of the antibiotic residues of tetracycline group by High Performance Liquid Chromatography — Mass Spectrometry" Retrieved from https://docs.cntd.ru/document/1200096573 Accessed December 11, 2025 (In Russian)

[5] GOST 32798-2014 "Food products, food raw materials. Method for determination of the aminoglycosides content by high performance liquid chromatography with mass spectrometry detector" Retrieved from https://docs.cntd.ru/document/1200112480 Accessed December 11, 2025 (In Russian)

Data were analyzed using the statistic program complex STATISTICA 9.0. Data are presented as an arithmetic mean ± standard deviation. Statistical significance was calculated using analysis of variance (ANOVA) with the use of Dunnett's test. A probability of 0.05 was chosen as a significant level.

Results

Concentrations of contaminating substances were chosen according to the requirements of the Technical Regulation of the Customs Union TR CU 021/2011[6], based on the specified MPL.

Confirmation of the increment of contamination of the model samples

Analysis showed that the recovered amounts of antibiotics calculated based on the established concentrations (Table 1) corresponded to their theoretical values. Deviations were not higher than 5 %, satisfying the criteria of accuracy specified for such types of analytical investigations and being sufficient for formalization of the goals of the research program.

Therefore, verification of the calculated concentrations by the actual determination using HPLC–MS/MS corroborated correctness of all declared contamination levels. This suggests high accuracy of dosing antibiotics into the model matrices and reproducibility of the procedure of their preparation.

Assessment of the correspondence of spiked and recovered amounts of antibiotics

For the developed panel of the model samples, a comparative assessment by the "spiked-recovered" method was performed using HPLC–MS/MS. Deviations between the calculated and determined values of the antibiotics content were not higher than 5 % with average losses varying in a range from 0.5 to 5 % (Table 2).

The results obtained correspond to the established analytical allowances and confirm the reliability of the method for preparation of model samples, which enables using them as validation matrices to assess accuracy and correctness of laboratory methods of analysis.

Influence of heat treatment on antibiotics stability in the model samples

Within the framework of the validation of analytical methods of the control (HPLC–MS/MS), an effect of technological operations that simulate conditions of storage and culinary treatment on stability of antibiotic residues in meat matrices was assessed. The study aimed at determining a degree of degradation of contaminants upon freezing (up to a temperature of –18 °C) and heating (up to a temperature of 72 °C in the sample center) in different types of raw materials: poultry meat, rabbit meat and chicken liver. The study was carried out at three levels of antibiotics incorporation to assess a dependence of the degradation on an initial concentration.

Stability of antibiotics during storage

The results of the assessment of the antibiotics stability during storage of the model samples at a temperature of –18 °C for 10 days show that freezing did not exert a significant effect on the concentration of the studied substances (Table 3). Concentrations of antibiotics after storage remained within the analytic error of the method, which suggests stability of these compounds at negative temperatures. The results obtained agree with the data of [14,21], which also show that low-temperature storage does not cause degradation of antibiotics in meat matrices. In most cases, the observed changes were within the accuracy of the analytical method, especially if weight losses upon freezing, which can create an illusion of an increase in the concentration, are taken into account.

Effect of heating on antibiotics concentration

When assessing an effect of heat treatment on antibiotics stability (heating to a temperature of 72 °C in the sample center), it has been established that a level of destruction of the compounds varied significantly both between different antibiotics (Table 3, Figure 1) and between types of studied matrices (meat and liver).

Discussion

In contrast to freezing, heating had a pronounced and multidirectional effect on the antibiotics stability depending on their chemical class, matrix type and contamination level.

Stability of different classes of antibiotics

Based on the data obtained, all studied antibiotics can be ranked by a degree of heat stability at a temperature of 72 °C in descending order:

- Most stable. The lowest decrease in a concentration upon heating was observed for streptomycin and bacitracin. Average losses were 30–33 % and 30–42 %, respectively, for three levels of contamination. At the same time, for individual liver samples with low levels of contamination with bacitracin destruction reached 65 %, which apparently is linked with matrix effects and higher lipophilicity of liver tissues. The lowest losses of bacitracin were noted in rabbit meat (20.4–37.7 %), while the minimum losses of streptomycin were recorded in the chicken liver (16.5 % for the samples with a concentration equal to MPL) and maximum in the muscle tissue samples (up to 33 %).
- Average stability. Penicillin antibiotics — penicillin and phenoxymethylpenicillin — demonstrated more pronounced thermal lability: a decrease in their concentration was from 46 % to 64.5 %. At the same time, the character of degradation was more even across the levels of contamination (spread of values did not exceed 10 %), which suggests a common mechamisms of the desrtruction.

[6] TR CU 021/2011 Technical Regulations of the Customs Union "TP TC 021/2011 "On food safety" (as amended as of April 22, 2024)" Retrieved from https://docs.cntd.ru/document/902320560 Accessed December 11, 2025 (In Russian)

Table 2. Mass spectrometric recovery of the spiked concentrations of antibiotics by the spike/recovery method

Analyte	Types of meat raw materials	Calculated concentration of antibiotic, mg/kg (No. of sample) SPIKED	Recovered concentration of antibiotic, mg/kg, RECOVERED		
			X_{av} 1	X_{av} 2	X_{av} 3
Bacitracin (as zinc bacitracin)	Poultry meat (chicken)	0.0100 (1.1)	0.0090 ± 0.0015	0.0096 ± 0.0017	0.0098 ± 0.0018
		1.00 (1.2)	0.93 ± 0.08	0.96 ± 0.09	0.94 ± 0.09
		10.00(1.3)	9.93 ± 0.94	9.89 ± 0.90	9.88 ± 0.90
	Rabbit meat	0.100 (2.1)	0.093 ± 0.008	0.090 ± 0.008	0.094 ± 0.009
		1.00 (2.2)	0.90 ± 0.08	0.91 ± 0.09	0.93 ± 0.09
		10.00 (2.3)	9.77 ± 0.84	9.89 ± 0.80	9.78 ± 0.86
	Chicken liver	0.100 (3.1)	0.092 ± 0.011	0.095 ± 0.013	0.092 ± 0.011
		1.00 (3.2)	0.95 ± 0.08	0.96 ± 0.09	0.92 ± 0.08
		10.0 (3.3)	9.73 ± 0.71	9.80 ± 0.78	9.85 ± 0.83
Penicillin (Penicillin potassium salt)	Poultry meat (chicken)	0.020 (4.1)	0.019 ± 0.001	0.017 ± 0.001	0.018 ± 0.001
		0.100 (4.2)	0.093 ± 0.008	0.010 ± 0.009	0.096 ± 0.009
		1.00 (4.3)	0.90 ± 0.08	0.91 ± 0.09	0.93 ± 0.09
	Chicken liver	0.020 (5.1)	0.018 ± 0.001	0.017 ± 0.001	0.020 ± 0.002
		0.100 (5.2)	0.090 ± 0.007	0.094 ± 0.008	0.096 ± 0.009
		1.00 (5.3)	0.93 ± 0.08	0.91 ± 0.08	0.96 ± 0.09
Phenoxymethylpenicillin (synonym: penicillin V)	Poultry meat (chicken)	0.020(6.1)	0.019 ± 0.001	0.019 ± 0.001	0.017 ± 0.001
		0.100 (6.2)	0.090 ± 0.008	0.094 ± 0.009	0.093 ± 0.009
		1.00(6.3)	0.91 ± 0.08	0.93 ± 0.09	0.92 ± 0.09
	Chicken liver	0.020 (7.1)	0.017 ± 0.001	0.017 ± 0.001	0.018 ± 0.001
		0.100 (7.2)	0.090 ± 0.007	0.088 ± 0.007	0.093 ± 0.008
		1.00 (7.3)	0.93 ± 0.08	0.90 ± 0.08	0.95 ± 0.09
Streptomycin/ Dihydrostreptomycin	Poultry meat (chicken)	0.20 (8.1)	0.19 ± 0.08	0.19 ± 0.08	0.17 ± 0.08
		1.00 (8.2)	0.91 ± 0.08	0.95 ± 0.09	0.92 ± 0.09
		10.00 (8.3)	9.88 ± 0.90	9.89 ± 0.90	9.86 ± 0.89
	Chicken liver	0.20 (9.1)	0.18 ± 0.08	0.19 ± 0.08	0.15 ± 0.07
		1.00 (9.2)	0.93 ± 0.08	0.91 ± 0.08	0.94 ± 0.09
		10.00 (9.3)	9.88 ± 0.90	9.89 ± 0.90	9.84 ± 0.87
Chloramphenicol	Chicken meat	0.000100 (10.1)	0.000090 ± 0.000010	0.000093 ± 0.000010	0.000088 ± 0.000010
		0.00100 (10.2)	0.00090 ± 0.00013	0.00093 ± 0.00016	0.00098 ± 0.00018
		0.0100 (10.3)	0.0090 ± 0.0015	0.0093 ± 0.0016	0.0098 ± 0.0018
Tetracycline	Chicken meat	0.0100 (11.1)	0.0089 ± 0.0015	0.0091 ± 0.0015	0.0094 ± 0.0016
		1.00 (11.2)	0.98 ± 0.09	0.97 ± 0.09	0.95 ± 0.08
		10.00 (11.3)	9.88 ± 0.90	9.89 ± 0.90	9.86 ± 0.89
Oxytetracycline	Chicken meat	0.0100 (12.1)	0.0099 ± 0.0019	0.0093 ± 0.0015	0.0095 ± 0.0017
		1.00 (12.2)	0.91 ± 0.08	0.93 ± 0.09	0.94 ± 0.09
		10.00 (12.3)	9.87 ± 0.94	9.89 ± 0.80	9.88 ± 0.95
Chlortetracycline	Chicken meat	0.0100 (13.1)	0.0087 ± 0.0014	0.0090 ± 0.0016	0.0091 ± 0.0017
		1.00 (13.2)	0.95 ± 0.08	0.93 ± 0.08	0.92 ± 0.08
		10.00 (13.3)	9.78 ± 0.80	9.82 ± 0.84	9.80 ± 0.80
Doxycycline	Chicken meat	0.0100 (14.1)	0.0090 ± 0.0015	0.0091 ± 0.0015	0.0088 ± 0.0014
		1.00 (14.2)	0.93 ± 0.08	0.95 ± 0.09	0.96 ± 0.09
		10.00 (14.3)	9.90 ± 0.94	9.88 ± 0.91	9.89 ± 0.91

Table 3. Effect of heat treatment of antibiotics stability in the model samples

Contaminating analyte	Types of meat raw materials	Calculated concentration of antibiotic, mg/kg (No. of sample)	Recovered concentration of antibiotic, mg/kg	Concentration after freezing at –18 °C	Concentration after heating to 72 °C in the sample center
Bacitracin (as zinc bacitracin)	Poultry meat (chicken)	0.0100 (1.1) 1.00 (1.2) 10.00(1.3)	0.0090 ± 0.0015^{a}	0.009 ± 0.002^{a}	0.0058 ± 0.0010^{b}
			0.93 ± 0.08^{ab}	0.94 ± 0.09^{ab}	0.45 ± 0.04^{b}
			9.93 ± 0.94^{b}	9.81 ± 0.89^{b}	6.18 ± 0.67^{c}
	Rabbit meat	0.100 (2.1) 1.00 (2.2) 10.00 (2.3)	0.093 ± 0.008^{ab}	0.088 ± 0.008^{a}	0.074 ± 0.006^{b}
			$0.90 \pm 0.08_{ab}$	0.90 ± 0.09^{a}	0.63 ± 0.07^{b}
			9.77 ± 0.84^{a}	9.91 ± 0.79^{b}	6.09 ± 0.56^{b}
	Chicken liver	0.100 (3.1) 1.00 (3.2) 10.0 (3.3)	0.092 ± 0.011^{a}	0.091 ± 0.011^{a}	0.032 ± 0.006^{b}
			0.95 ± 0.08^{a}	0.90 ± 0.08^{a}	0.71 ± 0.06^{b}
			9.73 ± 0.71^{a}	9.75 ± 0.76^{a}	7.75 ± 0.67^{b}
Penicillin	Poultry meat (chicken)	0.020 (4.1) 0.100 (4.2) 1.00 (4.3)	0.019 ± 0.001^{a}	0.018 ± 0.001^{a}	0.010 ± 0.001^{b}
			0.093 ± 0.008^{a}	0.091 ± 0.008^{a}	0.036 ± 0.006^{b}
			0.90 ± 0.08^{a}	0.86 ± 0.08^{b}	0.44 ± 0.05^{c}
	Chicken liver	0.020 (5.1) 0.100 (5.2) 1.00 (5.3)	0.018 ± 0.001^{a}	0.017 ± 0.001^{a}	0.009 ± 0.001^{b}
			0.090 ± 0.007^{a}	0.087 ± 0.007^{a}	0.016 ± 0.001^{b}
			0.93 ± 0.08^{a}	0.91 ± 0.08^{a}	0.36 ± 0.05^{b}
Phenoxymethylpenicillin	Poultry meat (chicken)	0.020(6.1) 0.100 (6.2) 1.00(6.3)	0.019 ± 0.001^{a}	0.017 ± 0.001^{a}	0.008 ± 0.001^{b}
			0.090 ± 0.008^{a}	0.091 ± 0.008^{a}	0.040 ± 0.005^{b}
			0.91 ± 0.08^{a}	0.88 ± 0.08^{a}	0.52 ± 0.06^{b}
	Chicken liver	0.020 (7.1) 0.100 (7.2) 1.00 (7.3)	0.017 ± 0.001^{a}	0.014 ± 0.001^{a}	0.009 ± 0.001^{b}
			0.090 ± 0.007^{a}	0.087 ± 0.007^{a}	0.047 ± 0.006^{a}
			0.93 ± 0.08^{a}	0.91 ± 0.08^{a}	0.53 ± 0.07^{b}
Streptomycin	Poultry meat (chicken)	0.20 (8.1) 1.00 (8.2) 10.00 (8.3)	0.19 ± 0.08^{a}	0.19 ± 0.08a	0.09 ± 0.08^{b}
			0.91 ± 0.08^{a}	0.95 ± 0.09^{a}	0.73 ± 0.08^{b}
			9.88 ± 0.90^{a}	9.89 ± 0.90^{a}	7.22 ± 0.67^{b}
	Chicken liver	0.20 (9.1) 1.00 (9.2) 10.00 (9.3)	0.18 ± 0.08^{a}	0.19 ± 0.08^{a}	0.15 ± 0.07^{b}
			0.93 ± 0.08^{a}	0.91 ± 0.08^{a}	0.54 ± 0.04^{b}
			9.88 ± 0.90a	9.89 ± 0.90^{a}	6.84 ± 0.42^{b}
Chloramphenicol	Chicken meat	0.000100 (10.1) 0.00100 (10.2) 0.0100 (10.3)	$0.000090 \pm 0.000010^{a}$	$0.000093 \pm 0.000010^{a}$	n.d.
			0.0009 ± 0.00013^{a}	0.00093 ± 0.00016^{a}	n.d.
			0.0090 ± 0.0015^{a}	0.0093 ± 0.0016	0.0036 ± 0.0008
Tetracycline	Chicken meat	0.0100 (11.1) 1.00 (11.2) 10.00 (11.3)	0.0089 ± 0.0015	0.0091 ± 0.0015^{a}	n.d.
			0.98 ± 0.09^{ab}	0.97 ± 0.09^{a}	0.078 ± 0.01^{b}
			9.88 ± 0.90^{a}	9.89 ± 0.90^{a}	$3.22^{b} \pm 0.34^{b}$
Oxytetracycline	Chicken meat	0.0100 (12.1) 1.00 (12.2) 10.00 (12.3)	0.0099 ± 0.0019^{a}	0.0094 ± 0.0015^{a}	0.0025 ± 0.0008^{b}
			0.91 ± 0.08^{a}	0.85 ± 0.07^{a}	0.44 ± 0.05^{b}
			9.87 ± 0.94^{a}	9.80 ± 0.76^{a}	5.44 ± 0.45^{b}
Chlortetracycline	Chicken meat	0.0100 (13.1) 1.00 (13.2) 10.00 (14.3)	0.0087 ± 0.0014^{a}	0.0089 ± 0.0015^{a}	n.d.
			0.95 ± 0.08^{a}	0.92 ± 0.08^{a}	0.05 ± 0.01^{b}
			9.78 ± 0.80^{a}	9.80 ± 0.82^{a}	2.15 ± 0.12^{b}
Doxycycline	Chicken meat	0.0100 (14.1) 1.00 (14.2) 10.00 (14.3)	0.0090 ± 0.0015^{a}	0.0088 ± 0.0013^{a}	n.d.
			0.93 ± 0.08^{a}	0.91 ± 0.07^{a}	0.18 ± 0.01^{a}
			9.90 ± 0.94^{a}	9.85 ± 0.89^{a}	3.44 ± 0.34^{b}

Letters a, b, c indicated a significant difference in the obtained results between the control and experimental groups.

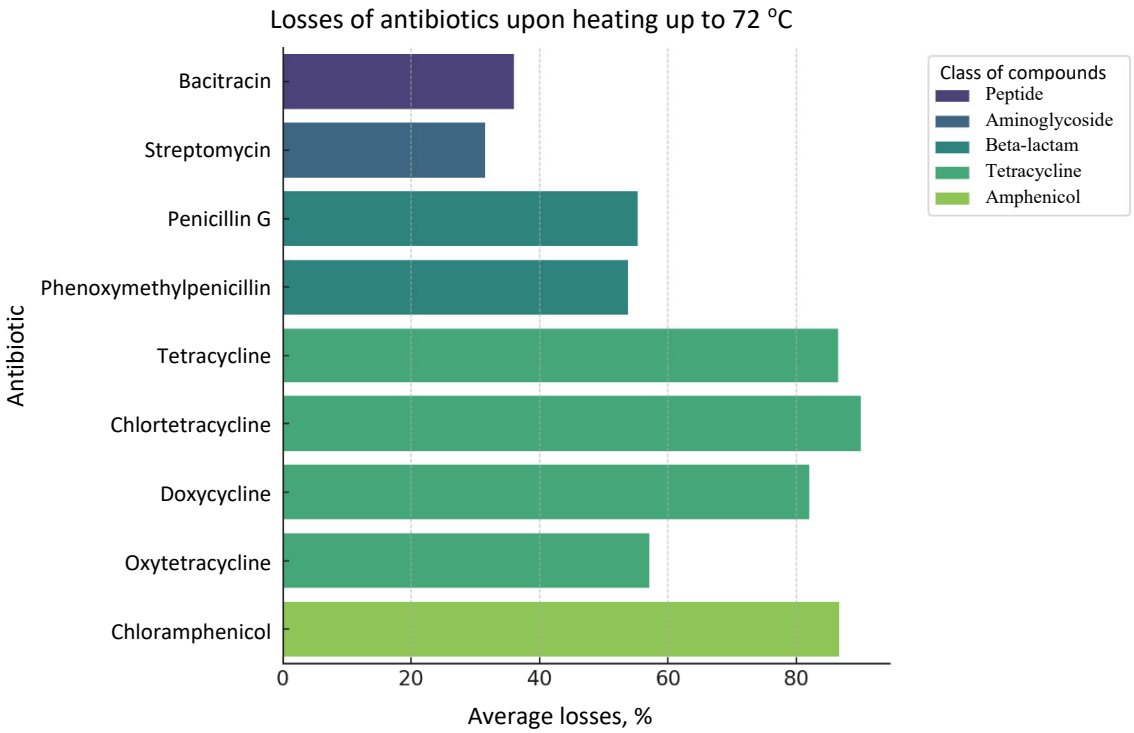

Figure 1. Average values of losses of different antibiotics upon heating to 72 °C in the analyzed matrix samples

Maximum penicillin losses were recorded in the liver, while phenoxymethylpenicillin showed higher destruction in muscle tissue, which corresponds to differences in their solubility and chemical stability.

- Least stable. The most pronounced losses were observed for tetracycline antibiotics. When heating up to 72 °C (heating was stopped upon reaching this temperature inside a sample), the following sequence of thermal stability was established: chlortetracycline (90.0 %) > tetracycline (86.5 %) > doxycycline (82.0 %) > oxytetracycline (57.1 %). It is worth noting that some concentrations, including MPL, showed 100 % destruction of all antibiotics, excluding oxytetracycline. Also, it is necessary to note a significant level of reduction in chloramphenicol residues, which was 86.7 % on average for three concentrations of contamination with account for 100 % destruction at the first two levels of contamination concentrations.

Effect of the matrix and contamination level

In most cases, the liver facilitated higher degradation of antibiotics (especially penicillin and bacitracin at lower levels) compared to muscle tissue, which can be explained by its complex chemical composition and role in metabolism. Rabbit meat showed itself as a matrix that preserved bacitracin slightly better upon heating than poultry meat.

A trend is clearly seen: at higher initial concentrations, a percent of degradation decreases as a rule. However, the absolute quantity of the destructed antibiotic is higher. This is especially important with respect to safety: high residual amounts can be significant even after considerable percentage degradation.

The results of the conducted research and literature data demonstrate that a degree of antibiotics destruction upon heat treatment depends both on their chemical structure and characteristics of the food matrix. Heat treatment significantly affects the content of antibiotics in meat tissues and a degree of degradation depends on a chemical structure of a compound, matrix nature and a method of heating.

Thermal stability of antibiotics has the following sequence: β-lactams = tetracyclines (less thermally stable) > lincomycin > amphenicols > sulfonamides > oxfendazole > levamisole (most thermally stable) [22]. Our results correspond to this trend: penicillins and tetracyclines were less stable to heating (Figure 1). For example, when heating to a temperature of 72 °C, concentrations of penicillin and phenoxymethylpenicillin reduced by 46–64 %, while losses were only 57 % for oxytetracycline. It has been noted that upon heating not only degradation of the initial antibiotic takes place, but also the development of the degradation products, some of which can retain the antibacterial activity [22]. Therefore, a decrease in the concentration of the initial compound is not always equivalent to the loss of the biological activity, which has a key significance for assessing food safety.

Hassani et al. [23] studied tetracycline antibiotics at a temperature of 110–140 °C. Upon low-temperature long-time heat treatment (conventional sterilization), more than 98 % of the initial amounts of antibiotics were destroyed, while short-term treatment at high temperatures left 50–90 % of residues. The authors found that doxycycline is the most thermally stable, while oxytetracycline the least. However, our results showed the opposite dependence: doxycycline was destroyed faster than oxytetracycline. This is probably can be explained by the characteristics of the used matrix (high content of proteins, lipids, moisture) and differences in pH and water activity. Meredith et al. [24]

showed that upon roasting, frying and autoclaving of poultry tissues, the residual amounts of chlortetracycline and oxytetracycline are completely destroyed even in the presence of the enhancing effect of terephthalic acid. These data are in agreement with the results of the present work, where chlortetracycline also turned to be the most thermally labile antibiotic. Chlortetracycline showed the lowest thermal stability, whereas oxytetracycline was the most stable among the studied tetracyclines. It is necessary to note that for some samples, especially with a concentration at MPL, 100 % destruction of antibiotics was observed with the exception of oxytetracycline, which retained residual traces even at the maximum heat treatment.

Bacitracin, which belongs to polypeptide antibiotics, revealed moderate stability to heating. Its high molecular weight (1486.06 Da) and spatial structure, which includes the heptapeptide ring, determine partial thermal stability. The results obtained correspond to the literature data [25], which indicate that bacitracin is poorly metabolized and is preserved mainly in the animals' intestine. Due to the limited amount of data on thermal stability of bacitracin, further study of this antibiotic in biological matrices is of scientific interest [26].

Studies by Gajda et al. [27] and Alaboudi et al. [28] confirm that processes of food preparation (boiling, frying, microwaving) lead to a decrease in residual amounts of antibiotics, but a degree of inactivation depends on time and temperature. However, our data demonstrate that common methods of preparation (frying, cooking, baking) do not ensure full inactivation of antibiotics, even those compounds that are most sensitive to heating. Only more tough regimes — autoclaving and long-term cooking — can lead to complete degradation. The results obtained by Tebbani et al. [29] and Alhendi et al. [30] show that the highest concentrations of antibiotics are found in the kidneys and liver — organs taking part in metabolism and excretion of compounds. Therefore, even after culinary treatment, the residual amounts of antibiotics may remain in biological tissues creating risks for consumers especially upon violation of withdrawal periods in animals.

An important confirmation is a conclusion about higher thermal stability of antibiotics in complex food matrices compared to water solutions [1]. Proteins, fats and other components of meat can exert a protective effect, binding molecules of antibiotics or creating a microenvironment that retard hydrolysis. However, our method did not enable controlling parameters such as water activity and pH, which according to Hassani et al. [23] are critical factors determining a rate of thermal degradation. This opens a prospect for further studies.

Conclusion

The conducted research enabled comprehensive assessment of the stability of the molecular structure and degradation kinetics of the active components of antibiotics from different pharmacological groups in conditions simulating storage and technological processing of meat raw materials. It has been established that low-temperature storage at –18 °C for 10 days practically did not affect the content of the studied compounds, while heat treatment up to 72 °C caused pronounced antibiotics degradation, which degree depends on their chemical structure, initial concentration and matrix type. The most thermally labile were chloramphenicol and tetracyclines, while bacitracin and streptomycin demonstrated higher stability to heating. With that, in several cases, the liver facilitated more intensive destruction of the compounds than muscle tissue.

The results obtained suggest that the standard regimes of culinary treatment (heating up to 72 °C) do not guarantee complete inactivation of the residual amounts of antibiotics, especially stable compounds. This justifies a need for strict control of the adherence to the withdrawal periods before animal slaughter. Furthermore, it is necessary to introduce systematic analytical control at all stages of the production process, which should correspond to the requirements of the Technical Regulation of the Customs Union 021/2011.

From the practical point of view, the developed panel of model samples with confirmed stability during storage can be a basis for validation of methods for controlling residual amounts of antibiotics in meat raw materials.

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IDENTIFICATION AND CHARACTERIZATION OF BIOFILMS AT PRODUCTION FACILITIES OF POULTRY PROCESSING AND MEAT PROCESSING ENTERPRISES

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Keywords: *biofilms, meat processing plant, poultry processing plant, microbial contamination of objects, transmission electron microscopy*

Abstract
Biofilm formation on food processing surfaces creates persistent reservoirs of microbial contamination, including opportunistic pathogens, posing a serious risk to product quality and safety, while standard monitoring programs are often ineffective at detecting them in hard-to-reach niches. The aim of the study was a comparative analysis of microbial contamination and biofilm prevalence on surfaces in a meat processing plant (MPP) and a poultry processing plant (PPP). Samples were collected by swabbing from various surfaces in the production environment. Significant differences in biofilm prevalence were revealed: at the PPP, biofilms were detected in 85.7 % of samples (6 out of 7), whereas at the MPP, only in 25 % (2 out of 8). The microbiota of both plants was characterized by the dominance of bacteria of the genera Pseudomonas, Serratia, Raoultella, and yeasts of the genus Candida. The presence of opportunistic pathogens (Enterococcus faecalis, Yersinia spp.) and indicator microorganisms (Escherichia coli) was established. Biofilms formed predominantly in hard-to-reach areas with moisture (transport wheels, bottom sides of equipment, and walls of chilling tunnels). The study demonstrates the systemic and uneven formation of biofilms, which act as persistent sources of contamination. Their detection in non-obvious locations not covered by standard monitoring indicates the need to revise sampling points and sanitation procedures for effective microbiological risk control in food processing facilities.

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Introduction

Assurance of microbiological safety and stable quality of products is one of the most urgent tasks of modern food biotechnology and food hygiene. A significant threat in this aspect is microbial contamination of objects of production environment and technological equipment, which can lead both to raw material spoilage and foodborne toxicoinfections in consumers. Over the last decade, the role of not only planktonic forms of microorganisms, but their structured communities, biofilms (BFs), has been actively discussed in scientific literature as a key factor of persistence and prevalence of microbial contaminants in food production facilities [1–3].

A biofilm is a complex structured consortium, in which cells are submerged into the self-formed extracellular polymeric matrix (EPM) consisting of exopolysaccharides, proteins, lipids and extracellular DNA (eDNA) [4,5]. This unique architecture provides resident microorganisms with resistance to an effect of physico-chemical stressors, including an action of disinfectants, temperature fluctuations, moisture, pH and mechanical impacts [6]. As a result, BFs transform into persistent microbial reservoirs acting as a constant source of secondary contamination of products and leading to cross-contamination throughout the technological chain [7].

An ability of several bacteria to form biofilms in specific conditions (lowered environmental temperature, high moisture, pH, and others) is of special concern in the meat industry. The case in point are not only psychrotrophic spoilage bacteria, such as *Pseudomonas* spp., *Brochothrix thermosphacta* и *Carnobacterium* spp., but also pathogenic/ opportunistic microorganisms, including *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella* spp. and certain strains of *Escherichia coli* [8]. The studies demonstrate that these microorganisms can retain viability and virulence in the composition of biofilms on materials typical of the food industry (stainless steel, polymers, rubber) even upon regular sanitary treatments [3,9].

Traditional programs of production control based on sampling from visually clean and easily accessible surfaces contacting with products as well as on using classic cultural methods often do not reflect a real picture. First of all, BFs are usually formed in "shadow" zones: in drainage systems, on the bottom surfaces of conveyors, in the places of condensate accumulation, on sealants and wheels of internal transport. Secondly, classic cultural methods allow for

detecting only an insignificant (less than 2 %) culturable part of microbiome, while the large proportion of bacteria in BFs can be in metabolically inactive, dormant state and are not revealed by standard detection methods [10].

Therefore, to form a real picture of microbial contamination at an enterprise, complex investigations that integrate modern analytical methods are necessary. Metagenomic next generation sequencing (NGS) enables assessing a complete taxonomic variety of a microbial community [11]. High-accuracy identification of culturable isolates by MALDI-TOF mass spectrometry makes it possible to correlate genetic data with particular species that have well-known technological or epidemiological significance. However, only methods of direct visualization, such as scanning electron microscopy and especially transmission electron microscopy are able to unambiguously corroborate the presence of an organized BF, characterize its morphology, structure of EPM and physiological state of cells [5]. This combined methodological approach used in the comparative aspect for different types of production facilities is represented insufficiently in the modern studies especially in the context of Russian meat processing plants. The aim of this study was a comparative analysis of microbial contamination and prevalence of biofilms on surfaces of a meat processing plant (MPP) and poultry processing plant (PPP).

Objects and methods

The objects of the research were samples taken from the surfaces of production facilities and technological equipment at different sites of a meat processing plant (MPP) and poultry processing plant (PPP). BF samples (Table 1) were taken by scraping off using a metal spatula from objects of production environment before the daily disinfection procedure.

The samples obtained were immediately placed into the sterile physiological solution for microbiological investigations and in the solution of glutaraldehyde in cacodylate buffer for investigation by electron microscopy.

Microbiological analysis of the samples

To destroy the biofilms' matrix, 1 cm^3 of BFR-enzymofilm (BFR Laboratories, Russian Federation) was added to each taken sample. After 20-min exposition, 0.1 cm^3 of suspension was applied to the surface of selective solid nutrient substrates: HiCrome Coliform agar (HiMedia Laboratories Pvt. Ltd., India), Yeast Glucose Chloramphenicol Agar (YGC agar) (Merck KGaA, Germany), Pseudomonas Cetrimide agar (OXOID, UK) and non-selective nutrient tryptic soy agar (TSA) (Merck KGaA, Germany). Plates with HiCrome Coliform agar were incubated at 37 °C for 24 hours, plates with Cetrimide agar and TSA at 30 °C for 72 hours, plates with YGC agar at 24 °C for 5 days. Incubation was carried out in thermostats (Binder, Germany). After incubation, colonies were transferred to non-selective nutrient agar. Species identification of isolated colonies was carried out on the MALDI-TOF-MS instrument using a mass spectrometer Autof MS 1000 (Autobio Diagnostics, China). To this end, bacterial mass of colonies was placed on a plate and dried at room temperature. Then, 1.2 µl of formic acid was introduced into each well with dried bacterial mass for 10 min and dried; after that, 1.2 µl of HCCA (α-cyano-4-hydroxycinnamic acid, 99 %) matrix was applied, and then dried again. The plate was placed into the instrument and the equipment for identification of microorganisms was started up using the program FlexControl. Results were analyzed using the software: if the value was lower than 6.0, a result was considered unreliable and was not used in the subsequent work. A result was considered reliable and was taken into account at values of 6.0–9.0 at the genus level and 9.0–9.5 at the species level.

Table 1. Identification of samples and points of sampling

No. of sample	Zone	Object for sampling
	MPP	
1	Carcass storage chamber (a temperature of +1.8 °C)	Drain in the floor
2	Raw material workshop, site of deboning (a temperature of +4 °C)	Mechanism of the container lifting device (bottom surface)
3		Lubricant from conveyor
4		Drain in the floor
5		Wet sealant peeled away from the wall
6		Internal surface of a frame for transporting boxes
7		Wheels of hydraulic trolley
8	Accumulator of comminuted raw materials (a temperature of +2 °C)	Ice with dirt from the floor
	PPP	
1	Workshop of poultry slaughter and primary processing	Ceiling
2		Bottom side of the planked footway of the stairs to the Morris water chiller
3		Ceiling near the Morris water chiller
4	Chilling tunnel (a temperature of 0 °C)	Wall in the carcass chilling tunnel
5		Plastic (Teflon) roller under the conveyor chain
6	Packaging site	Plastic (Teflon) wheels of a trolley at the beginning of the site
7		Plastic (Teflon) wheels of a trolley at the end of the site

Microscopic analysis

The structural organization of the samples was studied by TEM using ultra-thin sections. After taking a sample, it was placed in the 2.5 % solution of glutaraldehyde in cacodylate buffer (0.05 M sodium cacodylate solution, pH 7.0–7.5) and held at 4 °C for a day; then, it was washed three times with the same buffer solution over 5 min each time and fixed in the OsO_4 solution (1 % OsO_4 + 0.7 % ruthenium red solution in cacodylate buffer) at a temperature of 4 °C for 1.5 hours. After fixation, the samples were placed in 2 % agar-agar and held in succession in the 3 % uranyl acetate solution, then in 30 % ethanol for 4 h, and then in 70 % ethanol at a temperature of 4 °C for 12 h. The material was dehydrated in 96 % ethanol (2 times for 15 min. each time), then in absolute acetone (3 times for 10 min. each time). The samples were impregnated with EPON-812 resin (Epoxy Embedding Medium Epon® 812, Sigma-Aldrich, USA), and held in the mixture of resin: acetone in a 1:1 ratio for 1 h and then in a mixture of resin: acetone in a 2:1 ratio for 1 h. The obtained material was poured into capsules with resin and polymerized at a temperature of 37 °C for a day and then at a temperature of 60 °C for another day. Ultrathin sections were cut with a LKB-III microtome (LKB, Sweden) and contrasted in a 3 % aqueous uranyl acetate solution (30 min), then in a 4 % aqueous lead citrate solution (30 min). The ruthenium red stain (Sigma, USA) was used to detect acid mucopolysaccharides in BFs; ruthenium red stain was added in an amount of 0.7 % with OsO_4, with which it interacted. Using ruthenium red, the presence of extracellular polysaccharides in BFs of several bacteria was shown. The obtained preparations were analyzed using a JEM 100SHP electron microscope (JEOL, Japan) at accelerating voltage of 80 kV and operating magnification of 5000–50,000. Photo documentation of the materials was carried out with the use of the Morada G2 digital optical imaging system (Olympus Soft Imaging Solutions, Japan).

Results

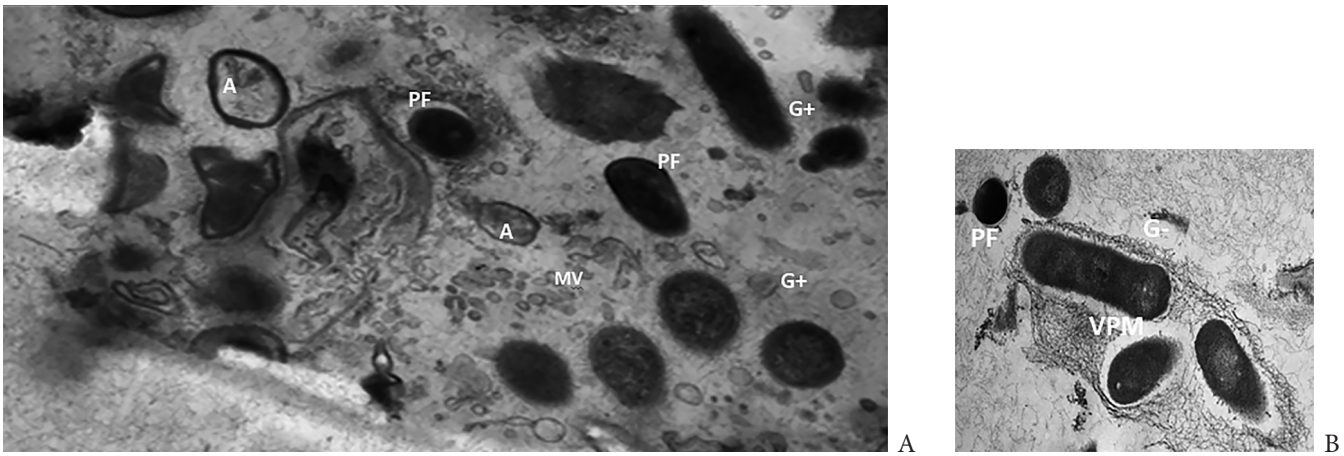
Identification and characterization of biofilms

For direct detection and characterization of biofilms, transmission electron microscopy was performed for the samples taken at the MPP and PPP.

At the MPP, eight samples from surfaces of production facilities and technological equipment (Table 1) were taken and analyzed. The presence of biofilms was confirmed only in two samples out of eight taken samples. Biofilms were detected in sample No. 2 taken from the bottom surface of the mechanism of the container lifting device and in sample No. 7 taken from wheels of hydraulic trolley. Sample No. 2 was represented by a well-formed biofilm (Figure 1). The biofilm was characterized by high morphological diversity of cells and their age heterogeneity: old cells of the Gram-negative type with inclusion of polyhydroxybutyric acid (type I); young Gram-positive, the so-called "hairy" cells with multiple pili (type II); rods with rounded ends (type III) (Figure 1A); large (~2 μm) Gram-negative cells with a well-pronounced periplasmic space (Figure 1B). Extracellular polysaccharide matrix (EPM) was well formed and included particles of organic matter and multiple membrane vesicles.

Sample No. 7 taken from the wheel of the hydraulic trolley was represented by a well-formed laminated biofilm, which consisted mainly of old cells with inclusions of polyhydroxybutyric acid (Figure 2) of two morphotypes differed by size: Gram-positive rods (~0.5 × 1.5 μm) and coccoid Gram-negative cells (~0.8 × 1.3 μm) growing as microcolonies; the microgranular matrix of the carbohydrate nature and membrane vesicles mainly linked with lyzed microcolonies of Gram-negative cells are clearly seen.

Other samples were represented by aggregates of organic and inorganic material and muscle cells of meat. With that, bacteria were either completely absent or presented in small amounts and were located individually. For example, sample No. 1 taken from the wall of the sewage drain in the storage chamber for porcine carcasses was represented



A B

Figure 1. A) Electron microscopy image of thin sections of the sample taken from the bottom surface of the mechanism of the container lifting device (raw material workshop). B) — microcolonies of Gram-negative cells surrounded by a fibrillar matrix of carbohydrates. Designations: **A** — autolyzed cells, **G-** — Gram-negative cells, **G+** — Gram-positive cells; **MV** — membrane vesicles, **PF** — resting cells, VPM — fibrillar extracellular polysaccharide matrix; **P** — pili. The scale bar is 1 micron

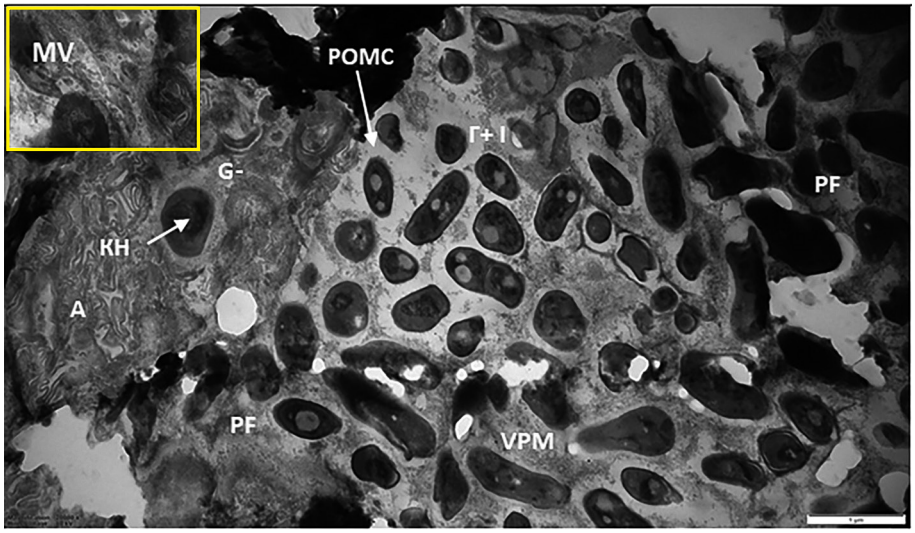


Figure 2. Electron microscopy image of thin sections of the sample taken from the wheels of the hydraulic trolley (raw material workshop). Designations: **POMC** — polyhydroxybutyric acid, **G-I** — old Gram-negative cells with a compacted nucleoid (KN), **A** — autolyzed cells, **VPM** — fibrillar extracellular polysaccharide matrix, **MV** — membrane vesicles. The scale bar is 1 micron

by aggregates of organic slimy material. A typical biofilm was not found. In sample No. 3 (lubricant from conveyor), only particles of organic material, individual cells and fat droplets were found. Aggregation of slimy material with inclusion of individual bacterial cells was detected in sample No. 4 taken from the wall of the sewage drain in the raw material workshop. Sample No. 5 (piece of sealant from the wall) did not contain the organic material. As the presence of slimy or paste-like organic material is one of the most important (significant) properties of biofilms, it was concluded that they were absent in this sample.

A mass of slimy organic material, in which bacterial cells were not detected, was found in sample No.6 taken from the internal surface of the frame for transporting boxes. Sample No. 8 taken from the floor (ice with dirt) in the workshop of accumulation of comminuted raw materials, represented fragmented muscle fibers and myocyte nuclei, bacterial cells were practically absent. Electron microscopy images, in which biofilms were not detected, are not presented in this paper.

At the PPP, seven samples from surfaces of production facilities and technological equipment (Table 1) were taken and analyzed. Among seven samples, six were represented by biofilms of different degree of maturity. Only sample No.5, taken from a Teflon roller of the conveyor in the chilling tunnel did not contain biofilm (electron microscopy image is not presented).

Old functionally inactive cells with the absence of vegetative forms of bacterial cells were revealed in three samples. These samples included: sample No. 1 taken from the ceiling in the workshop of poultry slaughter and primary processing; sample No. 2 taken from the bottom side of the planked footway of the stairs near the Morris water chiller; sample No.3 taken from the ceiling above the Morris water chiller.

Sample No. 1 represented the classic mature multispecies biofilm (Figure 3).

It can be assumed that conditions for BF formation are abundance of condensate and the surface of the room that is poorly sanitized. In this sample, the biofilm is characterized by the multilayer nature (zonality): in zone A (Figure 3) Gram-positive bacteria (G+) are present, in zone C — mainly Gram-negative bacteria, while a zone of organic material (B) is between them. In the sample, organic particles of different density, multiple membrane vesicles (MV) and dormant (resting) forms (PF) of Gram-positive and Gram-negative bacteria are seen; respectively, the first ones (PF I) are electron-dense, thick-walled, while the second ones (PF II) are small, moderately electron-dense and are surrounded by pili. Also, autolyzed cells (A) are

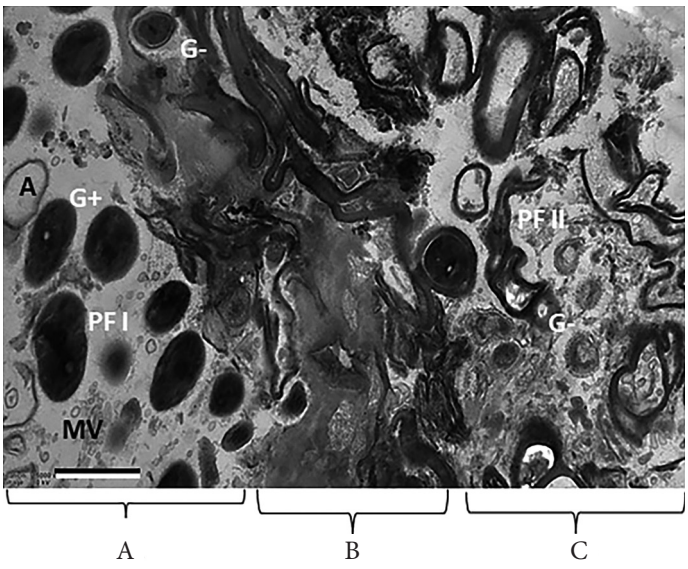


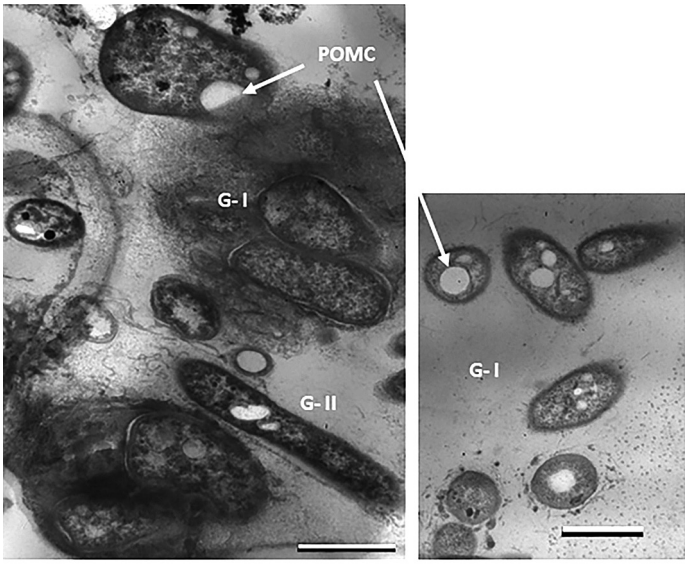
Figure 3. Electron microscopy image of thin sections of sample No. 1 taken at the PPP from the ceiling in the workshop of poultry slaughter and primary processing. Designations: **PF I** — resting forms, morphotype I; **PF II** — resting forms, morphotype II; **A** — autolyzed cells, **MV** — membrane vesicles. **G+** and **G-** are Gram-positive and Gram-negative cells, respectively. Three layers of the sample (A, B, C) are indicated below. The scale bar is 1 micron

present. The absence of vegetative forms of bacterial cells allows for characterizing this biofilm as inactive.

Sample No. 2 was taken from another uncommon sampling point from the bottom side of the planked footway of the stairs to the Morris water chiller. The sample represented a mature BF containing non-dividing (old) vegetative cells with abundant inclusions of polyhydroxybutyric acid (Figure 4) of several morphotypes: Gram-negative ovoid cells, often growing in the common capsules, and long rods.

In sample No. 2, autolyzed cells that differed in the structure organization of membranes were found.

The results of the electron microscopy analysis of sample No.3 taken from the ceiling near the Morris water chiller in the workshop of poultry slaughter and primary processing are presented in Figure 5.



A B

Figure 4. Electron microscopy image of thin sections of the sample taken from the bottom side of the planked footway of the stairs to the Morris water chiller in the workshop of poultry slaughter and primary processing.
A, B — old Gram-negative cells of two morphotypes (**G-I** and **G-II**). The scale bar is 500 nm.

In sample No. 3, an old, probably, functionally inactive BF consisting of old cells (Figure 5A) embedded into the matrix that was heterogeneous in terms of staining with ruthenium red was found, as well as sites with laminated, apparently calcified structure without cells. Also, thick-walled electron-dense dormant forms clustered in groups (microcolonies) were found.

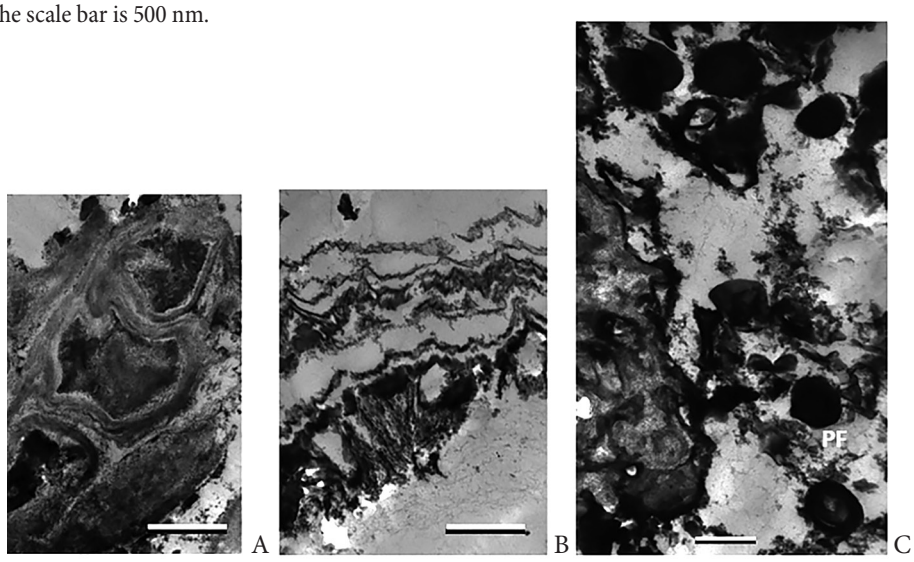
In samples No. 4, No. 6 and No. 7, biofilms with vegetative forms of cells were observed. Electron microscopy image of sample No. 4 taken from the wall in the carcass chilling tunnel is presented in Figure 6.

Temperature in the carcass chilling tunnel, as a rule, is not higher than 0 °C; therefore, the sample represented a frozen ice mass. Despite this, a laminated biofilm was found in it. This biofilm included zones of a typical biofilm with the matrix characterized by abundance of G- cells of a single morphotype, old with bio-crystalized nucleoid, cloddy texture of the cytoplasm, and zones of loose organic material unevenly stained with ruthenium red. Apparently, the biofilm was formed by a psychrophilic microorganism.

Sample No. 6 taken from the wheels of a trolley at the beginning of the packaging site (Figure 7) was represented by a heterogeneous material, part of which can be identified as a biofilm with the developed loose organic material, matrix that was unevenly stained with ruthenium red, in which the bacterial component was represented by cells of different morphotypes in terms of cell wall structure and with pili.

Cells were surrounded by capsular material of the non-polysaccharide nature, membrane vesicles were present (Figure 7A). Several sections contained individual cells without signs of the biofilm phenotype (polymer matrix and membrane vesicles, MV) (Figure 7B).

Sample No. 7 was taken from the wheels of a trolley at the end of the packaging site. Its composition was similar to sample No. 6: it contained two types of zones: biofilms and organic substance with individual cells (Figure 8).



A B C

Figure 5. Electron microscopy image of thin sections of the sample taken from the ceiling near the Morris water chiller:
A — a chain of cells surrounded by layered capsules and organic material; B — a multi-layered alternation of organic and probably mineral material. The scale bar is 500 nm

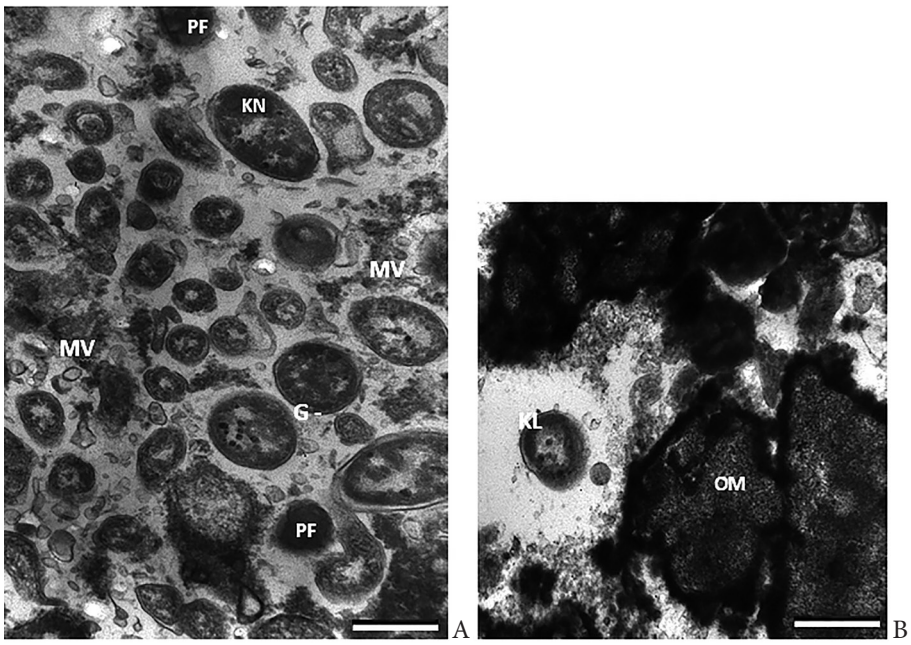


Figure 6. Electron microscopy image of thin sections of the sample taken from the wall in the carcass chilling tunnel: A — a monoid film of Gram-negative bacteria (**G -**), old with a compacted nucleoid (**KN**) and membrane vesicles (**MV**); B — a zone of loose organic material (**OM**) with single cells (**Kl**). The scale bar is 0.5 microns in A, and 1 micron in B

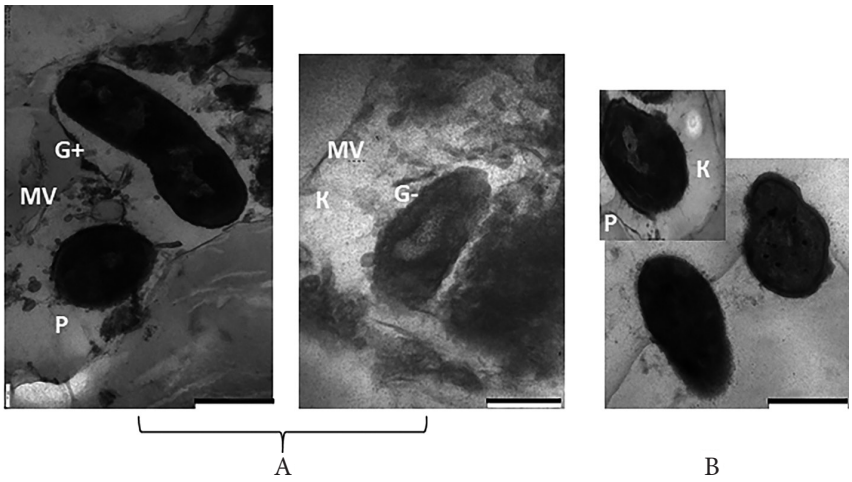


Figure 7. Electron microscopy image of thin sections of the sample taken from the wheels of a trolley at the beginning of the packaging site: A — individual cells with a biofilm phenotype of two morphotypes (**G+** and **G-**); B — individual cells of the G+ type cell. Designations: **MV** — membrane vesicles, **K** — capsule, **P** — pili. The scale bar is 0.5 microns

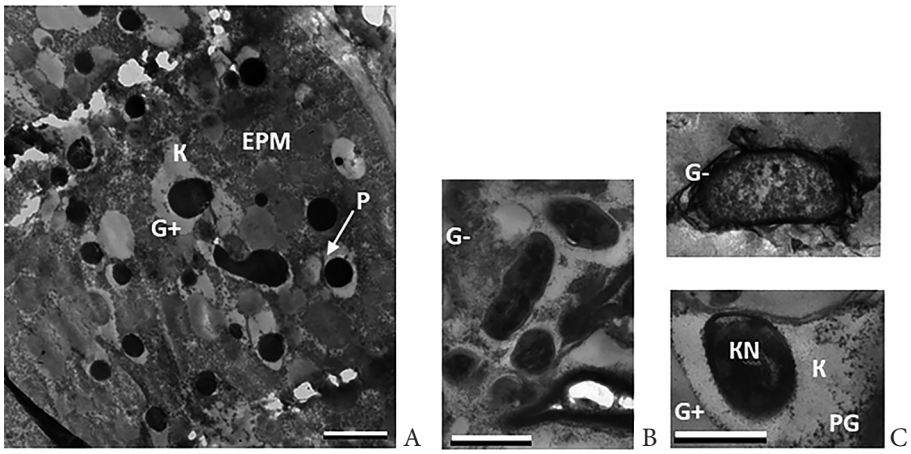


Figure 8. Electron microscopy image of thin sections of the sample taken from the wheels of a trolley at the end of the packaging site: A — old BF, with a dense matrix (EPM) of the polysaccharine nature, Gram–positive cells with short pili (**P**), some surrounded by a capsule of the non-polysaccharide nature (**K**); B — Gram-negative cells surrounded by capsules and matrix; C — Individual cells from the zone without biofilms: a resting cell of a Gram-positive bacterium (**G+**) with a compacted nucleoid (**KN**) surrounded by a two-component capsule of non-polysaccharide material (**K**) and polysaccharide microgranules (**PG**), a vegetative cell of the Gram-negative type (**G-**). The scale bar is 1 micron

Figure 8A presents a mature biofilm with the well-developed granular matrix that include Gram-positive cells with short pili; most cells are in the dormant state. Vegetative cells of the Gram-negative type were found in certain few zones (Figure 8B). The zone, where a biofilm was not detected (Figure 8C), showed both active bacterial cells and dormant forms with the thickened cell membrane and bio-crystalized nucleoid surrounded by a capsule of the non-polysaccharide nature separated from EPM by a layer formed by granular polysaccharide particles.

Results of the microbiological investigation of the samples at MPP and PPP

Comparative analysis of microbiota of the samples taken from the surfaces of the meat processing plant (MPP) and poultry processing plant (PPP) was carried out by classic microbiological methods with the following identification of colonies by the mass spectrometric analysis (MALDI-TOF MS). Microbiological investigation enabled establishing the presence and structure of biofilms (BFs) in the samples, which makes it possible to conduct the correlation analysis between the taxonomic composition and biofilm forming ability of microorganisms in the production conditions. Significant differences between two plants were revealed both in terms of the frequency of occurrence of biofilms and the structure of microbial communities. The composition of microflora of the samples taken at the MPP is presented in Table 2.

At the MPP, the presence of structurally organized biofilms was confirmed only in two samples (25 % of samples), which is in contrast with the total high level of microbial contamination and corresponds to the data about complex and heterogeneous ecology of microbial communities in such production facilities. Biofilms were detected exclusively in the samples taken from hard-to-clean mobile equipment used in the zones with high organic load, which emphasizes the role of such "hidden" niches as key reservoirs of microbial persistence. It is necessary to note that according to literature data microorganisms isolated from these points have the high biofilm forming ability [12,13]. In biofilm sample No. 2 taken from the bottom surface of the mechanism of the container lifting device in the raw material workshop, a consortium was revealed that was represented by yeast *Candida zeylanoides* and bacteria of the genus *Enterococcus* (*E. faecalis, E. malodoratus*). It is known that representatives of the genus *Candida*, including *C. zeylanoides*, actively form mono-species biofilms and *Enterococcus faecalis* is an opportunistic pathogen and classic biofilm former, which is distinguished by high tolerance to external impacts [14,15]. In biofilm sample No. 7 taken from the wheels of hydraulic trolley, the microbial community was formed mainly by Gram-negative microflora: *Pseudomonas brenneri, P. extremorientalis, P. veronii* and *Serratia fonticola*. The genus *Pseudomonas* is one of the key biofilm formers in the food industry [16], while *Serratia fonticola* also demonstrates a pronounced biofilm forming potential [17]. The presence of members of these genera in one sample creates conditions for formation of a complex multi-species (poly-microbial) biofilm, in which synergetic interactions can enhance the resistance of the whole community to sanitary treatments. Electron microscopy analysis of these samples demonstrated the presence of mature multi-layer biofilms with pronounced extracellular polymeric matrix containing cells of different morphological types and ages, which is a direct confirmation of the realization of the biofilm forming potential of these microorganisms and indicates a long-time process of formation and stability of these microbial communities.

The samples, in which biofilms were not revealed, are also of significant interest demonstrating another structure of microbial community. In sample No. 1 (drain in the floor of the storage chamber) and sample No.4 (drain in the floor of the raw material workshop), microorganisms either were not detected or were represented by few species

Table 2. Results of the microbiological analysis of the samples from the surfaces of the meat processing plant

№	Zone	Object for sampling	Presence of biofilm	Microorganisms
1	Carcass storage chamber (a temperature of +1.8 °C)	Drain in the floor	Not detected	—
2	Raw material workshop, site of deboning. (a temperature of +4 °C)	Mechanism of the container lifting device (bottom surface)	Detected	*Candida zeylanoides, Enterococcus faecalis, Enterococcus malodoratus*
3		Lubricant from conveyor	Not detected	*Escherichia coli, Raoultella terrigena*
4		Drain in the floor	Not detected	*Pseudomonas brenneri, Pseudomonas gessardii*
5		Wet sealant peeled away from the wall	Not detected	*Serratia fonticola*
6		Internal surface of a frame for transporting boxes	Not detected	*Candida parapsilosis, Candida zeylanoides, Enterococcus devriesei, Pseudomonas frederiksbargensis, Pseudomonas gessardii, Pseudomonas kilonensis*
7		Wheels of hydraulic trolley	Detected	*Pseudomonas brenneri, Pseudomonas extremorientalis, Pseudomonas veronii, Serratia fonticola*
8	Accumulator of comminuted raw materials (a temperature of +2 °C)	Ice with dirt from the floor	Not detected	*Aerococcus viridans, Alcalagenes faecalis, Pseudomonas gessardii*

(*Pseudomonas* spp.). Microscopy showed only individual cells, which can indicate the constant hydrodynamic loads or the absence of stable conditions for consolidation of a community. Sample No. 3 (lubricant from the conveyor) containing *Escherichia coli* and *Raoultella terrigena*, as well as sample No. 5 (sealant) with *Serratia fonticola* did not show the biofilm structure, possibly, due to the chemical composition of the substrate or other limiting factors that prevent adhesion and production of matrix. The most indicative is sample No.6 (internal surface of a frame of the trolley), which showed maximum taxonomic diversity, including yeasts, *Enterococcus* and three species of *Pseudomonas*. Despite the high biofilm forming potential of the isolates, a mature biofilm was not formed, which can be explained by the periodical drying of the surface, mechanical impact or competition within the heterogeneous community that prevented cooperation. Sample No. 8 (ice with dirt) represented an abiotic agglomerate of the material, where low temperature and, possibly, short time of existence did not allow for the development of a microbial community. These data highlight that the presence even of a significant number and diversity of microorganisms, including well-known biofilm formers, is a necessary but not sufficient condition for formation of structured biofilm, which is dramatically influenced by the local environmental conditions.

The composition of the microflora of the samples taken at the PPP is presented in Table 3.

Seven samples were investigated at the poultry processing plant (PPP) and mature biofilms were revealed in six of them (~86 %), which indicates systemic and widespread occurrence of biofilm communities at this production facility. The taxonomic composition of the samples at the PPP was characterized by the pronounced domination of the members of the genus *Pseudomonas*, which were isolated from all seven sampling points with *Pseudomonas gessardii* detected most frequently.

In sample No. 2 (bottom side of the planked footway of the stairs to the Morris water chiller) and sample No.4 (wall in the carcass chilling tunnel), members of the genus *Yersinia* (*Yersinia* spp. and *Y. enterocolitica*, respectively), as well as *Serratia* spp. were identified besides pseudomonades. *Yersinia enterocolitica* is able to form biofilms including at low temperatures, which is an important factor of its survival in the production conditions and is a significant problem for food safety [18]. The presence of these pathogens in the composition of complex poly-microbial communities that include active producers of extracellular matrix, such as pseudomonades, can significantly increase their resistance to sanitary treatments [19].

Samples No. 1 (ceiling in the slaughter workshop) and No. 3 (ceiling near the Morris water chiller) taken from the ceilings in the areas with high moisture contained communities which besides *Pseudomonas* spp. included bacteria of the genera *Kocuria* and *Staphylococcus*, as well as yeasts *Candida zeylanoides*. An ability of coagulase-negative staphylococci and yeasts to adhesion and biofilm formation complements the general potential of such consortia to colonize the surfaces [20]. Samples No.6 and No. 7 taken from plastic wheels of trolleys at different points of the package site demonstrated higher taxonomic diversity at the PPP, including *Enterobacter cloacae*, various species of *Pseudomonas, Serratia* and *Microbacterium*. This composition is typical of complex multi-species biofilms on mobile equipment, which acts as a vector of cross-contamination transmitting microflora between zones.

Table 3. Results of the microbiological analysis of the samples from the surfaces of the poultry processing plant

No. of sample	Zone	Object for sampling	Biofilm	Microorganisms
1	Workshop of poultry slaughter and primary processing	Ceiling	Detected	*Fictibacillus arsenicus, Kocuria rosea, Pseudomonas lundensis, Staphylococcus saprophyticus*
2		Bottom side of the footway of Morris water chiller	Detected	*Hafnia alvei, Lelliottia amnigena, Pseudomonas gessardii, Raoultella ornithinolytica, Serratia liquefaciens, Yersinia spp*
3		Ceiling near the Morris water chiller	Detected	*Candida zeylanoides, Kocuria polaris, Pseudomonas lundensis, Pseudomonas koreensis*
4	Chilling tunnel (a temperature of 0 °C)	Wall in the carcass chilling tunnel	Detected	*Pseudomonas gessardii, Pseudomonas grimontii, Pseudomonas libanensis, Serratia fonticola, Yersinia enterocolitica*
5		Plastic roller of the conveyor (at the top) in the chilling tunnel	Not detected	*Carnobacterium maltaromaticum, Lelliottia amnigena, Pseudomonas corrugata, Pseudomonas gessardii, Raoultella ornithinolytica, Serratia fonticola*
6	Packaging site	Plastic wheels of a trolley at the beginning of the workshop	Detected	*Enterobacter cloacae, Kocuria carniphila, Pseudomonas extremorientalis, Pseudomonas gessardii, Pseudomonas koreensis, Serratia proteamaculans*
7		Plastic wheels of a trolley at the end of the workshop	Detected	*Microbacterium liquefaciens, Pseudomonas brenneri, Pseudomonas gessardii, Pseudomonas libanensis, Pseudomonas tolaasii*

The only sample where a mature biofilm was not detected was sample No. 5 from the plastic roller from the conveyor in the chilling tunnel. Despite the presence in it of various and potentially psychrotrophic microflora (*Carnobacterium maltaromaticum, Lelliottia amnigena, Pseudomonas* spp., *Serratia fonticola*), structured biofilm community was not formed. This can be linked with physical properties of the Teflon surface, constant mechanical movement that prevented adhesion or other local conditions limiting consolidation of a biofilm even in the presence of suitable microorganisms.

Discussion

The conducted complex study that combined classic microbiological methods, MALDI-TOF mass spectrometry, and transmission electron microscopy (TEM) enabled not only identification of the taxonomic composition of microbiota of two production types, but also, for the first time, visualization of the structural organization of microbial communities at meat processing and poultry processing plants.

Comparative analysis revealed significant differences in the prevalence of mature biofilms: 85.7 % at the PPP versus 25 % at the MPP. This imbalance cannot be explained only by differences in the taxonomic composition as the basis of microbiota in both plants was formed by Gram-negative bacteria of the genera *Pseudomonas* and *Serratia*, for which the ability to active biofilm formation was well documented [21].

Results of the TEM analysis suggest that the combination of abiotic factors is of critical importance for the development of biofilms. At the PPP, mature biofilms were formed mainly in the zones with abundant and constant formation of condensate (ceilings, walls of chilling tunnels), which agrees with the data about the role of moisture as a key driver of biofilm formation [22]. At the MPP, both developed biofilms were located on the mobile equipment (wheels of trolleys, lifting devices), where significant moistening, presence of organic substrate and lowered frequency of mechanical cleaning are, apparently, combined. This emphasizes a role of "hidden" niches as main reservoirs of microbial persistence [23].

Detailed TEM analysis of the biofilm structure revealed their significant heterogeneity, which reflects different stages of maturity and local micro conditions. In sample No.1 from the PPP (ceiling from the slaughter workshop), a multi-layer biofilm consisted practically completely from Gram-positive and Gram-negative dormant forms with complete absence of vegetative cells. This research is in agreement with the results of other studies and corroborates the existence of inactive or dormant biofilms in the conditions of food production facilities, which are not always revealed by standard culture methods, but serve as a long-term reservoir for resumption of contamination when conditions are changed [23]. Detection of dormant forms of microorganisms in the samples from the MPP explains their persistence even after regular disinfections as the dormant state ensures phenotypic resistance to a wide spectrum of stressors [24].

Structural organization of the extracellular polymer matrix (EPM) was also variable and correlated with community's maturity. In the mature biofilms, such as those on the wheels of the hydraulic trolley (MPP, No. 7) and the walls of the chilling tunnel (PPP, No. 4), the dense, well-developed granular or fibrilar matrix was well visualized. This confirms its polysaccharide structure and indicates a high level of cooperation inside the community [25]. In the less mature samples (PPP, No. 6 and No. 7), only rudimentary capsular structures around individual cells were observed, which corresponds to the early stages of colonization. The abundance of membrane vesicles in the matrix, especially in the samples with domination of pseudomonades (MPP, No. 2; PPP, No. 1 and No.3) is an important observation. Membrane vesicles play a key role in intercellular communication, transfer of spoilage enzymes (lipases, proteases) and virulence factors as well as in neutralization of disinfectants, which directly facilitates survival and pathogenicity of the whole consortium [26].

Special attention deserves the detection of the zones with laminated, apparently, calcified structure in sample No. 3 from the PPP (ceiling near the Morris water chiller). This can indicate the matrix mineralization processes in old biofilms under the action of aggressive sanitary agents. Such calcification is described in the literature as a final stage of biofilm existence or as a mechanism of passive defense from biocides, when the mineral layer serves as an additional physical barrier [27]. This explains why eradication of such old depositions requires not only chemical disinfection, but also intensive mechanical cleaning.

Analysis of the taxonomic composition in correlation with TEM data allowed for establishing key factors of biofilm formation. In both plants, psychrotrophic *Pseudomonas* spp. dominated, which corroborates their role as the main agents of chilled meat and poultry spoilage [28,29]. Detection of *Pseudomonas gessardii*, *P. lundensis* and *P. brenneri* in all samples with biofilms confirms their adaptation to low temperatures and ability to quickly colonize surfaces [30]. Of critical importance is concomitant detection in mature biofilms of *Pseudomonas* spp. and pathogens such as *Yersinia enterocolitica* in sample No. 4 (PPP) and *E. faecalis* in sample No.2 (MPP). Pseudomonades producing abundant matrix provide a protective niche for less efficient biofilm formers, which was showed many times for poly-microbial biofilms with *Listeria monocytogenes* and *Salmonella* at food enterprises [31]. This synergy was morphologically confirmed in sample No.4, where *Yersinia* cells were submerged into the extensive matrix formed mainly by pseudomonades. Detection of *Y. enterocolitica*, a well-known psychrotrophic pathogen capable of biofilm forming at low temperatures, in the chilling tunnel presents a serious risk for food safety [18].

Comparison of the samples with biofilms and without them revealed a negative correlation with taxonomic diversity. The most heterogeneous samples in terms of the composition (for example, No. 6 from the MPP) did not demonstrate a mature biofilm according to the TEM data. This can be explained by the antagonistic interactions and strong competition in immature community that prevent cooperation [32]. At the same time, mature stable biofilms were characterized by dominance of a small group of species — "bio-engineers" capable of the cooperative synthesis of the matrix, which confirms the theory of the succession dynamics of microbial communities [33].

Detection of indicator microorganisms (*E. coli*) and opportunistic pathogens (*E. faecalis*, *Yersinia* spp.) in the composition of biofilms in non-obvious points can indicate a systemic problem of cross contamination. Wheels of trolleys act as a mechanical vector transmitting microorganisms from raw material zones to the zones with finished products. Walls and ceilings of chilling tunnels with condensate and a mature biofilm with *Y. enterocolitica* represent a constant aerosol risk. The data obtained actualize the need for revision of the standard points of sanitary control and inclusion of "hidden" niches into them, as well as validation of disinfectants precisely against biofilms and not planktonic cultures.

Conclusions

The conducted complex investigation of biofilms on the surfaces of the meat processing plant (MPP) and poultry processing plant (PPP) enabled making several important conclusions. Analysis shows that biofilms are a widespread but heterogeneous phenomenon in these production conditions. At the poultry processing plant, mature biofilms were detected in the vast majority of samples (86 %, or 6 out of 7 samples), while at the meat processing plant they were found only in 25 % of cases (in 2 out of 8 samples). This indicates a higher risk of formation of stable microbial communities in the specific conditions of the poultry processing plant.

Key factors facilitating biofilm formation were the presence of moisture (condensate) and hard-to-reach surfaces. With that, low positive temperatures (0...+4 °C) did not become a limiting factor for their formation. The most problematic zones turned to be mobile equipment (wheels of trolleys, frames), bottom sides of mechanisms (lifting devices, planked footway), sites of the condensate development on ceilings and walls of chilling tunnels. The structured communities with the developed extracellular polymer matrix were developed precisely at these sites.

The taxonomic composition of microbiota at both plants was characterized by dominance of Gram-negative bacteria, first of all, multiple species of the genus *Pseudomonas* (*P. gessardii, P. brenneri, P. lundensis* and others), which are classic and strong biofilm formers adapted to low temperatures. At the MPP, *Acinetobacter*, *Serratia fonticola*, enterococci (*Enterococcus faecalis*) and yeasts *Candida zeylanoides* also played a significant role. At the PPP, a spectrum of isolated microorganisms was wider and included besides pseudomonades the members of the genera *Yersinia* (including *Y. enterocolitica*), *Serratia*, *Kocuria*, *Carnobacterium*, *Hafnia* and *Lelliottia*.

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DIETARY CINNAMON AND CLAY EFFECTS ON GROWTH, MEAT QUALITY, AND BONES IN BROILERS

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Keywords: *broiler, cinnamon, clay, growth, meat, bone, quality*

Abstract

This study aims to investigate the effects of a diet supplementation of a mixture of cinnamon powder and clay (kaolin or marl) on growth performance, carcass traits, meat quality, and tibial morphometric characteristics of broiler chickens. A total of 120 broiler chicks were randomly assigned to three dietary treatment groups: control (CON), a mixture of 0.3 % cinnamon powder and 2.7 % kaolin (KCP), and a blend of 0.3 % cinnamon powder and 2.7 % marl (MCP), with five replicates of eight birds per treatment group. The results revealed that body weight, weight gain, and feed conversion ratio were significantly higher in birds receiving KCP or MCP mixture than in birds receiving CON ($p < 0.05$). Additionally, these groups exhibited superior carcass traits, including increased dressing percentage, higher breast and thigh weights, and reduced abdominal fat. The pH values at 24 h were higher in the KCP and MCP groups compared to the CON group. Additionally, these treatments resulted in a significant reduction in drip and cooking losses, indicating improved water-holding capacity and enhanced bone health in the tibia. Therefore, it was inferred that a combination of cinnamon powder and silicate minerals is a viable alternative to antibiotic growth promoters in broiler diets to improve growth performance, carcass traits, meat quality, and bone quality.

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Introduction

In recent years, the use of antibiotics as growth promoters in poultry has been restricted due to growing concerns about the development of antibiotic resistance and transfer of antibiotic residues in poultry products, which can cause negative effects on human health and food safety [1,2]. Therefore, different types of natural additives, such as phytogenics, also referred to as phytobiotics or phytochemicals, began to be included in poultry feed as a replacement for growth promoters to improve broiler health and production performance [3,4]. In addition, various studies demonstrated that the inclusion of clay as a natural additive to broiler diets can help improve production performance and product qualities [5].

Kaolin and marl are used as natural additives in broiler diets to enhance growth performance and carcass quality [6], due to the digestive properties, detoxifying capacity, and antibacterial effects of clay [7,8]. Kaolin is a type of clay containing a large proportion of kaolinite [$Al_2Si_2O_5(OH)_4$] particles (about 95 % of pure kaolinite), some quartz grains, and some traces of illite [9]. Some studies have shown that adding kaolin to broiler feeds improves feed intake, feed conversion ratio, and body weight gain [10,11], as well as bone characteristics [12]. Marl is a type of clay containing 35–65 % clay materials and 65–35 % carbonates [13]. Some studies have shown that adding 3 % marl to broiler feed significantly increases slaughter weight [14] and reduces abdominal fat by 5 % [15].

Cinnamon is an aromatic and ethnomedicinal herb belonging to the Lauraceae family. Cinnamon (*Cinnamomum cassia*) contains multiple phytochemicals, including cinnamaldehyde, camphor, caryophyllene, eugenol, and many other valuable bioactive substances [16]. Consequently, it has been reported that cinnamon can exert antifungal [17], antiviral [18], and antioxidant effects [19], as well as enhance digestive enzyme secretion, growth performance, and product quality in poultry [20]. Several studies have examined the effect of supplementing broiler feed with cinnamon powder alone and showed that it can improve growth performance and blood metabolites [21,22].

Despite the promising individual benefits of cinnamon and clay, no study has investigated the effectiveness of cinnamon powder combined with kaolin or marl on the production performance of broilers. Therefore, the present study

aimed to evaluate the effect of cinnamon powder combined with kaolin or marl on the growth performance, carcass traits, and tibial bone characteristics of broiler chickens.

Objects and methods

The experiment was conducted at the research station and in the Laboratory of Health, Animal Production and Environment, Institute of Veterinary and Agronomic Sciences at Batna 1 University, Algeria, from September to October 2025. Dry cinnamon bark was purchased from a local supermarket in Batna, Algeria, transported to the laboratory, and then ground using a grinder (Bomann, Germany) to a fine powder. Clays used in this study were white kaolin with a brown-colored surface and grey marl, abundant in the Aures region (Batna, Algeria). Kaolin contains 64 % kaolinite, 25 % micaceous materials and other clays, 8 % quartz and 3 % feldspar [12]. Marl contains 68 % clay, 13 % sand and a low rate of organic matter [14]. One hundred twenty (120) one-day-old mixed-sex broiler chicks (Cobb 500) were procured from a private hatchery.

Experimental design

On arrival, the chicks were weighed individually using a digital scale (OHAUS, USA), and then randomly assigned equally to 15 cages. They were then randomly allocated to three dietary treatments, each of which was replicated five times, with eight birds per replicate. The dietary treatments compared were: basal diet without any supplementation (control); basal diet supplemented with a mixture of cinnamon powder (3000 mg/kg feed) and kaolin (27000 mg/kg feed); and basal diet supplemented with a mixture of cinnamon powder (3000 mg/kg feed) and marl (27000 mg/kg feed). The starter (1–21 days) and finisher (22–49 days) experimental diets were formulated according to the recommendations of the National Research Council [23]. Throughout the experiment, the birds had *ad libitum* access to their respective diets and water. The ingredients percentage and calculated nutritional composition of the experimental broiler diets are listed in Table 1.

Growth performance

Weekly measurements of body weight and feed intake (FI) of the chickens were recorded, and at the end of the experiment, body weight gain (BWG) and feed conversion ratio (FCR) were calculated. BWG was determined by calculating the difference between the initial weight and the final weight of the birds in each replicate. FI was calculated by subtracting the residual feed from the total feed offered. The ratio of feed intake to body weight was calculated to determine the FCR. Mortality was recorded daily in each group.

Carcass traits

Ten birds from each treatment group were selected at the end of the experiment (two birds per pen with body weight close to the pen's average) and slaughtered after eight hours of fasting to empty the digestive tract. After dissection with a sharp knife, the visceral organs (gizzard and liver) were removed to measure the relative weight of the carcass and organs. The carcasses were cut after being stored at 2 °C for 24 hours. The abdominal fat was then removed by collecting the fat extending to the ischium and surrounding the cloaca, and was weighed. The right breast muscles, thigh, and drumstick were removed from the carcass to calculate their individual yields as percentages of the body weight at slaughter. Individual yields were estimated using the following formulas:

$$\text{Breast, \%} = \frac{\text{Weight of right breast}}{\text{Body weight at slaughter}} \times 100, \quad (1)$$

$$\text{Thigh, \%} = \frac{\text{Weight of thigh}}{\text{Body weight at slaughter}} \times 100, \quad (2)$$

Table 1. Composition and nutrients of the experimental diets

Ingredient, %	Finisher (22–42 days)	Starter (1–21 days)
Maize	59.69	52
Soybean meal	33.2	40.6
Soybean oil	3	2.79
Dicalcium phosphate	1.75	2.1
Limestone	0.95	1
Salt (NaCl)	0.1	0.1
Vitamin-mineral premix*	1	1
DL–Methionine	0.18	0.31
L–Lysine	0.13	0.1
Calculated composition, % **		
Metabolizable energy, kcal/kg	3029.55	2979.37
Crude protein, %	19.31	22.01
Lysine, %	1.00	1.20
Methionine + Cystine, %	0.73	0.91
Total phosphorus, %	0.39	0.45
Calcium, %	0.91	1.04

* Supplied per kilogram of vitamin + mineral premix: vitamin A: 300000 IU; vitamin D: 100000 IU; vitamin E: 1500 mg; vitamin K: 60 mg; cobalamin: 0.4 mg; thiamin: 78 mg; riboflavin: 200 mg; pantothenic acid: 350 mg; niacin: 1200 mg; B_6: 65 mg; folic acid: 40 mg; choline chloride: 12000 mg; Mn: 2000 mg; Zn: 3000 mg; Fe: 800 mg; I: 25 mg; Cu: 300 mg; Se: 6 g.

** Calculated from data provided by the INRAE-CIRAD-AFZ feed tables in France (2017–2024).

$$\text{Drumstick, \%} = \frac{\text{Weight of drumstick}}{\text{Body weight at slaughter}} \times 100. \quad (3)$$

Meat quality

At 24 hours post-mortem, the pH value (pH_{24}) of the right breast muscle was measured following the procedure outlined by El Rammouz et al. [24], using a portable pH meter (Crison Instruments SA, Spain) with direct electrode insertion in the muscle. Prior to measurement, the electrode was calibrated at ambient temperature in standard buffers at pH 4 and 7. Drip loss of raw meat was measured after storage to estimate the water-holding capacity of the breast muscle. The breast muscle samples were placed in plastic bags, hung on hooks, and weighed before and after storage at 2 °C for 4 days. Drip loss was calculated as a percentage of the initial sample weight after chilling, cleaning using absorbent paper, and then reweighing. Drip loss was calculated using the following formula, adapted from Honikel [25]:

$$\text{Drip loss, \%} = \frac{W_1 - W_2}{W_1} \times 100\,, \quad (4)$$

where W_1 — Initial weight of muscle sample before storage;
W_2 — Final weight of muscle sample (after storage/chilling).

To measure cooking loss, the breast subsample was weighed and then heated in plastic bags within a water bath maintained at 80–85 °C until temperature in the meat center reached 75 °C. After cooking, the samples were removed from the bath, blotted dry, left at room temperature for 15 minutes, and then weighed. Cooking loss was calculated as the difference in weight before and after cooking, expressed as a percentage of the initial weight. Cooking loss was calculated using the following formula, according to Honikel [25]:

$$\text{Cooking loss, \%} = \frac{\text{Initial Sample Weight} - \text{Sample weight after Cooking}}{\text{Initial Sample Weight}} \times 100, \quad (5)$$

Tibia bone parameters

The method outlined by Kocabağli [26] was used to measure the bone morphometric parameters: the right tibia was labeled, immersed in boiling water (100 °C) for 10 minutes, then cleaned of all adherent tissues, including cartilage caps and the patella. The tibia was weighed using a digital scale (OHAUS, USA), and its length and width were measured using a digital caliper (Mitutoyo, Japan). Tibial width was measured at the medial diaphysis, and the length was measured from the proximal end to the distal end.

The bone index (Seedor index) and the index of robusticity were calculated as indicators of whole-bone density and strength, using the following formulas:

- Seedor index + tibia weight/tibia length [27].
- Robusticity index + bone length/cube root of bone weight [28].

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS version 27.0). A one-way analysis of variance (ANOVA) was conducted to identify the differences between the treatment means at a 5 % significance level. Tukey's test was then used to conduct pairwise comparisons between the groups.

Ethics statement

The experiment was approved by the Scientific Council of the Institute of Veterinary and Agricultural Sciences at the University of Batna 1, Algeria.

Results

Growth performance

The effect of mixtures of cinnamon powder plus kaolin (KCP) or cinnamon powder plus marl (MCP) on broiler growth performance at day 42 is presented in Table 2. A significant improvement in growth performance traits was observed in broilers fed diets supplemented with the KCP or MCP mixtures. Final body weight (FBW), body weight gain (BWG), and feed conversion ratio (FCR) differed significantly across all groups ($p < 0.05$). Compared with the control (CON) group, FBW increased by approximately 10.31 % and 6.73 % in the KCP and MCP groups, respectively. In addition, an increase in BWG by around 10.94 % and 7.74 % was recorded among the KCP and MCP treatments, respectively. FCR decreased in both treatments: KCP by 9.49 % and MCP by 6.70 %. However, feed intake (FI) did not differ significantly among the groups ($p > 0.05$).

Carcass traits and relative weights of internal organs

The effect of KCP and MCP mixtures on carcass traits of broiler chickens at day 42 is presented in Table 3. Dressing percentage, breast and thigh meat yields, and abdominal

Table 2. Effects of dietary supplementation of the KCP or MCP mixture on broiler performance at 42 days of age

Parameters[1]	Treatments[2]			SEM[3]	*p*-value
	CON	KCP	MCP		
FBW, g/bird	2238.85[c]	2469.77[a]	2389.45[b]	15.9	0.02
BWG, g/bird	2191.60[c]	2431.40[a]	2361.35[b]	0.18	0.03
FI, g/bird	3934.24	3925.28	3943.03	0.97	0.66
FCR, g/g	1.79[a]	1.62[b]	1.67[b]	0.05	0.01

[1] FBW: final body weight; BWG: body weight gain; FI: feed intake; FCR: feed conversion ratio.
[2] CON: basal diet without supplementation; KCP: basal diet + 3 % of mixture of kaolin and cinnamon powder; MCP: basal diet + 3 % of mixture of marl and cinnamon powder.
[3] SEM: standard error of mean.
[a–c] Means within a row with different superscripts are different at $p < 0.05$.

fat showed significant improvements ($p < 0.05$) across treatments. Broilers fed diets supplemented with the KCP mixture showed the highest dressing percentage and the greatest relative weights of breast and drumstick, significantly exceeding those of the CON group. In addition, both KCP and MCP groups exhibited a significant increase in the relative weight of the thigh. By contrast, dietary treatments had no significant effect ($p > 0.05$) on the weights of internal organs (liver and gizzard). However, birds that received the KCP and MCP mixtures recorded significantly less abdominal fat than the CON group.

Meat quality traits

The effects of dietary supplementation with the mixtures of KCP or MCP on the quality traits of the breast meat (pH_{24}, drip loss, and cooking loss values) are presented in Table 4. Birds fed diets supplemented with the KCP or MCP mixtures showed significantly ($p < 0.01$) reduced acidity (pH_{24}) of breast meat compared to the CON group. Consequently, adding these mixtures led to a significant decrease in drip loss and cooking loss ($p < 0.01$).

Morphometric parameters of tibia

The effects of dietary supplementation with the mixtures of KCP or MCP on tibia morphometric parameters are presented in Table 5. The results demonstrate that, compared to the CON group, the groups receiving KCP and MCP exhibited significantly higher tibia weight, length, and Seedor index, with the MCP group showing the strongest effects ($p < 0.001$, 0.05, and 0.01, respectively). However, the robusticity index was not significantly different among the groups ($p > 0.05$).

Discussion

This study investigated the effect of supplementing the basal diets of broiler chickens with a mixture of phytogenics (cinnamon) and clay (kaolin and marl) to enhance production performance, meat quality, carcass traits, and bone characteristics. To the authors' knowledge, no previous studies have examined the impact of cinnamon and clay supplementation on broiler nutrition. The findings revealed that both mixtures of cinnamon powder plus kaolin (KCP) and cinnamon powder plus marl (MCP)

Table 3. Effects of dietary supplementation of the KCP or MCP mixture on carcass characteristics and relative organ weights of broiler chickens

Parameters	Treatments[1]			SEM[2]	p-value
	CON	KCP	MCP		
Dressing percentage	72.14[b]	74.91[a]	73.53[ab]	0.337	0.001
Breast, %	26.61[b]	29.25[a]	27.79[ab]	0.427	0.035
Thigh, %	13.16[b]	14.27[a]	14.31[a]	0.161	0.002
Drumstick, %	11.27[b]	12.39[a]	12.02[ab]	0.156	0.008
Gizzard, %	1.81	1.77	1.75	0.019	0.232
Liver, %	2.72	2.59	2.58	0.027	0.056
Abdominal fat, %	2.22[a]	1.87[b]	1.96[b]	0.052	0.014

[1] CON: Basal diet without supplementation; KCP: Basal diet + 3 % of mixture of kaolin and cinnamon powder; MCP: Basal diet + 3 % of mixture of marl and cinnamon powder.
[2] SEM: standard error of mean.
[a, b] Means within a row with different superscripts are different at $p < 0.05$.

Table 4. Effects of dietary supplementation of the KCP or MCP mixture on the meat quality of broiler chickens

Parameters	Treatments[1]			SEM[2]	*p*-value
	CON	KCP	MCP		
pH_{24}	5.79[b]	5.95[a]	5.93[a]	0.181	0.0001
Drip loss, %	2.32a	1.51b	1.54b	0.120	0.004
Cooking loss, %	29.85[a]	26.65[b]	26.57[b]	0.327	0.0001

[1] CON: Basal diet without supplementation; KCP: Basal diet + 3 % of mixture of kaolin and cinnamon powder; MCP: Basal diet + 3 % of mixture of marl and cinnamon powder.
[2] SEM: standard error of mean.
[a, b] Means within a row with different superscripts are different at $p < 0.05$.

Table 5. Effects of dietary supplementation of the KCP or MCP mixture on tibia bone characteristics of broiler chickens

Parameters	Treatments[1]			SEM[2]	p-value
	CON	KCP	MCP		
Weight, g	5.79[b]	6.68[a]	7.11[a]	0.150	<0.001
Length, cm	10.60[b]	11.035[ab]	11.14[a]	0.089	0.025
Width, mm	8.04	8.33	8.43	0.111	0.337
Seedor index	54.93[b]	59.96[ab]	64.45[a]	1.316	0.008
Robusticity index	5.30	5.62	5.61	0.110	0.455

[1]CON: Basal diet without supplementation; KCP: Basal diet + 3 % of mixture of kaolin and cinnamon powder; MCP: Basal diet + 3 % of mixture of marl and cinnamon powder.
[2] SEM: standard error of the mean.
[a, b] Means within a row with different superscripts are different at $p < 0.05$.

significantly improved the final body weight (FBW), body weight gain (BWG), and feed conversion ratio (FCR) of broilers ($p < 0.05$).

The observed enhancements in FBW, BWG, and FCR among broilers fed with a mixture of KCP or MCP are consistent with earlier research on the properties of these natural additives. The increase in FBW and BWG, and the decrease in FCR, are likely due to the properties of clay, which regulates digestion and improves nutrient absorption, thereby enhancing broiler performance [29,30]. Clay also enhances the digestive tract ecosystem during digestion, thereby promoting broiler growth [31,32]. In addition, the active compounds in cinnamon, such as cinnamaldehyde and eugenol, exert antibacterial effects against many pathogens in the poultry intestine and have antioxidant, anti-inflammatory, and antifungal properties. They also stimulate digestive enzymes in the intestines, thereby enhancing the efficiency of food utilization and growth rate [33,34]. Several studies have demonstrated that the supplementation of kaolin [11] and marl [12,15] significantly improves BWG and FCR. Furthermore, recent studies have shown that dietary supplementation with cinnamon powder improves BWG and FCR [34,35].

Improvements in carcass characteristics, including an increase in dressing percentage and muscle yields, and a decrease in abdominal fat, were also observed. These outcomes suggest enhanced protein synthesis, improved muscle deposition, and altered lipid metabolism, all of which are regulated by substances like cinnamaldehydes [36]. Cinnamon has been shown to promote lipid oxidation and suppress lipogenesis [37]. Similarly, clays have been reported to improve carcass yield and meat production while reducing abdominal fat [6,38,39]. These benefits may be attributed to the ability of clay to enhance feed conversion efficiency and gastrointestinal tract health [40].

In this study, the relative weights of the liver and gizzard were not affected by KCP or MCP supplementation. These observations are consistent with previous reports showing that the relative weights of these organs were unaffected by either clay [41,42] or cinnamon [43,44,45].

The pH of meat affects its color, tenderness, and water-holding capacity (WHC) [46,47]. The pH level of broiler meat is determined by the amount of muscular glycogen before slaughter, and its conversion level to lactic acid after slaughter [48]. The optimal pH range for broiler meat is 5.8–6.3. Poultry meat that appears pale typically has a pH below 5.8, whereas darker meat tends to have a higher pH, which may present health concerns for humans [48].

In our study, both KCP and MCP mixtures significantly reduced the acidity of meat. These results may be attributed to the antioxidant properties of cinnamon, particularly cinnamaldehyde and eugenol, which protect against free radical damage and oxidative stress [49]. Previous studies have demonstrated that the pH of the breast muscles in broiler chicken decreased with a rise in cinnamon doses [50] and its pH at 24 hours [51]. Additionally, clay minerals can influence meat quality, muscle oxidative level, and muscle mineral content [40,52]. Consistent with our results, An et al. [53] reported that incorporating zeolite into the broiler diet increased pH value and improved WHC. Castellini et al. [54] noted that a low pH in poultry meat is associated with decreased WHC, leading to higher cooking and drip losses, reduced shelf life, and decreased tenderness. This likely explains the reduced drip loss and cooking loss noted in breast meat in our study. The positive effects of KCP and MCP mixtures on meat quality parameters in broiler chicken may be attributed to the synergistic effects of cinnamon and clay.

In poultry production, the tibia is commonly considered as an indicator of skeletal health and growth [55]. The Seedor index is a simple measure to evaluate bone density. A higher index indicates denser bone. Conversely, a strong bone structure is indicated by a low robustness index. In the current study, the MCP supplement group had significantly greater tibia length, weight, and Seedor index than the CON group, while the KCP group was at an intermediate level.

Improved bone parameters in the groups receiving the cinnamon and clay supplements may be partially due to the mineral richness of clay, which influences mineral metabolism and electrolyte balance, thereby promoting bone formation [6]. These improvements can also be attributed to the small amounts of manganese and calcium in cinnamon, which are essential for chicken metabolism and bone health [56]. According to Safaeikatouli et al. [10], inclusion of 3 % zeolite or bentonite in broiler diets significantly enhanced robusticity, tibiotarsal weight/length, and tibiotarsal indices. Similarly, supplementation of 3 % kaolin has been reported to increase relative tibia weight [12]. Since calcium and phosphorus are vital for bone mineralization, their blood concentrations serve as useful markers of bone growth. In this context, Herzig et al. [57] found that the application of clinoptilolite had a positive effect on the lodgment of Ca and P in the bones (femur and tibiotarsus). The positive impact of clay on tibial bone features might result from improved dietary digestibility and increased intestinal absorption of Ca and P, which supports bone formation and remodeling. Although research on the direct effects of cinnamon powder on bone health of broiler and its properties is limited, the improvements observed in this study may be linked to the beneficial effects of cinnamon on gut health, nutrient digestibility, gut microbiota and immune response [34], which are considered to support skeletal health and bone mineralization in broiler chickens indirectly [58].

Conclusion

This study found that supplementing broiler diets with cinnamon and clay (kaolin or marl) significantly enhanced growth performance, feed efficiency, carcass yield, as well as meat and bone traits in broilers. These improvements may be attributed to the bioactive substances present

in cinnamon and the physicochemical properties of clay, which support intestinal health and enhance enzyme activity. These results suggest that a blend of cinnamon and clay (kaolin or marl) could be a natural alternative to growth promoters, particularly given the increasing restrictions on the use of antibiotic growth promoters in poultry production. Such supplementation can support sustainable and health-focused broiler production by boosting productivity without compromising animal welfare or product quality.

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INTELLIGENT ENGINEERING OF A SPECIALIZED MEAT-BASED PRODUCT

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Abstract
This article discusses the development and application of a digital twin (DT) for intelligent engineering of a specialized meat-based product. The focus is on the use of advanced intelligent optimization methods to create a virtual model capable of adaptively real-time managing the formula and process parameters, in accordance with the product's final purpose. To solve a food engineering problem (optimization of product formula and production modes), three metaheuristic intelligent algorithms were applied and compared: genetic algorithm (GA), particle swarm optimization (PSO), and sparrow search algorithm (SSA). The results of the comparative analysis demonstrated that SSA algorithm provided the best accuracy and convergence in solving the assigned optimization problems, making it the preferred tool for integration into the digital twin system. SSA algorithm demonstrates the ability to escape local optima, thereby avoiding the problem of premature convergence typical for some metaheuristic methods, such as GA and PSO algorithms. Based on an optimized digital twin, this approach enables dynamic prediction of physicochemical parameters, their compliance with the established medical and biological requirements for the final product, promptly adjusting its formula to meet the intended purpose of providing complete nutrition and enhancing the rehabilitation of patients with traumatic brain injuries (TBI), and ensures flexibility when scaling the technology or transferring production to another facility. Digital twins, integrating data from smart sensors and biosensors based on big data analysis, are the foundation for creating a safe, traceable, and adaptive food system. The study demonstrates that intelligent engineering based on a digital twin is a key technology for creating personalized, safe, and effective specialized food products within the framework of modern manufacturing principles and One Health concept.

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Introduction

Artificial intelligence (AI) and machine learning (ML) technologies enable the exploration and design of new food systems in a digital environment without need for extensive experimentation. Modern food formulation requires the expertise of researchers across multiple fields to achieve desired results through iterative development and innovation in the context of an intelligent engineering. To improve the result or bring parameters closer to the target, this approach requires significant time and funding for each iteration of product development, i.e. repeated data processing [1].

Algorithms, AI, and ML methods enable researchers to unlock vast opportunities for the scientific improvement (optimization) of traditional food formulas, as well as the creation of new food systems with defined compositions and properties for personalized (specialized) nutrition. With the increasing adoption of AI methods in the food industry, advances in food design increasingly move beyond traditional techniques, and the development of functional food formulas opens up entirely new possibilities [2].

The study by Lončar et al. [3] involves the use of advanced machine learning techniques in the development of dried peach cookie formulas to understand the complex relationships between input parameters (e.g., dehydrated peach and processing methods — lyophilization and lyophilization with osmotic pretreatment) and output variables representing various cookie quality characteristics. The authors used a support vector machine (SVM) and an artificial neural network (ANN) to optimize the cookie formulas. For each of the 32 output datasets, including the chemical composition of cookie samples, mineral content, moisture content, as well as baking characteristics (moisture loss, cookie thickness and diameter, hardness), color coordinates (L*, a*, b*, and ΔE), sensory characteristics and antioxidant properties, separate models were created using SVM and ANN. The obtained data demonstrated superior performance of ANN in predicting quality parameters with coefficient of determination (R^2) + 1, while when using SVM, R^2 + 0.981. Along with this, the optimal amount of dried peach (15 %) and the best technology for its dehydration (lyophilization with osmotic pre-treatment) were determined.

Pasta is primarily composed of wheat flour and water. However, flour may be partially replaced with fiber to provide the diet with additional functional ingredients. However, uneven fiber distribution may affect the technological characteristics of pasta. Typically, methods for determining pasta parameters are time-consuming and destructive. To improve the efficiency of fiber distribution analysis in pasta, Badaro et al. [4] used near-infrared hyperspectral imaging (NIR-HSI) in combination with machine learning methods (multivariate curvilinear regression, MCR-ALS). The method has high potential for application in non-destructive testing. The object of study was a fettuccine formula with the addition of 2 %, 3.5 %, 5 %, and 7 % of seven different fiber types to the dough mass. This method made it possible to evaluate both the fiber distribution depending on the formula and the spatial distribution of different fiber types. The results showed R^2 values ranging from 0.28 to 0.89, % LOF[1] < 6 %. The variance explained was greater than 99 %, and the similarity between the pure and reconstructed spectra was greater than 96 % and 98 % in the models using pure flour and control as initial estimates, respectively, demonstrating the applicability of NIR-HSI and MCR-ALS for identifying fibers in pasta. However, the significant variation in R^2 values (0.28 to 0.89) across models indicates instability in predictive ability depending on the analyzed component or initial conditions.

In a sociological study, Califano et al. [5] examined the consumer perception of food products (dishes and beverages) created using AI. The key finding of the study is that trust in AI-based formulas for innovative dishes is currently low.

Ali et al. [6] used the results of tomographic studies (more than 460 3D images) obtained from a synchrotron to study bread dough formulas during rising and baking. The aim of the research was to study the effect of different bread dough formulas, especially those including flour with different protein contents and salt additives, on the microstructure, texture, and porosity of bread during fermentation and baking in order to automate the process. The authors used automatic segmentation and analysis using U-Net deep learning algorithms. Automation of dough microstructure segmentation allows for an objective and efficient study of the formula effect on porosity. The trained U-Net model outperformed previously used segmentation methods. The developed automated data segmentation model may reduce the labor intensity of the process and open up opportunities for conducting experiments in 4D format. However, a critical limitation of this approach is the opacity of the neural network ("black box"): the high accuracy of the model's predictions is not accompanied by the ability to interpret the patterns embedded within it, which prevents the identification of cause-and-effect relationships between the formula and the formation of the product structure.

[1] LOF (Local Outlier Factor) is an algorithm for detecting anomalies in data.

Using artificial neural networks (ANN) and simplex-lattice mixture design (MD), Patil et al. [7] predicted and optimized the quality characteristics of functional cookies enriched with finger millet and jaggery as a natural sweetener. The authors used advanced optimization approaches to identify complex relationships between input variables, such as whole wheat flour (WWF) and finger millet flour (FMF), and output metrics representing various cookie quality characteristics. The resulting range of R^2 values for ANN is extremely wide (0.20 to 0.99). R^2 value of 0.20 indicates a virtually ineffective model, explaining only 20 % of the data variance. There is no explanation for such significant variation in ANN performance across different output variables. This may be due to insufficient training data, an incorrect network architecture, or the highly stochastic nature of the measured parameters themselves.

Designing specialized and functional food products based on intelligent simulation methods is a modern interdisciplinary paradigm. This approach combines advanced computational technologies with fundamental knowledge in food science, enabling the targeted creation of food systems with specified functional, technological, and consumer properties.

Lisitsyn et al. [8] and Ivashkin et al. [9] proposed a methodological approach to solving the problem of personalized (individualized) nutrition based on the principles of structural parametric simulation. A key aspect lies in the systematic formalization of accumulated data and expert knowledge of the product using modern digital technologies.

Türkmenoglu et al. [10] addressed the problem of developing personalized nutrition plans using multicriteria optimization methods. The proposed methodology is based on the application of multicriteria optimization, i.e. an evolutionary algorithm (non-dominated sorting genetic algorithm III or NSGA-III), to a real dataset consisting of prepared dishes or foods. In the presented study, the main criteria for diet optimization are: 1) the economic component (cost of food); 2) personal consumer preferences; 3) the technological aspect of dish preparation. Moreover, the model integrates daily nutrient restrictions for each meal, specified in the form of acceptable ranges (minimum — maximum) of nutrient intake. Key results demonstrate the ability of the algorithm to generate balanced daily menus, adapting them to individual user parameters: preferences, age, gender and body mass index, while adhering to recommended intake standards. The approach has a high degree of adaptability and can be expanded for individuals with special medical indications by introducing additional restrictions and modifying the regulatory requirements for the diet or dish.

Razali et al. [11] optimized menu planning for Malaysian adolescents (13 to 18 years old) using a self-adaptive hybrid genetic algorithm (SHGA). The main optimization factors in the study were: 1) minimizing the cost of the diet per student; 2) maximizing the variety of daily meals; 3) taking into account the capabilities of the caterer;

4) maintaining the structure of dishes in meals; 5) fulfilling standard recommendations for nutrient intake. The methodological novelty of the study lies in the development and hybridization of two new local search methods: insertion search (IS) and insertion search with delete-and-create (ISDC). ISDC, unlike IS, expands the search area, which ensures the generation of 100 % feasible solutions. An additional improvement is the implementation of a self-adaptive strategy for adjusting the parameters of the mutation operator, which significantly reduces the computation time (to several minutes) and prevents the problem of premature convergence. The developed SHGA approach demonstrates a significant performance improvement compared to the traditional genetic algorithm due to a balanced combination of exploitation (search intensification) and exploration (diversification) strategies. The algorithm effectively avoids local optima and ensures the generation of high-quality and feasible solutions in the conditions of a high variability in menus.

The paper [12] presents a range of AI technologies applied in manufacturing optimization. One subsection of the paper examines in detail two classes of AI optimization methods:

Class 1 — machine learning as a tool for analyzing big data from sensors (Industry 4.0/5.0). This tool identifies complex parameter-response relationships and supports real-time decisions to improve efficiency and accuracy.

The paper [13] defines available machine learning methods, focusing on potential benefits and examples of successful application in manufacturing environments. Kumar et al. [14] analyzed the current state of machine learning technology, with an emphasis on modern manufacturing methods, specifically additive manufacturing. The paper [15] examines a relevant problem of the production management: effective decision-making regarding rescheduling of a flexible manufacturing system. The authors note that in real-world stochastic environments, rescheduling decisions are often made based on simplified (greedy) heuristics, which does not guarantee an optimal balance between the frequency of equipment rescheduling and the accumulation of delays in the production schedule. As a solution, an integrated framework combining machine learning (ML) methods and optimization algorithms is proposed. The framework aims to justify the feasibility of rescheduling at a specific point in time. The review [16] presents the synergy between deep reinforcement learning (DRL) algorithms and the concept of digital twins. Digital twins are considered as a critical component compensating for the lack of an accurate analytical model of a complex production environment. Their use allows for the creation of a virtual testing ground for training and tuning DRL agents, potentially improving the performance, safety, and velocity of algorithm deployment.

Class 2 — evolutionary algorithms (genetic algorithm (GA), particle swarm optimization (PSO), differential evolution (DE)) are bioinspired metaheuristics effective for solving multicriteria (multi-objective) optimization problems in manufacturing. Their ability to work in changing conditions and integrate into the concept of Industry 5.0 is emphasized [17,18].

Genetic algorithm is a heuristic method for finding solutions based on the principles of natural selection and genetic operations [19]. Genetic algorithms are a class of evolutionary algorithms made popular by Holland in the 1970s [20]. They are used to find exact or approximate solutions to optimization problems, as demonstrated in the works by Goldenberg [21] and Sivanandam and Deepa [22]. The classical genetic algorithm includes the following stages: population initialization, fitness assessment, selection, recombination, mutation, formation of a new population, and testing of stopping criteria. This approach enables efficient search for optimal solutions in complex parameter spaces.

PSO algorithm models social interaction within a swarm, where each particle adjusts its trajectory based on individual and collective experience [23,24]. PSO was first introduced by Kennedy and Eberhart in 1995 [25]. This method is based on the concepts of social behavior found in groups of birds, fish, or insects. The algorithm's strategy is to use this group intelligence to find the best solution in a complex one-dimensional area, where each agent, called particle, changes its direction based on personal and social experience to navigate the search area. PSO uses particle swarm, where each particle points to a possible solution [26]. These particles "fly" through the search space and change their position depending on the best position found and the position best known to their neighbors. Using this collaborative mechanism, PSO can effectively perform both exploration and exploitation: exploration by allowing particles to move to different areas, preventing them from premature convergence, and exploitation by concentrating the search in promising areas to improve the search. This balance is necessary to cover complex search spaces and efficiently find solutions. In the context of the food product formula selection, each particle is a vector whose components correspond to the mass fractions of ingredients. After each iteration, the vector is normalized to ensure consistency with the percentage composition. Formula quality is assessed using a fitness function that accounts for deviations from the specified protein, carbohydrate, and fat content recommendations.

Sparrow search algorithm (SSA) is a bioinspired swarm intelligence method that models the behavior of a flock of sparrows during foraging and threat avoidance. The algorithm was presented in 2020 by Xue and Shen [27]. It divides the population into functional groups, each of which plays a specific role in the optimization process, ensuring a balance between exploring new search areas and exploiting identified promising solutions. The same fitness function as in PSO algorithm is used to evaluate solutions.

A systematic analysis of relevant scientific publications led to a categorization of the key intelligent methods used in this field:

1. Artificial intelligence and machine learning methods
- application of artificial neural networks to predict the degradation kinetics and stability of functional ingredients in heterogeneous food matrices;
- development of formula optimization algorithms aimed at simultaneously achieving target nutritional value and sensory characteristics (taste, texture);

These methods, based on the analysis of big data describing the properties of ingredients, their synergistic and antagonistic interactions, and their impact on physiology, serve as the basis for the creation of personalized food products adapted to individual genetic predispositions or metabolic profiles.

2. Bioinformatics and omics technologies
- integration of genomics, proteomics, metabolomics, and transcriptomics methods for screening and studying the bioactive components of food raw materials;
- molecular modeling (docking, molecular dynamics) to study the mechanisms of interactions at the molecular level, for example, the binding of biologically active peptides or polyphenols to cellular or taste receptors.

These approaches enable the targeted identification and selection of compounds with target properties, such as peptides with antihypertensive or antimicrobial activity.

3. Mathematical simulation and optimization methods
- use of bioinspired metaheuristic algorithms (genetic algorithms, swarm intelligence methods) to solve combinatorial optimization problems when searching for rational ratios of multicomponent mixtures;
- formalization and solution of systems of balance equations for nutrients (proteins, fats, carbohydrates, vitamins, minerals) taking into account their bioavailability.

These methods enable multicriteria optimization of product composition based on specified fitness functions, enabling the identification of compromise solutions between often conflicting requirements.

4. Computer-aided design (CAD) and digital twin technologies
- development of virtual simulations (*in silico*) of key production processes (mixing, heat treatment, drying) to analyze their impact on the final product properties;
- predictive modeling of shelf life, component migration, and the physicochemical stability of products under various storage conditions.

A comparative analysis revealed the key advantages of these methods: 1) significant reduction in time and resources during the research and development phase by minimizing real experiments; 2) the ability to create targeted products for specific population groups (patients with various diseases, children, older adults, athletes, etc.); 3) improved safety through proactive modeling of potential risks (microbiological, chemical, and technological ones).

However, there are also drawbacks (limitations) to the application of these methods: 1) the critical dependence of the accuracy and reliability of the models on the volume, representativeness, and quality of the input data; 2) the need for mandatory experimental validation of the *in silico* results under real-world conditions; 3) high capital costs for the implementation and support of the corresponding technological platforms, as well as the need for skilled personnel.

The introduction of intelligent methods marks the digital transformation of the food industry, shifting the development of functional products from an empirical to a knowledge-intensive and targeted approach.

The goal of our study is to develop and validate (verify) an optimization model based on genetic algorithms for solving the problem of multicriteria selection of optimal combinations and quantitative ratios of ingredients in a specialized meat-based product.

Objects and methods

The object of the study was a digital model of a specialized meat-based product (SMP) intended for the rehabilitation of individuals with traumatic brain injuries. The formula of the SMP included 14 functional ingredients selected based on their nutritional potential, biochemical and technological compatibility. Particular attention was paid to the content of target proteins and peptides in the meat raw materials, as these components play a key role in neural regeneration and metabolic restoration in this category of patients. Product quality criteria were developed based on the medical and biological requirements developed for the specific product and regulating the composition of specialized food products for neurorehabilitation. Restrictions included: 1) ranges of protein, fat, and carbohydrate content; 2) acceptable levels of macro- and micronutrients, as well as bioactive compounds.

The digital model of the formula was developed not only for technological feasibility but also considering the physiological and metabolic characteristics of the target consumer group.

A digital model of a food product typically represents a parametric description or algorithm focused on individual properties (e.g., composition, formula, sensory properties) and used to solve specific design problems. In contrast, a digital twin is a dynamic, continuously updated virtual copy of a physical product throughout its entire lifecycle. A digital twin integrates real-time data (from raw materials to consumer experience), uses feedback for self-learning, and is designed for comprehensive modeling, forecasting, and optimization.

Digital twin

Evolutionary optimization methods, specifically genetic and swarm algorithms, were used in the research on developing a digital twin of a food product.

Genetic algorithm is an evolutionary numerical optimization method based on the principles of natural selection, recombination, and mutation (Figure 1).

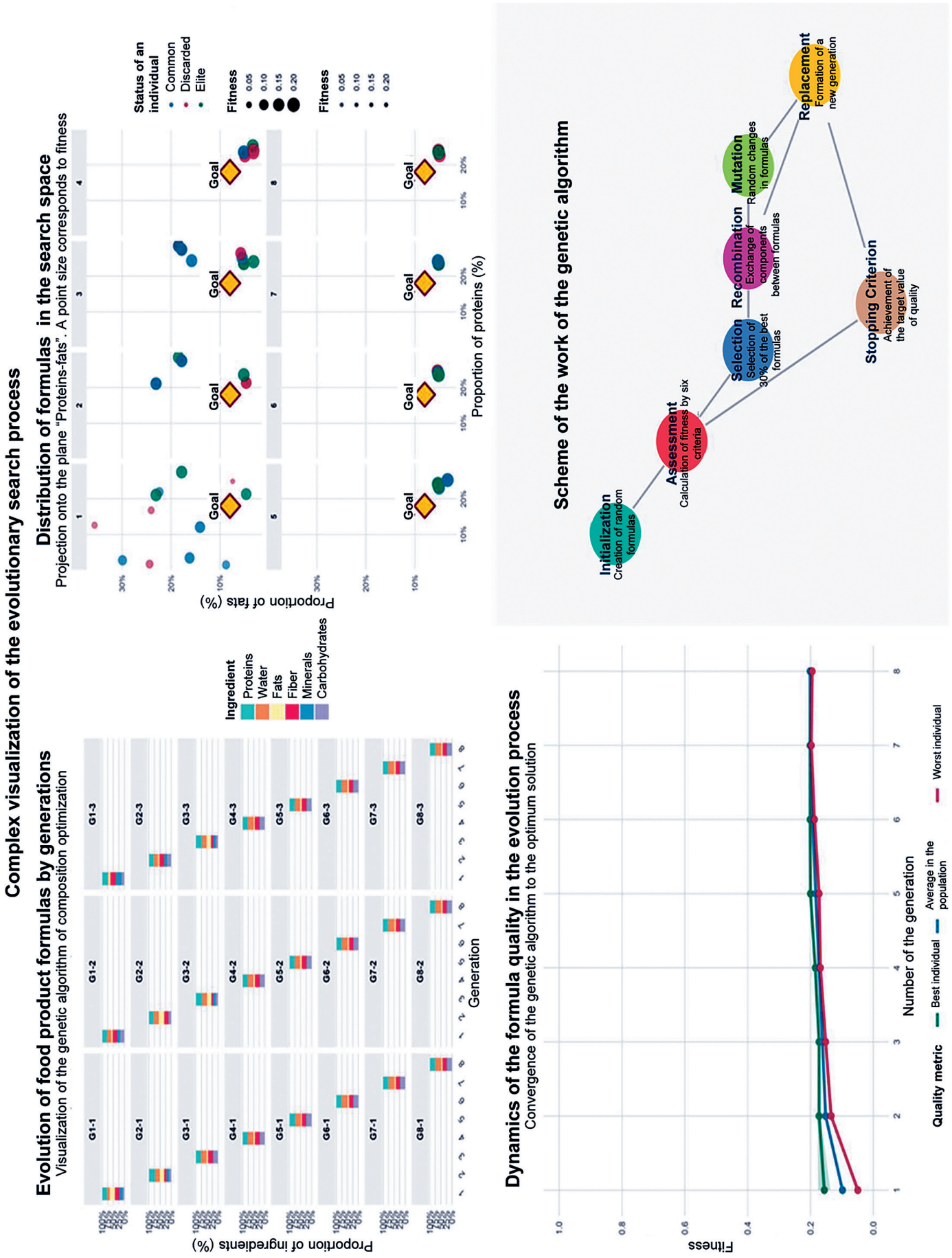


Figure 1. Genetic algorithm for food product formula optimization

In the context of food product formula optimization, the algorithm functions as an iterative process for finding the optimal composition and proportions of ingredients, minimizing or maximizing a given fitness function. The sequential implementation of the algorithm's stages forms a closed evolutionary cycle, ensuring targeted improvement of the developed formula.

The initial stage, "Initialization", involves forming an initial population, i.e. a set of formulas (individuals) whose parameters (mass fractions of the components) are randomly generated within technologically acceptable limits. This creates the initial diversity necessary to initiate the optimization process.

Stage 2, "Fitness Assessment", is the key stage for the formula synthesis problem. For each individual (formula variant), a fitness function is calculated, which quantitatively evaluates the quality of the solution. In food technology, such a function may integrate multiple criteria: compliance of the nutrient profile with specified regulatory requirements, sensory assessments, raw material cost, process parameters (e.g., viscosity, pH), and predicted digestibility. Thus, the fitness function formalizes the complex technical, economic, physiological, and hygienic requirements for the final product.

Stage 3, "Selection", selects parent formulas to create the next generation. The probability of selecting an individual is proportional to the value of its fitness function, implementing the principle of "survival of the fittest". The frequently used tournament selection method, in which a random subset of individuals competes, effectively combines selective pressure with stochasticity.

Stage 4, "Recombination (Crossing)", simulates biological crossing: selected pairs of parent formulas exchange parts of their parameters, generating new recombinant offspring formulas. This operator allows for combining successful combinations of ingredients from different solutions, exploring central areas of the search space.

Stage 5, "Mutation", introduces random changes to the offsprings with a specified low probability (for example, a minor adjustment to the proportion of one component). This operator maintains population diversity and provides the ability to escape local optima by exploring new, potentially promising areas of the formula space.

At stage 6, "Formation of a New Population", elitism occurs, i.e. keeping the few best formulas of the current generation unchanged and replacing the least fit individuals with new offsprings. This strategy guarantees a monotonic (non-degrading) improvement in the quality of solutions from generation to generation.

At stage 7, "Checking the Stopping Criterion", the optimization process terminates when one of the following conditions is reached: 1) exhaustion of the computational resource (maximum number of iterations); 2) stagnation, i.e. no significant improvement in the fitness function over a specified number of generations. If this condition is not met, the cycle repeats from stage 2, "Fitness Assessment", for a new generation of formulas.

Thus, the sequential implementation of genetic algorithm provides a systematic, directed, yet stochastic search in the multidimensional space of formula parameters. This enables the finding of compromise solutions that simultaneously satisfy multiple, often conflicting, requirements (sensory, nutritional, technological, and economic), making this method a powerful tool for computer-aided design and optimization of food products.

In addition to genetic algorithm, swarm intelligence algorithms were used in the study, i.e. particle swarm optimization (PSO) and sparrow search algorithm (SSA).

Unlike genetic algorithm, PSO algorithm includes the following stages.

At stage 1, "Population Initialization", an initial swarm of particles is created. Each particle corresponds to a possible solution to the problem. In the context of formula selection, this is a vector whose components represent the proportions of ingredients. The initial values are randomly assigned within acceptable limits, after which normalization is performed to ensure the correct percentage ratio.

At stage 2, "Initialization of Individual and Global Experience", each particle remembers its best position achieved over the entire duration of the algorithm's execution. Simultaneously, the best position among all particles in the population is determined, which is considered the current global optimum.

Stage 3, "Iteratively Update of Particle Velocity and Position". At each iteration of the algorithm, each particle adjusts its motion, taking into account three components: 1) inertial component, i.e. maintaining the previous direction of motion; 2) individual experience, i.e. the desire to return to its best historical position; 3) collective experience, i.e. the desire to approach the globally best position found by the entire swarm. Based on the updated velocity, the particle changes its position in the solution space.

Stage 4, "Formula Normalization". After changing the position, the vector is normalized so that the sum of all components remains equal to a specified value, for example, 100 %, ensuring the physical correctness of the formula.

At stage 5, "Solution Quality Assessment", a fitness function is calculated for each particle, reflecting the quality of the corresponding solution. In the context of the food product composition problem, the function takes into account compliance with target protein, carbohydrate, and fat content and may also include penalties for deviations from the standard.

Stage 6, "Updating Individual and Global Experience". If a particle's current position is better than its previous best position, the individual experience is updated. After evaluating all particles, the globally best solution for the entire population is adjusted if necessary.

Stage 7, "Checking the Stopping Condition". The algorithm continues iterating until the specified number of steps is reached or until the quality of the global solution stabilizes within an acceptable error. When the stopping criterion is met, the best solution found is returned.

PSO algorithm combines elements of global and local search, enabling efficient exploration of multidimensional solution spaces and finding balanced optima in problems with multiple constraints, such as food formula optimization.

Another alternative metaheuristic algorithm used in this study was the sparrow search algorithm. Its implementation is as follows.

At stage 1, "Population Initialization", an initial population of sparrows is created, each representing a possible solution to the problem. In this case, this is a vector specifying the proportions of ingredients in the formula. Initial values are assigned randomly within acceptable ranges, after which normalization is performed to ensure correct percentages.

At stage 2, "Sparrow Role Classification", the population is divided into three groups based on their role in the search process: 1) producers, the fittest sparrows, responsible for actively searching for new areas with high-quality solutions; they act as leaders, directing the movement of the flock; 2) scroungers, sparrows that follow the producers and utilize the resources they have discovered; their behavior is aimed at local exploration of areas around the current best solutions; 3) watchers, a randomly selected subset of the population whose task is to detect potential threats and prevent premature convergence to suboptimal solutions.

Stage 3, "Updating Producer Positions". The positions of the producers are updated based on the "alarm" level, which simulates the presence of a threat. At low alarm levels, producers intensively explore areas around their current best positions. At high alarm levels, they may make random movements to find new, safer areas.

At stage 4, "Updating Scrounger Positions", the scroungers adjust their positions based on two strategies: 1) some scroungers in unfavorable conditions leave their current positions in search of new areas; 2) the remaining scroungers approach the producers and explore the surrounding space, which allows them to refine their solutions.

At stage 5, "Updating Watcher Positions", the positions of the watchers are adjusted so that sparrows move away from potentially dangerous areas while simultaneously moving closer to the current best solutions. This mechanism helps maintain a balance between safety and search efficiency.

Stage 6, "Formula Normalization". After each position update, the vectors are normalized to ensure the sum of all components remains constant, which satisfies the physical requirements for the formula composition.

At stage 7, "Solution Quality Assessment", a fitness function is calculated for each sparrow. This function evaluates the quality of the solution based on specified criteria, such as compliance with protein, fat, and carbohydrate content standards. This function includes both the target component and penalty components for deviations from the requirements.

Stage 8, "Checking the Stopping Condition". The algorithm continues iterating until the specified number of generations is reached or until the fitness function values stabilize. Upon completion, the best solution found is returned.

SSA algorithm combines elements of social interaction, adaptive behavior, and threat avoidance mechanisms, ensuring a high convergence velocity, search accuracy, and resistance to premature stabilization in local optima. This makes it an effective tool for solving multicriteria optimization problems, such as complex food formula selection.

All computational experiments were implemented in R Studio software using the R programming language, which ensured reproducibility and flexibility in algorithm parameters [19,28–30].

Results and discussion

To design a food system with the target composition and characteristics, genetic algorithm was used, the pseudocode for which is shown in Figure 2.

The use of evolutionary algorithms to optimize the formula for a specialized meat-based product (SMP) involved three key steps: 1) coding the solutions; 2) defining the fitness function; 3) assigning the search operators.

Step 1, coding the solutions. Each solution (individual) was represented by a vector of the proportions of 14 ingredients in the formula, formed based on their composition in terms of key nutrients: proteins, fats, carbohydrates, and other functional components (Figure 3).

Algorithm Genetic algorithm pseudocode

```
# Run the genetic algorithm
GA <- ga(
 type = "real-valued",
 fitness = fitness,
 lower = min_proportions,   # Vector of minimum values
 upper = max_proportions,   # Vector of maximum values
 popSize = 100,             # Population size
 maxiter = 500,             # Number of iterations
 pcrossover = 0.8,          # Crossover probability
 pmutation = 0.1,           # Mutation probability
 elitism = 6,               # Number of best individuals to keep
 run = 50,                  # Stop after 50 generations
 seed = 123,                # For reproducibility
 monitor = gaMonitor,       # Enable monitoring
keepBest = TRUE             # Keep the best solutions
```

Figure 2. Genetic algorithm pseudocode

```
# Ingredient Data
ingredients <- matrix(
 c(23.37, 3.60, 0.00, 64.33, # Ingredient_1
 85.00, 5.00, 3.00, 7.00, # Ingredient_2
 0.00, 99.85, 0.00, 0.00, # Ingredient_3
 0.00, 99.00, 0.00, 0.00, # Ingredient_4
 5.00, 0.00, 28.00, 0.00, # Ingredient_5
 0.00, 0.00, 93.00, 4.00, # Ingredient_6
 40.1, 0.20, 26.40, 0.00, # Ingredient_7

 29.50, 8.50, 0.00, 0.00, # Ingredient_13
 0.00, 0.00, 0.00, 100.00), # Ingredient_14
 nrow = 14, byrow = TRUE
```

Figure 3. A fragment of "Ingredient Data" R code listing

Step 2, defining the fitness function. The energy value of the product was used as the target criterion. The fitness function assessed caloric content and included penalty points for deviations from the specified medical and biological requirements for nutrient content.

Step 3, assigning the search operators. Standard operators were used for evolutionary search: 1) crossover (cxBlend) — combining the solutions; 2) mutation — introducing random changes using a normal distribution; 3) selection — selecting the best solutions using a tournament method.

Mathematical model for SMP included: 1) optimization criterion; 2) a system of constraints on formula components and elemental composition; 3) balance equations with coefficients accounting for thermal losses, obtained experimentally.

The optimization criterion is presented as the minimization of the sum of the squared deviations of the calculated values from the target values:

$$P(z) = \sum_{j=1}^{m} \left(x_j^0 - x_j^{calcul.} \right)^2 \rightarrow \min . \quad (1)$$

The model includes four calculation blocks reflecting the main characteristics of the food system:

1. Nutritional value block (protein, fat, and carbohydrate content, taking into account loss factors).

$$\textbf{\textit{Protein}} = (23.37 \cdot x_1 + 85 \cdot x_2 + 5 \cdot x_5 + 40.1 \cdot x_7 + 29.5 \cdot x_{13}) \cdot \mathbf{0.96} \quad (2)$$

$$\textbf{\textit{Fat}} = (3.6 \cdot x_1 + 5 \cdot x_2 + 99.85 \cdot x_4 + 99 \cdot x_5 + 0.2 \cdot x_7 + 100 \cdot x_{12} + 8.5 \cdot x_{13}) \cdot \mathbf{0.92} \quad (3)$$

$$\textbf{\textit{Carbohydrates}} = (3 \cdot x_2 + 28 \cdot x_5 + 93 \cdot x_6 + 26.4 \cdot x_7) \cdot \mathbf{0.99} \quad (4)$$

Nutritional value block restrictions: (a) protein is 6–6.5 g; (b) fat is 4.5–5.5 g; (c) carbohydrates are 14.5–15.5 g.

2. Biological value block (content of essential amino acids: valine, leucine, isoleucine, methionine + cystine, threonine, lysine, phenylalanine + tyrosine, tryptophan).

$$\textbf{\textit{Valine}} = (4.11 \cdot x_1 + 6.5 \cdot x_2 + 8.92 \cdot x_5 + 6.48 \cdot x_7 + 4.02 \cdot x_{13}) \cdot \mathbf{0.82} \quad (5)$$

$$\textbf{\textit{Leucine}} = (9.88 \cdot x_1 + 5.7 \cdot x_2 + 8.84 \cdot x_5 + 11.97 \cdot x_7 + 7.58 \cdot x_{13}) \cdot \mathbf{0.83} \quad (6)$$

$$\textbf{\textit{Isoleucine}} = (4.96 \cdot x_1 + 9.6 \cdot x_2 + 4.18 \cdot x_5 + 5.24 \cdot x_7 + 2.41 \cdot x_{13}) \cdot \mathbf{0.52} \quad (7)$$

$$\textbf{\textit{Methionine + Cysteine}} = (4.07 \cdot x_1 + 4.3 \cdot x_2 + 2.2 \cdot x_5 + 9.48 \cdot x_7 + 2.71 \cdot x_{13}) \cdot \mathbf{0.24} \quad (8)$$

$$\textbf{\textit{Threonine}} = (5.95 \cdot x_1 + 4.1 \cdot x_2 + 5.42 \cdot x_5 + 6.23 \cdot x_7 + 3.91 \cdot x_{13}) \cdot \mathbf{0.92} \quad (9)$$

$$\textbf{\textit{Lysine}} = (11.81 \cdot x_1 + 4.9 \cdot x_2 + 3.92 \cdot x_5 + 10.72 \cdot x_7 + 6.9 \cdot x_{13}) \cdot \mathbf{0.46} \quad (10)$$

$$\textbf{\textit{Phenylalanine + Tyrosine}} = (11.21 \cdot x_1 + 10.2 \cdot x_2 + 5.42 \cdot x_5 + 22.94 \cdot x_7 + 7.22 \cdot x_{13}) \cdot \mathbf{0.56} \quad (11)$$

$$\textbf{\textit{Tryptophan}} = (2 \cdot x_2 + 0.96 \cdot x_5 + 2 \cdot x_7) \cdot \mathbf{0.88} \quad (12)$$

Biological value block restrictions for essential amino acids: (a) isoleucine is 4.20–4.64 g/100 g protein; (b) leucine is 7.63–8.43 g/100 g protein; (c) lysine is 7.44–8.22 g/100 g protein; (d) methionine + cystine are 3.30–3.64 g/100 g protein; (d) phenylalanine + tyrosine are 7.78–8.60 g/100 g protein; (e) threonine is 3.95–4.35 g/100 g protein; (g) tryptophan is 1.12–1.24 g/100 g protein; (g) valine is 5.31–5.87 g/100 g protein.

3. Vitamin block (B_1, B_2, B_3, B_5, B_6, E, C, D_3).

$$\textbf{\textit{B1}} = (0.16 \cdot x_1 + 375 \cdot x_{10} + 1.31 \cdot x_{13}) \cdot \mathbf{0.55} \quad (13)$$

$$\textbf{\textit{B2}} = (283 \cdot x_{10} + 1.88 \cdot x_{13}) \cdot \mathbf{0.30} \quad (14)$$

$$\textbf{\textit{B3}} = (6.98 \cdot x_1 + 2571 \cdot x_{10} + 14.25 \cdot x_{13}) \cdot \mathbf{0.55} \quad (15)$$

$$\textbf{\textit{B5}} = (0.28 \cdot x_1 + 1985 \cdot x_{10}) \cdot \mathbf{0.37} \quad (16)$$

$$\textbf{\textit{B6}} = (286 \cdot x_{10}) \cdot \mathbf{0.74} \quad (17)$$

$$\textbf{\textit{E}} = (0.1 \cdot x_1 + 3.62 \cdot x_3 + 23.32 \cdot x_4 + 1900 \cdot x_{10} + 8.18 \cdot x_{12} + 1.44 \cdot x_{14}) \cdot \mathbf{0.66} \quad (18)$$

$$\textbf{\textit{C}} = (50000 \cdot x_{10} + 21.9 \cdot x_{13}) \cdot \mathbf{0.56} \quad (19)$$

$$\textbf{\textit{D3}} = (0.95 \cdot x_1 + 2660 \cdot x_{10}) \cdot \mathbf{0.74} \quad (20)$$

Vitamin block restrictions: (a) thiamine (vitamin B1) is 0.15–0.2 mg; (b) riboflavin (vitamin B2) is 0.17–0.21 mg; (c) niacin (PP, vitamin B3) is 1.83–2.7 mg; (g) vitamin C is 10–15 mg; (d) vitamin B6 is 0.17–0.3 mg; (e) pantothenic acid (vitamin B5) is 0.6–0.7 mg.

4. Mineral substances block (Ca, K, Na, Mg, Fe, Zn, Cu, I).

$$\textbf{\textit{Ca}} = (8.681 \cdot x_1 + 4706 \cdot x_9 + 4.5 \cdot x_{14}) \cdot \mathbf{0.91} \quad (21)$$

$$\textbf{\textit{K}} = (368.188 \cdot x_1 + 11176 \cdot x_9) \cdot \mathbf{0.72} \quad (22)$$

$$\textbf{\textit{Na}} = (108.002 \cdot x_1 + 12 \cdot x_7 + 43 \cdot x_8 + 7059 \cdot x_9 + 0.9 \cdot x_{14}) \cdot \mathbf{0.86} \quad (23)$$

$$\textbf{\textit{Mg}} = (28.537 \cdot x_1 + 1353 \cdot x_9 + 1 \cdot x_{14}) \cdot \mathbf{0.96} \quad (24)$$

$$\textbf{\textit{Fe}} = (3.673 \cdot x_1 + 94 \cdot x_9 + 0.0012 \cdot x_{14}) \cdot \mathbf{0.93} \quad (25)$$

$$\textbf{\textit{Zn}} = (4.979 \cdot x_1 + 1200 \cdot x_{10}) \cdot \mathbf{0.85} \quad (26)$$

$$\textbf{\textit{Cu}} = (0.126 \cdot x_1 + 150 \cdot x_{10} + 0.0006 \cdot x_{14}) \cdot \mathbf{0.99} \quad (27)$$

$$\textbf{\textit{I}} = (15.96 \cdot x_{10}) \cdot \mathbf{1.00} \quad (28)$$

Mineral substances block restrictions: (a) sodium (Na) is 120–134 mg; (b) potassium (K) is 190–234 mg; (c) calcium (Ca) is 80–112 mg; (d) magnesium (Mg) is 23–30 mg; (d) iron (Fe) is 1.6–2.4 mg; (e) zinc (Zn) is 1.2–1.8 mg; (g) copper (Cu) is 0.15–0.27 mg; (h) iodine (I) is 13.3–20 μg.

Each equation includes weighting factors obtained and verified during real experiments under real food production conditions. These factors quantitatively characterize the losses of specific nutrients at various stages of the technological process (e.g., during heat treatment).

For each block, restrictions were imposed based on medical and biological requirements for the composition of products for patients with traumatic brain injuries.

Execution of the program code resulted in 479 iterations (generations) of the evolutionary algorithm. Generation 479 proved to be the best, achieving the target

ranges of key nutrient content based on the specified optimization criteria. Maximizing the energy value of the product was used as the fitness function. The resulting optimization yielded a caloric value of approximately 129 kcal per 100 g of the product. The calculated nutritional value of the optimized composition per 100 g: 1) protein is 6.22 g; 2) fat is 4.91 g; 3) carbohydrates are 15.00 g.

These values comply with the established restrictions and confirm the effectiveness of the applied algorithm in solving the problem.

Figure 4 shows a 3D visualization of the dependence of the fitness function on the content of proteins and fats, reflecting the trajectory of solution improvement during the evolution of the population.

The 3D model visualizes the evolutionary trajectory of the system, showing formula changes across generations, and also allows tracking the values of key nutritional parameters (protein, fat, and caloric content) at each point in space. The latter parameter is used as the fitness function of the algorithm. The model also supports the visualization of other food nutrients.

For example, protein may be characterized by its profile of essential and nonessential amino acids. Figures 5 and 6 show point clouds demonstrating all possible combinations of amino acid content obtained during the genetic algorithm's transition from the initial random formula to the optimized one. Examples include branched-chain amino acids (BCAAs), as well as aromatic amino acids and their precursors.

The red line indicates the trajectory of formula improvement toward the optimum.

To compare the effectiveness of the classical genetic algorithm, two evolutionary algorithms were also used: particle swarm optimization (PSO) and sparrow search algorithm (SSA). Table 1 presents the semantic analogy between PSO/SSA terminology and the elements of food system simulation.

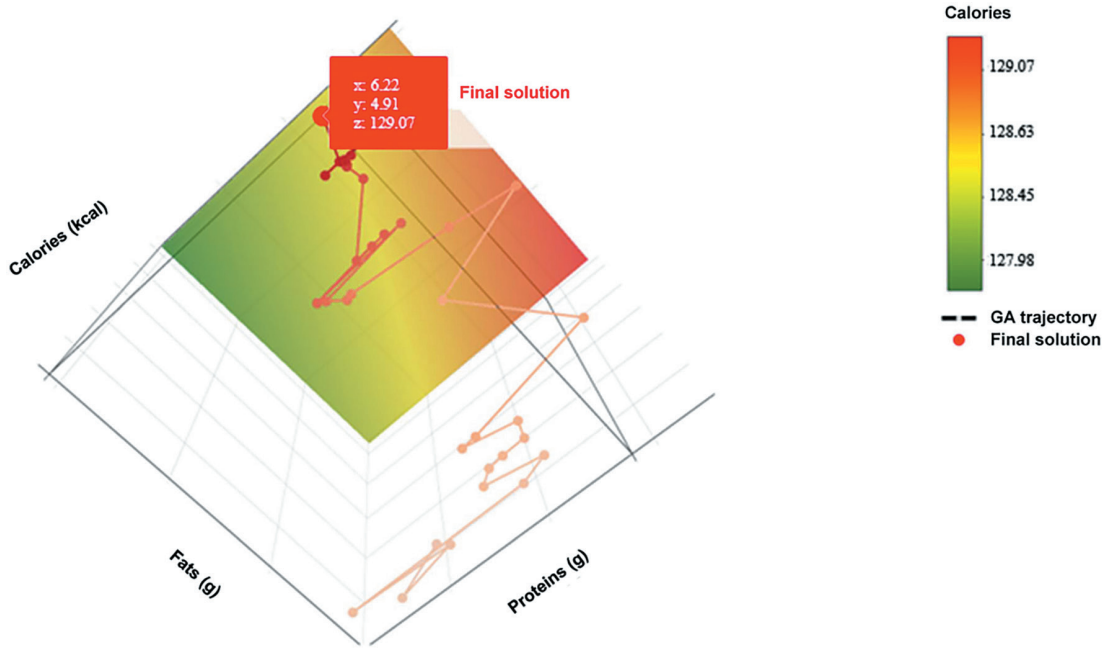
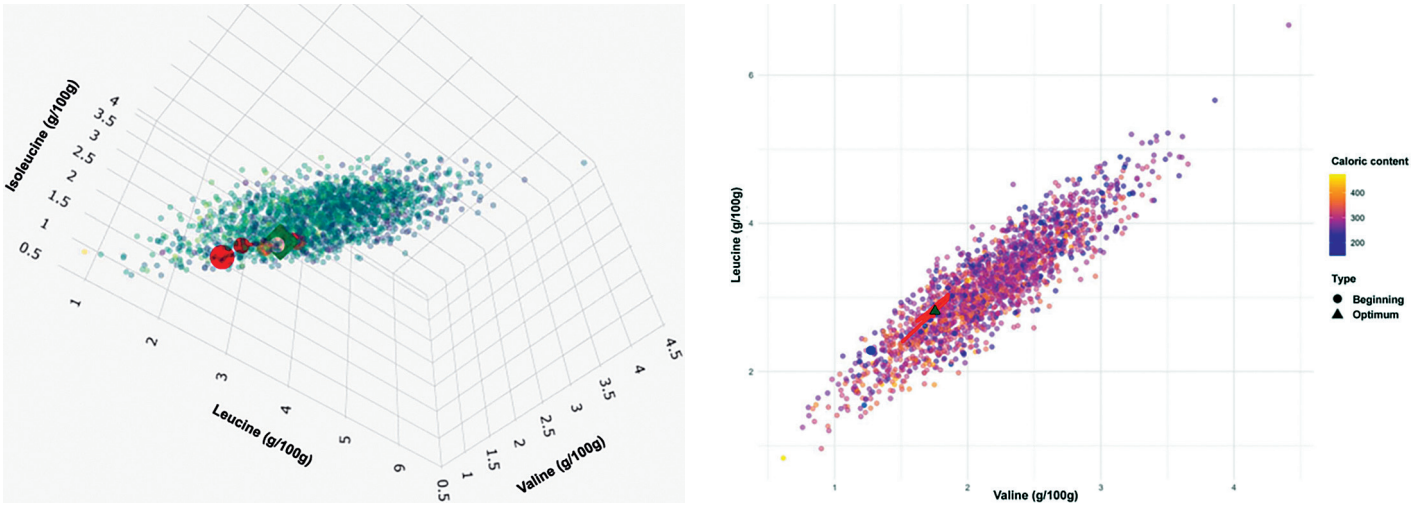


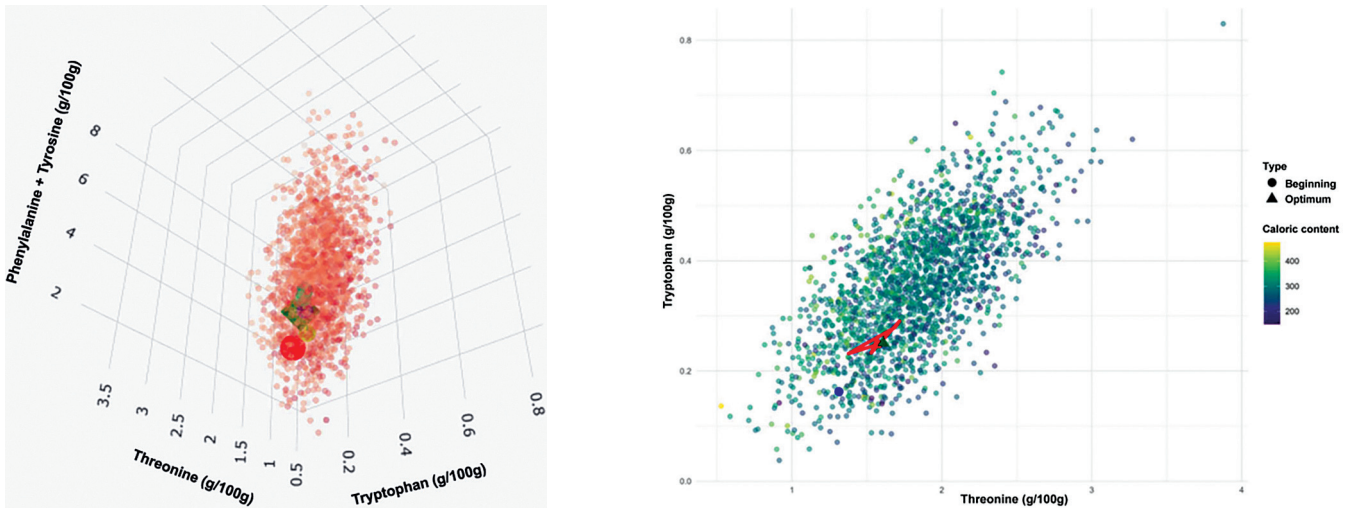
Figure 4. 3D chart of the fitness function versus fat and protein content in the food system: generational improvement trajectory and final solution



Valine-Leucine-Isoleucine

Valine-Leucine 2D cross-section

Figure 5. Virtual space of amino acids



Tryptophan-Threonine-Phenylalanine + Tyrosine

Tryptophan-Threonine 2D cross-section

Figure 6. Virtual space of amino acids

Table 1. Correspondence between PSO/SSA terminology and food system simulation concepts

PSO term	SSA term	Interpretation in the food system model
Particle	Sparrow	Formula variant (set of ingredients, process parameters)
Position	Position	Point in parameter space (specific values of nutrient content, mass fractions, processing modes) or Vector of component percentages
Velocity	— (movement without a direct velocity analog)	Formula change step from the current state to a new one or Direction and magnitude of the composition change
Individual best experience (pbest)	— (functionally corresponds to the producer's memory of the best state)	Best fitness function value achieved by a given formula variant in previous iterations
Global best experience (gbest)	Best producer	Optimal formula found so far among all variants
—	Producer	Active search for a new solution, i.e. a scenario in which the formula is purposefully modified (e.g., increasing protein)
—	Scrounger	Adoption of a successful solution, i.e. copying or slightly modifying the best formula
—	Scout	Random search/anti-stagnation, i.e. abrupt formula change to escape the local optimum
Fitness	Fitness	Target product quality indicator: maximizing nutritional value, minimizing losses, cost, and deviation from the standard
Premature convergence	Local optimum trap	Stopping the search on a suboptimal formula without further improvement
Search space	Search area	Set of acceptable combinations of ingredients and processing modes (restrictions regarding safety, sensory properties, regulations)

PSO algorithm yielded an optimal solution, visualized in Figure 6. The highlighted area (red square) shows the nutrient values for the optimal formula: protein (x) + 6.30 g, fat (y) + 5.10 g, and carbohydrates (z) + 14.81 g per 100 g of product. The calculated energy value is 130 kcal per 100 g.

These values meet the medical and biological requirements for specialized nutritional products intended for the rehabilitation of patients with traumatic brain injury (TBI). Compared to the solution obtained using a classical genetic algorithm (GA), the optimum achieved by PSO method is characterized by higher target nutrient values: protein content increased by 0.78 g and fat increased by 0.19 g (per 100 g). The resulting increase in nutritional value is statistically significant ($p < 0.05$) and has an apparent dietary value in the context of nutritional support for TBI.

The gray dots in Figure 7 represent the set of intermediate formulas (2,341 iterations in total) explored during the directed search for the optimum. The search trajectory,

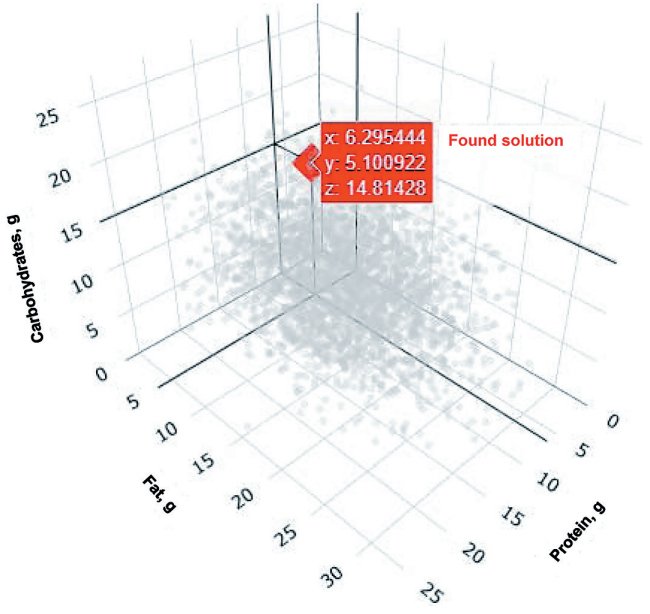


Figure 7. Visualization of the search space for the optimal solution

represented by the set of gray dots, demonstrates the stable convergence of PSO algorithm to the global optimum areas, while the spread of fitness function values at the final iterations does not exceed 2.1%. The absence of pronounced local optimum clusters indicates the effectiveness of the chosen inertial weighting and cognitive-social balancing strategies.

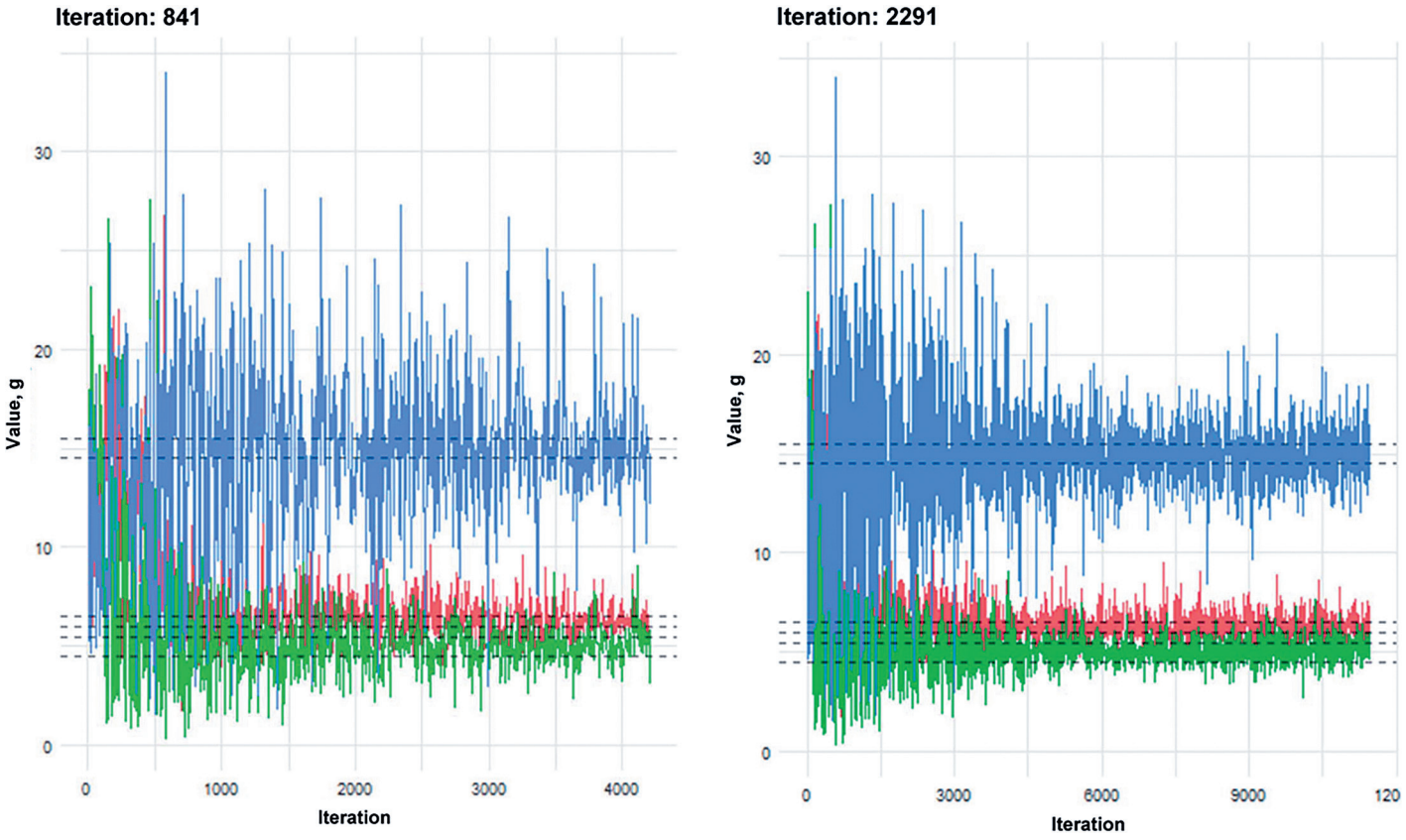
Using SSA algorithm, we obtained an alternative optimal SMP formula with the following composition (per 100 g of product): protein ≈ 6.50 g, fat ≈ 5.40 g, carbohydrates ≈ 14.50 g. The energy value of this variant was ≈ 133 kcal/100 g. Comparative analysis showed that the solution found by SSA algorithm exceeds the results obtained using GA and PSO in protein content by 0.28 g and 0.20 g, respectively. In terms of fat content, the advantage is 0.49 g relative to GA and 0.30 g relative to PSO. Moreover, the carbohydrate content was lower by 0.50 g and 0.31 g, respectively, which does not conflict with the dietary requirements for products of this class and is compensated by the increased proportion of the protein and lipid component.

Figure 8 shows the dynamics of changes in the content of key macronutrients (protein, fat, and carbohydrates) during the iterative search for the optimal formula using SSA algorithm. Visualization of the trajectories allows to trace the algorithm's convergence and its ability to maintain a balance between exploration and exploitation of the solution space.

SSA algorithm demonstrated the highest efficiency in synthesizing an optimal SMP formula under conditions of uncertainty in the input raw material characteristics recorded in real time by the sensor system. Its high adaptability and resilience to variability in initial parameters make SSA a promising tool for intelligent decision support in the development of personalized food products.

A comparative analysis of GA, PSO, and SSA performance in the SMP formula optimization problem revealed significant differences in their search behavior, due to the conceptual features of each approach. The classical genetic algorithm, which simulates the mechanisms of hereditary variation and natural selection, demonstrated the ability to effectively explore the discrete space of formula combinations. Crossover and mutation operators generated new composition variants, including unconventional ones, as evidenced by the wide spread of intermediate solutions in early iterations (Figure 4). However, a relatively low convergence velocity was observed: reaching the optimum required over 800 generations, and the final solution was inferior in protein and fat content to the results obtained using swarm intelligence methods (PSO, SSA). This observation is consistent with a well-known limitation of GA, i.e. the need to fine-tune crossover and mutation probabilities to prevent premature stagnation or excessively slow drift [31,32].

In contrast, PSO algorithm ensured faster localization of promising search areas. Thanks to the cognitive-social learning mechanism and the inertial component, particle motion in the space of technological parameters was directed, which allowed an energy value of 130 kcal per 100 g to be achieved by the 340th iteration. However, as suggested by literature data [33,34,35], a decrease in swarm diversity and a tendency to concentrate around suboptimal areas were observed at



a) b)

Figure 8. Iterative process for changing nutrient content in a formula: red line, protein; green line, fat; blue line, carbohydrates, where a) 841 iterations, b) 2291 iterations

the final stage, limiting further improvement of the formula. The protein (6.30 g) and fat (5.10 g) contents in the solution obtained by PSO were lower than those obtained using SSA, confirming the vulnerability of classical PSO to premature convergence in multi-extreme landscapes.

The best results across all criteria were achieved using SSA algorithm. The hierarchical swarm structure it reproduced, including producers, scroungers, and watchers, ensured a qualitatively different balance between global exploration and local intensification. Producers actively explored new areas of the space, while scroungers concentrated around the most promising solutions, accelerating convergence. The watching detection mechanism played a critical role: upon detecting stagnation, some agents initiated random movement, allowing the population to leave the local optimum area and continue improving the formula. As a result, a composition variant was developed with increased protein (6.50 g) and fat (5.40 g) content while maintaining energy density within the specified range (≈ 133 kcal per 100 g). The obtained values meet stringent medical and biological requirements for products for the rehabilitation of patients with traumatic brain injury and outperform the results of GA and PSO in terms of the integral indicator of nutritional adequacy.

Thus, the comparative analysis confirms that SSA implements a more complex and adaptive model of collective intelligence, ensuring targeted management of diversification and search intensification. Given the variability of raw material characteristics recorded by the sensory system, this property takes on particular significance, allowing SSA to be considered a preferred intellectual support tool for the design of personalized food systems.

In addition, other key ratios were calculated. For example, the SFA: MUFA: PUFA ratio was found to be 1:3.39:1.81, and the linoleic (C18:2n-6) to linolenic (C18:3n-3) fatty acid ratio was 4.24:1. Maintaining these proportions is crucial for the physiological adequacy of the diet for patients with TBI, as the efficiency of their metabolism and, in particular, the normalization of lipid metabolism depend on the balance of fatty acids.

In addition to achieving the standard levels of macro- and micronutrients established in MR 2.3.1.0253-21[2], the study also determined the optimal proportions of mineral substances. The ratio of calcium, phosphorus, and magnesium was 1:0.9:0.29, respectively. This balance is a significant factor determining the efficiency of absorption of these elements in the body and the maintenance of acid-base balance.

Under natural conditions, there are no foods containing the full spectrum of nutrients necessary to meet human physiological needs. Therefore, combining various foods in the diet allows for optimal satisfaction of the body's need for physiologically active components [36].

[2] Methodological recommendations MR 2.3.1.0253-21 "Standards of physiological needs for energy and nutrients in various population groups of the Russian Federation" (approved by the Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing on July 22, 2021). Replaced MR 2.3.1.2432-08.

As Aguilera [37] notes, most modern food products are developed without a systematic engineering approach, which involves moving from a conceptual idea to a finished product based on scientifically valid methods and rigorous technological procedures. Over the past decade, the term "intelligent food design" has become established in scientific literature, implying the design of products that meet both functional nutritional requirements and consumer preferences [38,39].

Creating food systems with specified nutritional and functional health properties requires the application of modern scientific knowledge and technological solutions. In this study, metaheuristic algorithms were used to create one stage of the lifecycle of SMP digital twin.

Verification of the developed model by comparing the results of virtual simulation with data from a real experiment revealed a difference of 0.5–3.3 % for individual nutrient groups. The obtained convergence indicates that all input parameters, restrictions, and weighting factors were correctly considered in the model. The proven model allows for real-time prediction of changes in the chemical composition of a food system when any ingredient is varied, and in the event of an unsatisfactory result, it promptly suggests alternative formula modifications.

The digital twin is a quantum leap in the evolution of food engineering (food combinatorics), overcoming the key limitations of traditional simulation approaches [40]. Unlike classical simulation, which operates with static, predefined models and scenarios, the results of which are analyzed and implemented post-factum, the digital twin functions as a dynamic, "living" entity. Its fundamental difference lies in the ability to continuously update based on data flow from the physical object, as well as the ability to implement conclusions directly into the operational circuit. This is achieved through feedback via actuators or personnel, enabling prompt corrective measures and immediate action in real-world production conditions. Thus, the digital twin transforms simulation from an analysis and preliminary design tool into an active component of the control system, ensuring real-time process optimization and improving product quality.

Along with this, the digital twin significantly expands the conceptual and applied boundaries of computer simulation. While traditional methods such as virtual prototyping and "What... If..." scenario analysis primarily focus on the process and product design stages, the digital twin extends simulation and data processing capabilities to the entire product lifecycle. This technology provides a comprehensive, holistic understanding of not only the product's design but also its behavior throughout its entire lifecycle. Consequently, the digital twin may be considered as a way to bridge the gap between the design-oriented digital space and the operation-oriented physical world, creating a continuous digital circuit that supports decision-making at both the strategic (design, planning) and tactical (operational management, optimization) levels.

Conclusion

Using the digital twin, a highly detailed virtual dynamic model of the SMP, enables real-time prediction of the final product's characteristics. This enables rapid adjustments to formulas and process parameters to meet the medical and biological requirements for a food system designed for the rehabilitation of patients with TBI. The digital twin enables rapid formula transformation for transferring production between facilities and adapting it to new equipment. The model simulates the product's physicochemical properties at all stages of production, creating the basis for predictive analysis and optimization of process modes. Thus, the use and implementation of the digital twin improve the flexibility, manageability, and efficiency of specialized food production, ensuring compliance with strict medical standards and accelerating adaptation to changing production conditions.

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OPTIMIZING JAPANESE BAMBOO LEAF PULP TREATMENT OF FRESH BEEF

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Abstract
This study evaluated the preservative potential of Japanese bamboo leaf (Dracaena surculosa Lindl.) pulp to extend the refrigerated shelf life of beef. Beef cubes were soaked in aqueous pulp at 50 % and 75 % (w/w) for 1, 2, or 3 hours, then stored at 5 °C and tested on days 0, 5, and 10. A test for saponin availability produced a stable foam (2 cm for 5 minutes), thus confirming presence of saponins in the pulp. Measured parameters were color (CIE L, a*, b*), texture (hardness, gf), pH, total protein and total plate count (TPC). The experiment used a two-factor completely randomized design of the trial, with data analysis of ANOVA followed by Honestly Significant Difference (HSD) test at the 5 % significance level. The results showed that pulp concentration, soaking time, and their interaction significantly affected several parameters ($P < 0.05$). The 75 % pulp reduced texture loss versus control sample, with the 75 % and 2 h soaking as the most effective combination. Although pH increased during storage for most samples, pH remained comparatively stable in 75 % cases (5.58–5.85 to day 5). Higher pulp concentration delayed microbial growth: on day 10 TPC in the 75 % treatments was 2.5×10^6 CFU/g compared with 4.5×10^6 CFU/g in the control. In conclusion, Japanese bamboo leaf pulp at 75 % with a 2 h soaking shows promising capacity as a natural shelf-life extender for chilled beef; further work on sensory evaluation and mechanistic studies is recommended. These findings support sustainable, plant-based preservation strategies for meat industry.*

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Introduction

Beef is considered an essential food product for satisfying human protein requirements due to its high-quality protein content. It contains a complete profile of essential amino acids that are vital for numerous physiological functions [1]. Since these amino acids cannot be synthesized endogenously, they must be obtained through dietary intake. Proteins found in beef are not only well-balanced in amino acid composition, but are also highly bioavailable, thus making them an effective source for supporting tissue development and resilience, maintenance and functioning of immune system [2]. Thus, the incorporation of beef into a balanced diet plays a significant role in enhancing nutritional status and promoting overall health.

Highly available protein of beef profoundly influences its microbiological quality and shelf life. Beef provides a rich supply of nutrients (amino acids, water, and fat) that support rapid microbial growth [3]. Microbial activity mostly driven by proteolytic metabolism that cleave muscle protein of beef into peptides and amino acids, which are further broken down into sulfurous and nitrogenous compounds (amines, hydrogen sulfide) that in their turn produce off-flavors and mucus associated with spoilage [4]. Due to these attributes, fresh beef typically features a limited shelf life. To address this issue, various preservation methods have been explored, including the use of chemical preservative agents. Several previous studies have shown that certain natural ingredients such as ginger (*Zingiber officinale*) [5], *Citrus microcarpa* fruit extract [6], bay leaf (*Syzygium polyanthum*) [7], teak leaf (*Tectona grandis*) [8], and bamboo leaf (*Gigantochloa apus)* [9] can be applied for beef preservation. Another natural ingredient with potential application in beef preservation is Japanese Bamboo leaf (*Dracaena surculosa* Lindl).

Japanese Bamboo leaf (*Dracaena surculosa* Lindl), as by-products, are often underutilized. Their application as natural preservatives in meat products have already received limited attention within current preservation practices. Among the few studies addressing this topic [9], promising results were reported for using *Gigantochloa apus* bamboo leaf extract to prolong the shelf life of beef stored under refrigerated conditions. This finding suggests that bamboo-derived compounds may possess antimicrobial or antioxidant activities beneficial for reducing spoilage and maintaining meat quality. While direct studies on properties of *D. surculosa* are limited, research on close relatives within the Dracaena genus, namely *Dracaena indivisa* leaf extracts, demonstrates its notable potential as an antibacterial agent [10].

Japanese bamboo leaf (*Dracaena surculosa* Lindl.) contains a rich profile of phytochemicals, such as flavonoids (e.g. quercetin, kaempferol), tannins, oleanolic acid, and

notably steroidal saponins that exhibit significant antioxidant and antimicrobial activities [11,12]. Studies of various plant saponins in meat or food systems highlight that its source and structure matter. For instance, quillaja (Soapbark tree) and guar (*Cyamopsis*) saponin extracts showed the strongest antibacterial action in one study, significantly inhibiting growth of *S. aureus* and other bacteria, whereas yucca and soybean saponins were much less active, especially against Gram-negative microbes [13].

In general, Japanese bamboo leaves show strong potential as a natural beef preservative due to leveraging their saponin-rich profile to inhibit spoilage microbes and oxidation. Given its potential, the current study aims to explore the preservative efficacy of *Dracaena surculosa* leaf pulp when applied to fresh beef under refrigerated storage conditions. Specifically, this research will investigate the antimicrobial activity of the pulp against common spoilage bacteria, its ability to delay oxidative degradation, and its influence on key physicochemical parameters such as texture, color, pH, protein, and total plate count of microbes in refrigerated fresh beef.

Material and methods

Samples preparation

Fresh Japanese bamboo leaves were sorted, rinsed under running water to remove debris, then crashed and blended with distilled water at a 1:2 (w/v) ratio until a homogeneous pulp formed. Beef samples were trimmed of visible fat and connective tissue, then cut into uniform cubes measuring 2×2×1 cm to standardize marinade absorption.

Soaking treatment, storage, and analysis

Beef cubes were immersed in the bamboo leaf slurry at a meat-to-slurry ratio of 1:3 (w/v) for 30 minutes at ambient temperature. After being soaked, samples were drained on a sterile rack, individually wrapped in aluminum foil, and stored at 5 °C for 10 days. Quality assessments including instrumental color (L*, a*, b*), texture profile analysis (hardness), pH measurement, total protein content (Kjeldahl method), and total microbial count (Total Plate Count) were conducted on days 0th, 5th, and 10th of storage according to standard procedures [14], with minor adjustments to culture media and pH calibration.

Saponin qualitative analysis

Japanese bamboo leaf pulp was vigorously shaken with distilled water for 30 seconds. The presence of saponins can be detected by observing the formation of stable foam lasting for minimum 30 seconds, with a thickness ranging from 1 cm to 3 cm high [15].

Color analysis

Color analysis was performed using a color reader device model CR-400 (Konica Minolta, Japan) based on the CIE Lab method to measure L*, a*, b*, and ΔE* values. The color reader was first turned on and calibrated by placing the optical head against a standard white plate. Next, the L*, a*, and b* function buttons were activated from the menu options. The sample was then placed in a transparent container (clear plastic), positioned against the optical head, and the start button was pressed. Finally, the L*, a*, and b* values displayed on the device were recorded [16].

Texture analysis

Texture measurement for hardness was conducted using a texture analyzer (Brookfield LFRA Texture Analyzer, AMETEK Brookfield, USA). First, a needle-type probe (TA 9) was attached to the texture analyzer. The sample was then placed directly beneath the needle probe. The analyzer was set to a speed of 0.1 mm/s, with a penetration distance of 5 mm and a trigger force of 10 g. The probe pierced the surface of the sample, and the final load value, expressed in gram force (gf), displayed on the device was recorded.

pH analysis

pH (Mettler Toledo, FiveEasy F20, Metler Toledo, Switzerland) was determined in the following way [17]. Twenty grams of sample were mixed with 50 mL of distilled water, then homogenized until getting uniform and smooth condition. The pH of the resulting homogenate was measured with a pH meter that had been calibrated immediately beforehand using standard buffer solutions at pH 4.0 and pH 7.0. For each measurement the electrode was immersed into the sample solution and the pH value was recorded once the reading value got stabilized.

Protein content analysis

Protein content value was determined by the Kjeldahl method [17]. A sample with mass of 0.20 g was dissolved with 10 mL H_2SO_4 and 2 g catalyst until clear, diluted, turned to alkaline with 5 mL 45 % NaOH, distilled into 10 mL 2 % boric acid containing indicator, and titrated with 0.01 N HCl; protein (%) was calculated as % of protein.

Microbiology analysis (total plate count)

Total bacterial count was determined following the standard SNI 2897:2008 [18]. Plate Count Agar (PCA) was prepared and sterilized. A 10 g sample was placed in a sterile bag with 90 mL Buffered Peptone Water (BPW 0.1 %) and homogenized (stomacher) for 1 min to obtain a 10^{-1} suspension. Serial decimal dilutions were made to 10^{-6} by transferring 1 mL into 9 mL BPW. For dilutions 10^{-4}–10^{-6}, 1 mL aliquots were pour-plated with 15 mL molten PCA. Plates were allowed to solidify and incubated in inverted position at 36 °C for 24 h. Plates with 25–250 colonies were counted. Bacterial count was calculated as:

$$\frac{CFU}{g} = colony\ count\ x\left(dilution\ factor\right)^{-1}. \quad (1)$$

Statistical analysis

This study was conducted using a completely randomized design (CRD) with a two-factor factorial arrangement. Factor A is the concentration of Japanese bamboo leaf pulp at two levels (A_1 = 50 % w/w, A_2 = 75 % w/w) and factor B is

the soaking time at three levels (B_1 = 1 h, B_2 = 2 h, B_3 = 3 h). Data were analyzed using analysis of variance (ANOVA) to assess treatment effects and their interaction. Significant differences ($P < 0.05$) were further evaluated using the Honestly Significant Difference (HSD) test at the 5 % level.

The experiment used a two-factor completely randomized design (CRD), with factor A is the concentration of Japanese bamboo leaf pulp at two levels (A_1 = 50 % w/w, A_2 = 75 % w/w) and factor B the soaking time at three levels (B_1 = 1 h, B_2 = 2 h, B_3 = 3 h). Each treatment combination was replicated in a randomized method.

Results and discussion

Saponin qualitative analysis

Figure 1 illustrates the qualitative detection of saponins in Japanese bamboo leaf pulp (*Dracaena surculosa* Lindl). A fresh Japanese bamboo leaf pulp was vigorously shaken with distilled water for 30 seconds; the immediate formation of a uniform foam layer confirmed the presence of saponin compounds. Notably, the foam reached an approximate height of 2 cm and remained stable for approximately 5 minutes before gradually dissipating, a duration that is characteristic of medium-to-high saponin content in plant extracts.

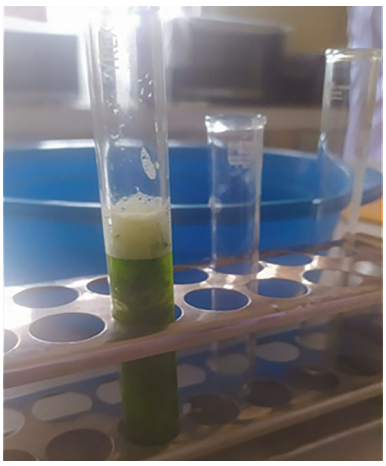

Figure 1. Saponin qualitative analysis of Japanese bamboo leaf pulp

The qualitative screening for saponins presence in Japanese bamboo leaf pulp was carried out to confirm the presence of these surface-active glycosides in the sample. These qualitative observations provide a foundation for subsequent quantitative assays (e.g., spectrophotometric or chromatographic techniques) to determine actual saponin concentrations and to correlate foam stability dynamics with bioactivity profiles. The immediate formation of a stable foam layer, typically exceeding 1 cm in height and persisting for at least 5 minutes, served as a positive indication of saponin compound presence in the sample. The persistence of the foam is attributable to the amphiphilic nature of saponins, which reduces surface tension and trap air bubbles, and serves as a convenient preliminary indicator of saponin abundance and surface-active potential [19].

Texture

Mean texture values of beef treated with various concentrations of Japanese bamboo leaf pulp during various soaking time are presented in Table 1.

Table 1. Texture value (gf) of beef sample during storage

Treatments	Observation day		
	Day 0	Day 5	Day 10
A_0 (Control)	115.50	98.96	82.23
A_1B_1	71.98^a	64.43^b	58.60^b
A_1B_2	76.46^a	56.53^a	48.16^a
A_1B_3	72.13^a	56.63^a	55.66^b
A_2B_1	84.4^a	65.46^b	56.90^b
A_2B_2	89.06^a	67.26^c	58.66^b
A_2B_3	88.93^a	64.96^b	58.93^b

A_1: 50 % pulp concentration; A_2: 75 % pulp concentration; B_1: 1 hour soaking time; B_2: 2 hours soaking time; B_3: 3 hours soaking time. Values followed by different letters within the same column indicate statistically significant differences based on 5 % Honestly Significant Difference (HSD) post hoc test.
Numbers followed by different letters indicate a significant difference ($P < 0.05$).

Texture condition decreased progressively in all treated samples throughout storage. Statistical analysis showed that the combination of pulp concentration and soaking time significantly affected texture on day 5 ($P < 0.05$), the highest texture value was observed in the sample A_2B_2 (75 % pulp concentration, 2 h soaking time), whereas the samples A_1B_2 and A_1B_3 exhibited the lowest values. On day 10, only the sample A_1B^2 differed significantly from the other treatments, while the remaining samples showed comparable texture values.

These results indicate that bamboo leaf pulp delays beef tenderization, as higher marinade concentrations result in firmer beef during chilled storage. Beef texture deteriorates during storage as a result of several factors, including the duration of storage, chilling temperature, and any treatments applied to the beef. Extended storage periods lead to measurable declines in shear force values and overall firmness which in this study stated as texture. Incorporating Japanese bamboo leaf pulp into beef soaking has shown its ability to enhance texture values. The protective effect likely stems from the leaf's tannins/flavonoids inhibiting muscle proteases, as seen by the firmer texture of 75 % pulp samples (e.g. A_2B_2 on day 5). In poultry models, dietary bamboo leaf flavonoids significantly improved meat texture profiles, boosting elasticity and gumminess while modulating protein secondary structures [20].

However, extended soaking times, even in the presence of inhibitor-rich bamboo leaf, eventually lead to a net decrease in texture (gf), as prolonged soaking disrupts protein cross-links and promotes partial hydrolysis of muscle fibers and connective tissues [21]. Natural tenderization occurs throughout refrigerated storage due to endogenous proteolytic enzymes, particularly cathepsin B and L that remain active at low temperatures. Through proteolytic hydrolysis of myofibrillar and connective-tissue proteins, these enzymes progressively break down structural muscle

components and thus soften the beef [22]. Moreover, saponins present in Japanese bamboo leaf further promote tenderization through membrane-permeabilizing effects, which enhance enzyme access to intracellular substrates, thus contributing to texture changes during marination [23]. Practically, marinating with sufficient bamboo pulp can maintain beef firmness during early storage, but extended marination or aging will eventually reduce shear force. Future work should quantify pulp phenolics, include replicate measurements for taking error into consideration, and assess sensory impact (e.g. flavor, color) alongside with texture.

Color

Lightness

Table 2. Lightness values of beef samples during storage

Treatments	Observation day		
	Day 0	Day 5	Day 10
A_0 (Control sample)	35.98	34.48	25.69
A_1B_1	33.63^a	36.94^d	26.03^b
A_1B_2	37.83^b	34.12^c	23.73^a
A_1B_3	38.54^b	41.51^e	28.30^c
A_2B_1	26.82^a	21.31^a	35.15^d
A_2B_2	28.03^a	25.64^b	37.92^d
A_2B_3	25.09^a	24.65^b	36.98^d

A_1: 50 % pulp concentration; A_2: 75 % pulp concentration; B_1: 1 hour soaking time; B_2: 2 hours soaking time; B_3: 3 hours soaking time. Values followed by different letters within the same column indicate statistically significant differences based on 5 % Honestly Significant Difference (HSD) post hoc test.
Numbers followed by different letters indicate a significant difference ($P < 0.05$).

The CIELAB lightness parameter (L*) quantifies sample brightness on a continuous scale from 0 (black) to 100 (white) [24]. In the present study, the mean L* values of control sample and treated beef samples on day 0 ranged from 25.09 to 38.54, indicating a moderate baseline lightness. After five days of refrigerated storage, high-pulp samples remained darkest (L* ≈ 21–26), while the sample A_1B_3 (50 % pulp, 3 h) was brightest (41.51) and significantly higher than all others. By day 10 the pattern reversed, the 75 % pulp groups became the brightest (L* ≈ 35–38) and were significantly higher than most 50 % groups (the sample A_1B_2 remained lowest at 23.73). Thus, higher bamboo-pulp marinade yielded darker beef initially but lighter color after prolonged storage. The effects of Japanese bamboo leaf pulp concentration and soaking time on L* across the three storage intervals are detailed in Table 2.

The results indicate that prolonging storage time leads to a decrease in the lightness (L*) of beef. Numerous studies have reported a progressive decline in L* values along with increasing storage time, as oxidation of the surface pigments and shifts in myoglobin chemistry reduce reflectance (i.e., brightness) over the beef surface [25]. This reduction in L* is closely related to beef pH. Beef with higher pH values (> 6.0) tends to exhibit darker, less-bright surfaces. For instance, the sample A_1B_2 (50 % Japanese bamboo leaf pulp, 2 h marination) exhibited an L* of 23.73 together with a comparatively high pH of 6.42. Elevated pH stabilizes myoglobin in its reduced or deoxygenated forms and promotes metmyoglobin accumulation, both of which absorb more light and lower L* value [25]. Adding higher concentrations of Japanese bamboo leaf pulp causes a further drop in beef's L* value.

Redness

Table 3. Redness values of beef samples during storage

Treatments	Observation day		
	Day 0	Day 5	Day 10
A_0 (Control)	15.58	15.7^a	10.07
A_1B_1	4.82^a	8.24^a	15.04^c
A_1B_2	4.58^a	1.31^a	-1.2^a
A_1B_3	2.14^a	10.39^a	1.75^b
A_2B_1	-4.83^a	0.47^a	0.05^a
A_2B_2	-6.99^a	-7.44^a	1.24^b
A_2B_3	-5.70^a	-4.37^a	0.92^a

A_1: 50 % pulp concentration; A_2: 75 % pulp concentration; B_1: 1 hour soaking time; B_2: 2 hours soaking time; B_3: 3 hours soaking time. Values followed by different letters within the same column indicate statistically significant differences based on 5 % Honestly Significant Difference (HSD) post hoc test.
Numbers followed by different letters indicate a significant difference ($P < 0.05$).

The effect of Japanese bamboo leaf pulp concentration and soaking time on the measured parameter (e.g., Δ value) was dependent on both treatment and storage (Table 3). The untreated control (A_0) maintained relatively stable values of redness on day 0 (15.58), day 5 (15.70), and declined modestly by day 10 (10.07), indicating only minor change over time. Over time, A_1B_1 (50 % pulp, 1 h) showed a strong increase in a*, becoming highest by day 10 (15.04) significantly above all others. In contrast, A_1B_2 (2 h) declined to negative a* (–1.20). The 75 % pulp samples (A_2 series) started with negative a* (greenish) and remained near zero. Overall, a* increased in early storage (consistent with oxymyoglobin formation) but treatments with more bamboo pulp showed less redness (or even greenness) in all cases.

Table 3 indicates that a* values of beef samples increased throughout refrigerated storage. This phenomenon is closely related to the presence of myoglobin, the primary heme pigment responsible for the red coloration in muscle tissues. Myoglobin, when exposed to oxygen during storage, undergoes oxidation to form oxymyoglobin, the ferrous heme form responsible for the bright red "bloom" under aerobic, low-temperature conditions [26]. The findings of this study align with these mechanisms. For instance, in the A_1B_1 treatment group (50 % Japanese bamboo leaf paste concentration with 1-hour soaking), the a* value increased notably from 4.82 to 15.04 over the storage period. This rise indicates the enhanced formation of oxymyoglobin in the early stages of storage. By day 10, however, further oxidation of oxymyoglobin to ferric metmyoglobin (MetMb) induced a brownish-red discoloration characteristic of aged

beef [27]. Furthermore, increasing the concentration of Japanese bamboo leaf paste resulted in a reduction of the a* value, indicating a decline in redness and a shift towards greenness. This effect can be attributed to the high chlorophyll content in the dark green leaf of bamboo. Chlorophyll, a green pigment predominant in plant tissues, can provide a greenish hue to the surface of beef products, particularly when applied in high concentrations.

Yellowness

Table 4. Yellowness values of beef samples during storage

Treatments	Observation day		
	Day 0	Day 5	Day 10
A_0 (Control)	5.59	5.79	5.54
A_1B_1	12.46^a	14.78^a	12.71^b
A_1B_2	12.44^a	16.19^a	22.77^d
A_1B_3	12.19^a	17.54^b	17.95^c
A_2B_1	29.96^b	24.99^c	10.92^a
A_2B_2	28.90^b	29.63^e	11.43^a
A_2B_3	34.68^c	26.96^d	10.65^a

A_1: 50 % pulp concentration; A_2: 75 % pulp concentration; B_1: 1 hour soaking time; B_2: 2 hours soaking time; B_3: 3 hours soaking time. Values followed by different letters within the same column indicate statistically significant differences based on 5 % Honestly Significant Difference (HSD) post hoc test.

Numbers followed by different letters indicate a significant difference ($P < 0.05$).

The CIELAB chromaticity coordinate b* quantifies the yellow-blue axis, with values ranging from –70 (blue) to +70 (yellow) [24]. On day 0 the 75 % pulp groups had the highest yellowness (A_2B_3 + 34.68, A_2B_1 + 29.96), being significantly above the 50 % groups (≈12.2–12.5). Day 5 showed a similar ranking, when the sample A_2B_2 was highest in yellowness (29.63), followed by the sample A_2B_3 (26.96), then the sample A_2B_1 (24.99), while A_1 samples were intermediate (14–17). By day 10, the trend inverted: the sample A_1B_2 (50 % pulp, 2 h) became the most yellow (22.77), whereas all A_2 samples fell to ≈10–11 and were significantly lower than the samples A_1B_2-B_3. In summary, bamboo pulp initially boosted beef yellowness (due to leaf carotenoids), but this effect faded over 10 days, with higher-pulp samples ending up less yellow than some low-pulp processed samples.

Table 4, illustrates that increasing concentrations of Japanese bamboo leaf pulp resulted in a higher b* value (yellowness) of beef on day 0 of storage. This initial increase in b* is likely due to the presence of carotenoid pigments in the bamboo leaf, which can be transferred to the beef surface during the marination process. Carotenoids are natural pigments responsible for yellow to orange coloration in plants and are known to influence the color attributes of beef when applied externally. Tushar et al. [28] also reported that adding bamboo sprouts dietary fiber increased the b* value of emulsified sausages. The initial enhancement of yellowness can be attributed to the abundant carotenoids in the bamboo leaf pulp, which, when being combined with the relatively low myoglobin content of freshly treated beef, amplify the yellow chromaticity [29,30]. However, by day 10th of storage, an inverse trend was observed, with a decrease in b* values in the samples treated with higher bamboo leaf pulp concentrations. This may be due to increased accumulation of myoglobin in the muscle matrix and progressive depletion of β-carotene from the bamboo leaf this way causing a decrease in the b* value [30]. First, the progressive oxidation of myoglobin into metmyoglobin reduces yellow tones. Metmyoglobin formation is well documented to shift beef color toward brownish hues, diminishing both redness (a*) and brightness, which can indirectly suppress b* values [31]. Indeed, a study on beef treated with chitosan-lycopene coatings found that increased metmyoglobin content correlated with a substantial 23.21 % reduction in b* after 8 days [31]. Table 4.15 further supports the role of soaking time, showing that longer soaking periods result in increased b* value (yellowness). This suggests that prolonged immersion facilitates deeper penetration of leaf-derived carotenoids into beef's muscle, thus providing a stronger yellow hue. Supporting studies underscore that plant extracts rich in pigments (and possessing antioxidant activity) can influence the final color of beef products, by safeguarding these pigments during processing and perhaps enhancing their binding to muscle tissues [12].

pH parameter

pH is a crucial indicator of meat quality, reflecting post-mortem biochemical changes and potential microbial activity during meat storage. Post-mortem pH decline in red meat is regulated by anaerobic glycolysis which converts glycogen to lactic acid, with the ultimate pH modulated by pre-slaughter stress and residual glycogen reserves [32]. pH rose from 5.50–5.83 (day 0) to 5.75–6.47 (day 10) in the control sample due to proteolytic release of basic metabolites by spoilage microflora [33]. The addition of Japanese bamboo leaf saponin at 25 %, 50 %, and 75 % significantly buffered this trajectory, with 75 % Japanese bamboo leaf pulp able to maintain pH within the optimal 5.3–5.8 range throughout till the day 5th. This effect arises from Japanese bamboo leaf flavonoids, tannins, and saponins, which provide mild acidification and inhibit microbial proteolysis [34,35]. Moreover, soaking time 1–3 h modulated saponin uptake via osmotic mechanisms, such as 2 h soaking time at 75 % of Japanese bamboo leaf pulp produced the least pH fluctuation (5.58–5.85) throughout till the day 5th. Maintaining muscle pH between 5.5 and 5.8 is essential for preserving water-holding capacity, textural integrity, and the redox state of myoglobin, thereby limiting metmyoglobin formation and color deterioration [31].

Based on Figure 2, both the concentration of Japanese bamboo leaf pulp and the soaking time significantly affected the pH values of beef stored for 0, 5, and 10 days. At the beginning of storage (day 0), pH values remained within the normal range (5.50–5.83), in accordance with SNI-01-3948-1995 [36]. During storage, a gradual increase

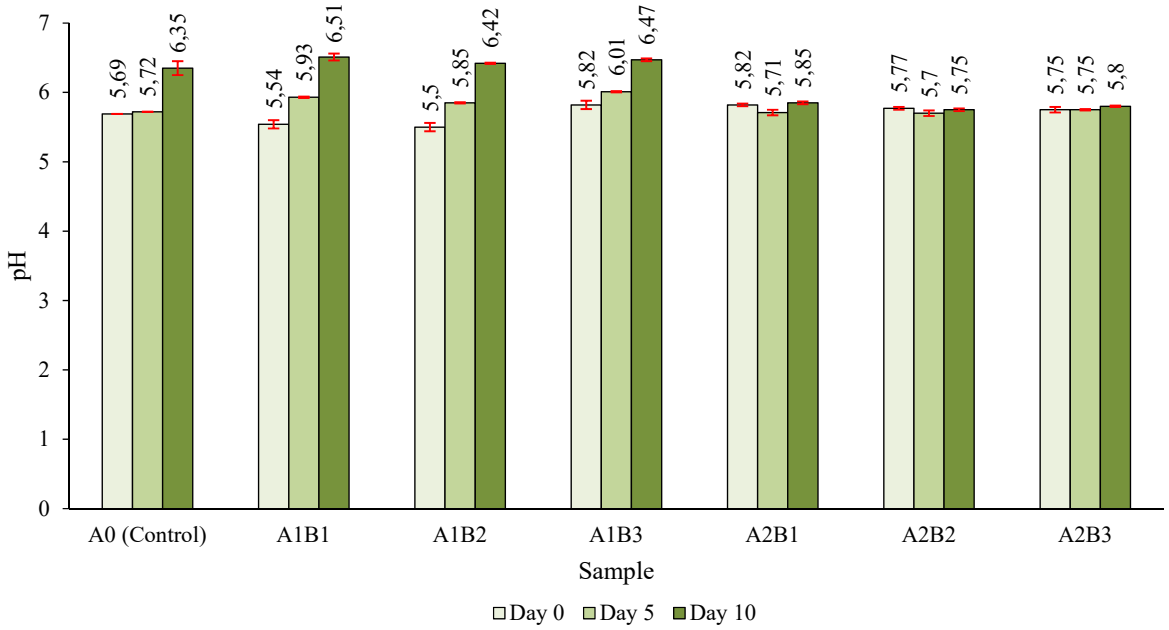

Figure 2. pH value of beef during cold storage. A0: control (0 % Japanese bamboo leaf pulp, 0 h soaking time); A_1: 50 % pulp concentration; A_2: 75 % pulp concentration; B_1: 1 hour soaking time; B_2: 2 hours soaking time; B_3: 3 hours soaking time

in pH was observed, reaching up to 6.47 by day 10. Soaking time also played a crucial role in changing pH value of the meat, where longer durations led to increasing pH. The correlation between concentration and soaking time was significant on days 0 and 5, indicating that both variables synergistically influenced the early phase of storage. However, by day 10, this correlation was no longer significant. The pH of the bamboo leaf pulp itself (5.58) may have contributed slightly to the initial increase in meat pH, particularly at higher treatment concentrations.

Table 5. Mean pH values of beef treated with Japanese bamboo leaf pulp concentrations during cold storage

Japanese bamboo leaf pulp concentrations	Day-0	Day-5	Day-10
A_1 (pulp concentration 50 %)	5.61[a]	5.93[b]	6.46[a]
A_2 (pulp concentration 75 %)	5.78[b]	5.71[a]	5.80[b]

A_1: 50 % pulp concentration; A_2: 75 % pulp concentration; B_1: 1 hour soaking time; B_2: 2 hours soaking time; B_3: 3 hours soaking time. Values followed by different letters within the same column indicate statistically significant differences based on 5 % Honestly Significant Difference (HSD) post hoc test.
Numbers followed by different letters indicate a significant difference ($P < 0.05$).

The concentration of Japanese bamboo leaf pulp significantly influenced beef pH throughout storage (Table 5). On day 0, the sample of the beef treated with 75 % pulp concentration exhibited a higher pH (5.78) than the sample of the beef treated with 50 % concentration (5.61). however, during storage, the samples treated with 75 % pulp maintained significantly lower pH values than those treated with 50 % pulp, reaching 5.71 and 5.80 on days 5 and 10, respectively. In contrast, the samples treated with 50 % concentration showed a continues increase in pH, reaching 6.46 by day 10. These findings suggest that the higher concentration of pulp was more effective in slowing pH deterioration during storage.

Similarly, soaking time significantly affected pH values at all observation periods (Table 6). On day 0, the sample treated with 3-hour soaking resulted in the highest pH (5.78), whereas the sample that was treated with 2-hour soaking duration produced the lowest pH (5.63). During storage, beef samples that were soaked for 2 hours consistently maintained lower pH values than the other samples, resulting pH values of 5.77 and 6.08 on days 5 and 10, respectively. Conversely, the samples processed with 1-hour soaking showed the greatest pH increase, reaching 6.18 by day 10. These results indicate that a moderate soaking duration may facilitate optimal absorption of bioactive compounds from bamboo leaves into the muscle tissue, thereby enhancing pH stability during storage [33].

Table 6. Mean pH values of beef samples as affected by soaking time during cold storage

Soaking time	Day-0	Day-5	Day-10
B_1 (soaking time 1 hour)	5.68[a]	5.81[b]	6.18[c]
B_2 (soaking time 2 hours)	5.63[a]	5.77[a]	6.08[a]
B_3 (soaking time 3 hours)	5.78[b]	5.88[b]	6.13[b]

A_1: 50 % pulp concentration; A_2: 75 % pulp concentration; B_1: 1 hour soaking time; B_2: 2 hours soaking time; B_3: 3 hours soaking time. Values followed by different letters within the same column indicate statistically significant differences based on 5 % Honestly Significant Difference (HSD) post hoc test.
Numbers followed by different letters indicate a significant difference ($P < 0.05$).

Protein content

The lowest protein content was observed in the samples treated with 50 % Japanese bamboo leaf pulp, 1 h soaking time, this reduced level of protein content may be due to water absorption into the muscle fibers, causing partial protein denaturation and dilution within the intra and extracellular spaces [37]. In contrast, the samples treated with higher concentrations of bamboo leaf pulp exhibited a consistent increase in protein content during storage.

This may be attributed to the presence of Japanese bamboo leaf, which are known to interact with proteins and increase protein-associated enzymatic activity [38]. Soaking time also affected protein levels which longer soaking time allowed greater diffusion of bioactive compounds and may have triggered structural changes in muscle proteins that increased content of detectable protein and its substrates [39]. Soaking time significantly influence protein retention and potential enhancement of its content in beef during storage.

As shown in Figure 3, protein content in beef increased along with increasing storage time. On day 0, protein values ranged from 13.77 % to 20.91 %, increasing to 16.91 % to 28.86 % on the day 5th and reaching 18.66 % to 28.42 % by the day 10th. The lowest protein content was observed in the sample A1B1 (50 % Japanese bamboo leaf pulp, 1 h soaking time), with a value of 13.77 %.

Table 7. Protein content in beef samples during storage

Treatments	Observation Day		
	Day 0	Day 5	Day 10
A_0 (Control)	20.93	25.02	23.00
A_1B_1	13.84[a]	16.95[a]	18.73[a]
A_1B_2	16.94[c]	19.43[b]	22.17[c]
A_1B_3	17.37[d]	21.71[c]	27.39[e]
A_2B_1	15.51[b]	17.53[a]	19.47[b]
A_2B_2	18.84[e]	20.99[bc]	22.94[d]
A_2B_3	24.25[f]	28.58[d]	29.15[f]

A_1: 50 % pulp concentration; A_2: 75 % pulp concentration; B_1: 1 hour soaking time; B_2: 2 hours soaking time; B_3: 3 hours soaking time. Values followed by different letters within the same column indicate statistically significant differences based on 5 % Honestly Significant Difference (HSD) post hoc test.
Numbers followed by different letters indicate a significant difference ($P < 0.05$).

Table 7 shows that the interaction between Japanese bamboo leaf pulp concentration and soaking time significantly affected ($P < 0.05$) the protein content of beef during cold storage. The results indicate that the effect of bamboo leaf pulp concentration depended on the soaking time applied. At both concentration levels, increasing the soaking time generally resulted in higher protein content. For example, at the 50 % concentration, protein content increased from 13.84 % in the sample A_1B_1 to 17.37 % in the sample A_1B_3 on the day 0 and from 18.73 % to 27.39 % on the day 10th. A similar trend was observed at the 75 % concentration, where protein content increased from 15.51 % in the sample A_2B_1 to 24.25 % in the sample $_2B_3$ on day 0 and from 19.47 % to 29.15 % on the day 10th. These findings suggest that longer soaking times enhanced the effectiveness of the bamboo-leaf pulp treatment.

The significant correlation indicates that the greatest protein retention was achieved when a higher pulp concentration was combined with a longer soaking duration. This may be attributed to increased absorption of bioactive compounds such as flavonoids, tannins, and saponins into the muscle tissue, which could reduce protein degradation during storage. Therefore, the combination of 75 % Japanese bamboo-leaf pulp and a 3-hour soaking time was statistically identified as the most effective treatment for maintaining beef protein content throughout refrigerated storage.

Total plate count

Total plate count was measured on the days 0, 5, and 10 at 4 °C, the result shown that on the day 0, counts ranged from 1.4×10^5 to 9.9×10^5 CFU/g below the SNI limit (1×10^6 CFU/g). By the day 5th, bacterial counts in control samples surged past the threshold of 1.9×10^6 — 2.2×10^7 CFU/g, all samples exceeded the commonly cited spoilage threshold of 10^6 CFU/g. By day 10, the microbial counts ranged from 1.7×10^7 — 9.9×10^8 CFU/g. Although total plate count increased during storage, no significant

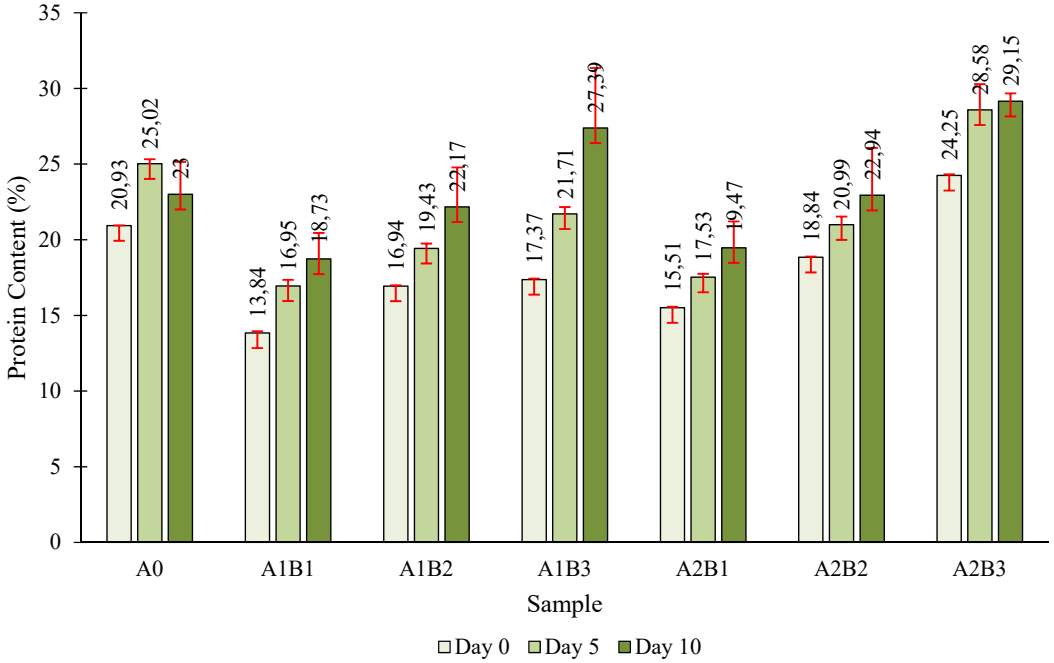

Figure 3. Beef protein content during cold storage. A_0: control (0 % Japanese bamboo leaf pulp, 0 h soaking time); A_1: 50 % pulp concentration; A_2: 75 % pulp concentration; B_1: 1 hour soaking time; B_2: 2 hours soaking time; B_3: 3 hours soaking time

effect ($P > 0.05$) from Japanese bamboo leaf pulp concentration nor soaking time was detected. The wide ranges at each time point likely reflect differences in initial contamination and treatment effects, thus indicating that under the tested conditions microbial proliferation substantially compromises beef quality within five to ten days of its refrigerated storage.

Table 8. Total plate count of microorganisms (CFU/g) in beef during cold storage

Treatment	Observation Day		
	Day 0	Day 5	Day 10
A_0 (Control)	9.2×10^6	7.35×10^8	3.94×10^9
A_1B_1 (50 %, 1 h)	8.96×10^6	1.64×10^7	1.95×10^8
A_1B_2 (50 %, 2 h)	9.43×10^6	4.51×10^7	1.26×10^8
A_1B_3 (50 %, 3 h)	6.0×10^6	5.49×10^7	1.16×10^8
A_2B_1 (75 %, 1 h)	9.32×10^6	7.85×10^7	2.01×10^8
A_2B_2 (75 %, 2 h)	9.76×10^6	1.89×10^7	1.72×10^8
A_2B_3 (75 %, 3 h)	8.18×10^6	1.33×10^7	6.69×10^9

A_1: 50 % pulp concentration; A_2: 75 % pulp concentration; B_1: 1 hour soaking time; B_2: 2 hours soaking time; B_3: 3 hours soaking time. Values followed by different letters within the same column indicate statistically significant differences based on 5 % Honestly Significant Difference (HSD) post hoc test.
Numbers followed by different letters indicate a significant difference ($P < 0.05$).

The progressive increase in total plate count (TPC) during refrigerated storage reflects the canonical bacterial growth, in which microbial populations enter an exponential phase as long as nutrients and favorable conditions persist [40]. Control samples, lacking any microbial contamination by day 5, highlight the high initial bioburden typical of freshly processed beef caused by non-sterile slaughter and handling [41]. In contrast, treatments incorporating Japanese bamboo leaf pulp at 50 % and 75 % concentrations effectively delayed microbial proliferation, particularly in the 75 % group where TPC remained below or just above the threshold until day 5. Beef samples treated with Japanese bamboo leaf pulp, especially at the 75 % concentration, showed reduced microbial loads compared to the control sample. This suggests the presence of antimicrobial compounds such as saponin, tannins, flavonoids, and phenolics, which have been detected in Japanese bamboo leaf [42]. Saponins exhibit antibacterial effects by inhibiting microbial glucose metabolism and protein synthesis, thus leading to cellular death [43]. However, despite the presence of these compounds, microbial counts still increased during extended storage periods. This may be due to the specificity of saponin's antibacterial activity, which tends to be more effective against certain pathogens like *Escherichia coli* and *Staphylococcus aureus* [42].

Conclusion

Applying Japanese bamboo leaf pulp (*Dracaena surculosa*) positively affected the quality of refrigerated fresh beef by slowing down microbial growth and by better preserving texture and pH compared with the untreated samples. The 75 % pulp concentration combined with a 2-hour soaking time showed the most consistent benefit across the measured parameters (TPC, instrumental texture, pH and color) among the samples, thus indicating a clear dose-and-time correlation effect. Qualitative saponin screening suggests surface-active bioactive compounds likely contributed to the antimicrobial and antioxidative action, although the active constituents were not quantified. Importantly, the treatment of beef with Japanese bamboo leaf pulp only delayed spoilage, but did not completely prevent microbial proliferation or physicochemical changes by the day 10th, so it is best viewed as a shelf-life extender rather than a full preservative.

Future research should quantify and characterize the active compounds, determine optimal dosages and exposure times, assess customers' sensory acceptability and safety (including potential saponin toxicity), and test synergies with other preservation strategies (packaging or lower storage temperatures). Overall, *Dracaena surculosa* leaf pulp, particularly at 75 % concentration combined with a 2-hour soaking time, offers a promising, plant-based approach to improve short-term stability of refrigerated beef and deserves further optimization for its practical use.

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SCREENING FOR ANTIMICROBIAL RESISTANCE (AMR) IN POULTRY MEAT AND PUBLIC HEALTH IMPLICATIONS: A BIBLIOMETRIC ANALYSIS OF LITERATURE

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Abstract
Antimicrobial resistance (AMR) remains a persistent threat in this century. While public debate often focuses on human medicine, the agricultural sector plays a crucial role in the emergence and mitigation of antimicrobial resistance. The growth of intensive livestock farming has encouraged antibiotic use across all animal sectors, including poultry production. Research on poultry meat in various regions of the world has revealed high levels of AMR, exceeding required standards. Encouragingly, significant progress has been made in recent years in reducing antibiotic use in livestock farming, particularly in poultry production. Despite ongoing efforts, AMR continues to spread in many countries, highlighting the urgent need to implement antibiotic reduction strategies. Robust surveillance systems and responsible antimicrobial use are essential. Without them, progress made in some regions risks being undone by uncontrolled practices. This article summarizes scientific research on antimicrobial resistance in poultry meat in different countries and its consequences for public health.

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Introduction

Antibiotics are used as the primary means of combating bacterial infections in veterinary medicine, whether in livestock farming or for treating companion animals. In poultry farming in particular, antimicrobial therapy is an essential tool for reducing the enormous losses in the poultry industry caused by bacterial infections [1–4].

In this context, the use of antibiotics has two objectives: therapeutic and zootechnical. Antibiotics are used therapeutically to eradicate an existing infection or prevent a potential infection, such as during transport, vaccination, or other stressors. The main classes of antibiotics are represented, but the number of molecules is very limited compared to those used for human purposes. Alongside this therapeutic use, there is a zootechnical application specific to livestock. Small amounts of antibiotics or coccidiostats are incorporated into the feed during the animals' growth period to improve weight gain [5–9]. This effect is primarily observed in farms with poor hygiene and tends to decrease as sanitary conditions improve. The choice of antibiotics is still too often made without prior antibiogram testing. The immediate consequence of AMR in livestock is treatment failure [10–14]. Furthermore, there is growing concern that the use of antimicrobials in animals could impact human health if resistant bacteria develop in these animals and are transmitted to humans through the food chain or the environment. However, there is still no scientific consensus on the precise role of antibiotics administered to animals in the development of resistance and its transfer to bacteria that can affect humans [6,15–17]. Any indiscriminate use of antibiotics leads sooner or later to the selection of resistant bacteria. Constant changes have been observed in recent years. These include, firstly, an increase in the frequency of resistant bacteria and an increase in multi-resistance. Currently, in intensive livestock farming, bacteria isolated during disease outbreaks are mostly resistant to several antibiotics from different families. Thus, if a bacterium is resistant to several antibiotics from different families, the use of just one of these antibiotics will promote the selection and spread of that bacterium, as well as the various resistance mechanisms to other families. This is known as co-selection. AMR is widespread in bacterial isolates worldwide. Continuous monitoring of these resistances provides useful data for choosing which molecules to use. The gut microbiota is the main reservoir for resistance genes. In short, the more antibiotics are used irrationally, the greater the likelihood that bacteria will acquire resistance. This is how some techniques for producing chicken without antibiotics were developed. The future will reveal their success or failure [18].

The objective of this article is to demonstrate the impact of AMR in poultry meat and the consequences for public health, thanks to the different investigations carried out by different researchers around the world.

Objects and methods

A bibliometric analysis was conducted to systematically map the scientific literature on quantitative microbial risk assessment (QMRA) applied to foodborne hazards. Reference bibliographics were extracted from various online databases (Scopus, Web of Science, Google Scholar, ResearchGate). Inclusion criteria were defined based on the type of study, the relevance of the topic, the language (English or French), and the period studied. No restrictions were applied regarding the geographical region or specific food matrices. Only research articles published between 1990 and 2025 were included.

After an initial extraction (n = 1985) using keywords specific to microbial risks in food, selection filters based on publication year, document type, and article language were applied, resulting in a final set of 106 references. All references addressing microbiological quality and QMRA of poultry meat were included in the selection phase, as all remaining studies met the criteria. The study design and writing were carried out in accordance with the PRISMA 2020 guidelines for systematic reviews [19]. The complete selection process is illustrated in the flowchart PRISMA 2020 (Figure 1).

The PRISMA 2020 statement provides updated reporting guidance for systematic reviews that reflects advances in methods to identify, select, appraise, and synthesize studies. The PRISMA 2020 statement consists of a 27-item checklist, an expanded checklist that details reporting recommendations for each item, the PRISMA 2020 abstract checklist, and revised flow diagrams for original and updated reviews.

Antimicrobial resistance (AMR) of bacteria contaminating poultry meat

Salmonella resistance to antibiotics

Salmonella is the second leading cause of foodborne illness in humans and remains the most frequent cause of foodborne outbreaks of bacterial origin in Europe, with contaminated poultry eggs and meat products as major sources of *Salmonella* infection in humans [20]. The main reservoir of *Salmonella* is the gastrointestinal tract of mammals and birds. Transmission to humans occurs primarily through the consumption of contaminated raw or undercooked food. For the most susceptible individuals, salmonellosis is treated with antibiotics. However, bacteria can acquire antibiotic resistance and thus resist treatment. This phenomenon poses a threat to public health [21–25].

Controlling *Salmonella* in the field to ensure food safety is a daunting and costly task. Several authors have demonstrated that *Salmonella* control on the farm is largely ineffective if product integrity is not maintained between the farm and the consumer's table [26–29]. Achieving food safety (meat, eggs) must be a shared responsibility between the poultry farmer, the slaughterhouse, and the consumer. Epidemiological studies of these pathogens must consider their significant persistence in the environment [30–33]. Although these *Salmonella* strains were susceptible to various disinfectants, several authors attest to the difficulty of controlling environmental contamination in the field, particularly during disinfection operations in poultry houses and slaughterhouses [34,35]. With current technology, the probability of eradicating all pathogens that cause foodborne illnesses is very low. Even the development of new

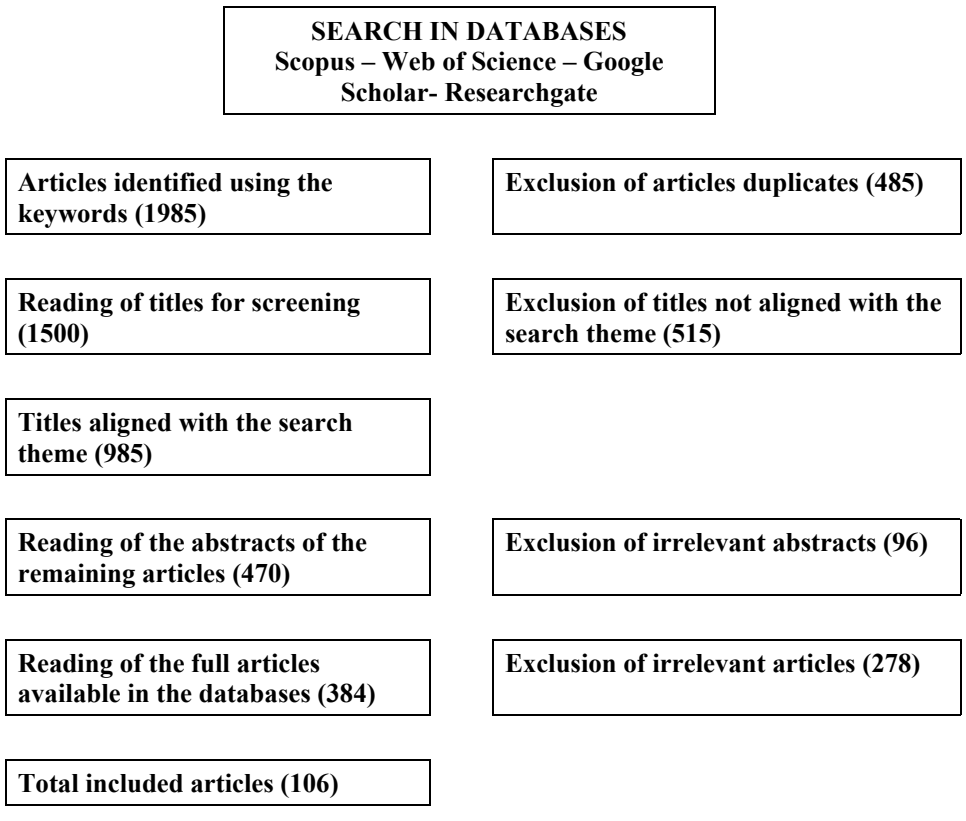

Figure 1. Flow diagram of the study selection process according to PRISMA

technologies to ensure rigorous biosecurity can sometimes fail, especially during cooking [36].

The level of resistance to antibiotics, as exhibited by *Salmonella* isolates from poultry, is detailed in Table 1.

Table 1. Antibiotic resistance of *Salmonella* isolated from poultry meat taken from butcher shops

Groups	Antibiotics	Prevalence, %	Country	Reference
Quinolones + Fluoroquinolones	Ciprofloxacin	18.9	China	[37]
		17.24	Bangladesh	[38]
		3.5	Vietnam	[39]
	Nalidixic Acid	100	Brazil	[32]
		72.3	China	[37]
	Enrofloxacin	20	Brazil	[32]
		21.1	China	[37]
	Norfloxacin	0	Egypt	[40]
Aminoglycosides	Amikacin	7.5	China	[37]
		34.48	Bangladesh	[38]
	Kanamycin	19.2	China	[37]
	Kanamycin	3.1	Vietnam	[39]
	Streptomycin	95	Brazil	[32]
		48.7	China	[37]
		80.65	Egypt	[40]
	Gentamicin	17.9	China	[37]
		3.23	Egypt	[40]
		13.79	Bangladesh	[38]
		5.7	Vietnam	[39]
Penicillins	Ampicilin	85	Brazil	[32]
		55	China	[37]
		41.6	Vietnam	[39]
	Amoxilin	85	Brazil	[32]
		67.8	Egypt	[40]
		44.83	Bangladesh	[38]
Carbapenem	Imipenem	0.3	China	[37]
Cephalosporins	Ceftiofur	75	Brazil	[32]
		14.5	China	[37]
	Cefotaxime	85	Brazil	[32]
		14.8	China	[37]
	Cefoxitin	85	Brazil	[32]
		1.9	China	[37]
B- Lactam/ B- Lactamase Inhibitor	Amoxilin-Clavulanate	85	Brazil	[32]
		9.7	China	[37]
		83.88	Egypt	[40]
Nitrofurans	Nitrofurantoin	45	Brazil	[32]
Phenicol	Chloramphenicol	25.8	China	[37]
		37.4	Vietnam	[39]
Tetracycline	Tetracycline	100	Brazil	[32]
		47.8	China	[37]
		66.67	Bangladesh	[38]
		59.1	Vietnam	[39]
Sulfonamides	Sulfamethoxazole-Trimethoprim	93.55	Egypt	[40]
		75.86	Bangladesh	[38]
		34.6	Vietnam	[39]

Antibiotic resistance of Escherichia coli

Escherichia coli (*E. coli*) are Gram-negative bacilli belonging to the *Enterobacteriaceae* family and the *Escherichia* genus. The majority of *E. coli* strains are simple commensals of the digestive tract of humans and warm-blooded animals. However, some *E. coli* strains are enteropathogenic. They can also cause extraintestinal diseases (meningitis, urinary tract infections) [41].

E. coli can be detected in animal feed, in animals used for food production, and in animal products intended for human consumption. This bacterium is frequently used as an indicator in surveillance and monitoring programs because it provides information on potential reservoirs of antibiotic resistance genes that can be transferred to pathogenic bacteria [42].

In poultry, while some strains of *Escherchia coli* are truly pathogenic, this bacterium is most often opportunistic, associating with other pathogens. Antibiotic treatment greatly improves the health of the animals, but careful antibiotic selection is crucial because resistance is common [43].

Multidrug resistance appears to be common for *E. coli* and geographically widespread. The emergence of multiple antimicrobial-resistant strains is often coupled with resistance to quinolones and third-generation cephalosporins in some regions [44,45].

The emergence of strains carrying extended-spectrum beta-lactamases (ESBLs) was first observed in clinical isolates of *E. coli* and then, shortly thereafter, through monitoring systems at slaughterhouses. Resistance rates to cefotaxime in *E. coli* are increasing very rapidly in poultry production, as this type of resistance was not observed before 2005 and affected more than 4 % of strains isolated from ceca of broiler chickens randomly sampled at slaughterhouses in 2007. The risks associated with the emergence of this new resistance phenotype are twofold. For animal health, there is a risk of loss of clinical efficacy of beta-lactams used to treat colibacillosis in animals, with the possibility of developing multidrug-resistant strains that are impossible to treat. For public health, the spread to humans of resistant *E.coli* and/or transmissible resistance genes occurs either through contact with animals or via food. Indeed, a large proportion of *E. coli* present in the human intestinal flora originates from our diet [18,46].

The level of resistance to antibiotics, as exhibited by *E. coli* isolates from poultry, is detailed in Table 2.

Antibiotic resistance of Staphylococcus aureus

While it is one of the most common commensal bacteria in our normal flora, *Staphylococcus aureus* is a formidable pathogen that has developed resistance to every new antibiotic introduced over the past half-century [51,52]. The plasticity of its genome allows it to adapt to all environmental conditions, and in particular to acquire antibiotic resistance genes and develop regulatory mechanisms to adapt to increasing antibiotic concentrations. Thus,

Table 2. Antibiotic resistance of *E. coli* isolated from poultry meat taken from butcher shops

Groups	Antibiotics	Prevalence, %	Country	Reference
Quinolones and Fluoroquinolones	Ciprofloxacin	96	India	[47]
		26.66	Egypt	[48]
		33.33	Romania	[49]
	Nalidixic acid	75.5	Korea	[50]
		33.33	Egypt	[48]
		44.44	Romania	[49]
	Enrofloxacin	13.33	Egypt	[48]
Aminoglycosides	Kanamycin	0	Romania	[49]
	Streptomycin	60	Egypt	[48]
	Gentamicin	11.11	Romania	[49]
		23	India	[47]
Penicillins	Ampicilin	69.1	Korea	[50]
		80	Egypt	[48]
		27.7	Romania	[49]
	Amoxilin	27.7	Romania	[49]
Cephalosporins	Cefotaxime	40	Egypt	[48]
		0	Romania	[49]
	Ceftazidime	33.33	Egypt	[48]
	Ceftriaxone	20	Egypt	[48]
B- Lactam/ B- Lactamase Inhibitor	Amoxilin-Clavulanate	66.66	Egypt	[48]
Phenicol	Chloramphenicol	9	India	[47]
		20	Egypt	[48]
		22.22	Romania	[49]
Tetracycline	Tetracycline	64	Korea	[50]
		80	Egypt	[48]
		93	India	[47]
		66.66	Romania	[49]
Sulfonamides	Sulfamethoxazole-Trimethoprim	61	India	[47]
		66.66	Egypt	[48]
		22.22	Romania	[49]

penicillin-resistant staphylococci appeared as early as 1941, thanks to the acquisition of a plasmid-mediated penicillinase, an enzyme that degrades penicillin. Penicillin resistance initially confined to hospital settings, spread very quickly in the community and now affects more than 90 % of *S. aureus* strains. During the 1950s, multidrug-resistant strains of *Staphylococcus aureus* emerged: resistance to penicillin was associated with resistance to streptomycin, erythromycin, tetracycline, chloramphenicol, and sulfonamides. The introduction in 1959 of methicillin, a semi-synthetic derivative of penicillin, for the treatment of staphylococcal infections raised great hopes. But barely a year later, the first hospital-acquired strains of methicillin-resistant *Staphylococcus aureus* (MRSA) appeared in a hospital in Great Britain [53].

Apart from spontaneous mutations, *S. aureus* diversifies its genome through the exchange of genetic material with other bacterial species via horizontal gene transfer [54].

In recent years, studies conducted in the European Union have shown the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) strains in animals. Numerous studies carried out in the Netherlands and Germany have contributed to a better characterization of this risk. Most strains belong to a specific clonal complex (CC398) found among pigs and other animal species, posing a risk of carriage for people working in contact with animals (farmers, veterinarians, slaughterhouse workers). The potential risk to public health is that these strains may acquire virulence factors, making them more pathogenic to humans or animals [53–55].

The level of resistance to antibiotics displayed by *Staphylococcus aureus* isolates from poultry is detailed in Table 3.

Table 3. Antibiotic resistance of *Staphylococcus aureus* in poultry meat samples taken from butcher shops

Groups	Antibiotics	Prevalence, %	Country	Reference
Beta-lactams	Methicillin	0	Italy	[54]
		76.4	Turkey	[56]
	Oxacillin	7.89	Thailand	[57]
		70	Spain	[58]
		66.66	Italy	[54]
	Ampicillin	55.26	Thailand	[57]
		58.33	Italy	[54]
	Penicilin G	25	Italy	[54]
		52.9	Turkey	[56]
	Cefalotin	0	Italy	[54]
Quinolones	Ciprofloxacin	7.89	Thailand	[57]
		17.8	Spain	[58]
Aminosides (Aminoglycosides)	Gentamicin	13.15	Thailand	[57]
		0	Spain	[58]
		16.66	Italy	[54]
	Streptomycin	18.42	Thailand	[57]
Phenicol	Chloramphenicol	21.05	Thailand	[57]
		2	Spain	[58]
Sulfonamides	Sulfamethoxazole/ trimethoprim	28.94	Thailand	[57]
		8.33	Italy	[54]
	Sulfisoxazole	24.8	Spain	[58]
Tetracyclines	Tetracycline	44.73	Thailand	[57]
		8.33	Italy	[54]
	Doxycycline	58.4	Spain	[58]
Lincosamides	Clindamycin	67.3	Spain	[58]
		8.33	Italy	[54]
Macrolides	Erythromycin	20.8	Spain	[58]
		8.33	Italy	[54]
		5.8	Turkey	[56]
Nitrofurans	Nitrofurantoin	28.7	Spain	[58]
Glycopeptides	Teicoplanin	0	Italy	[54]
	Vancomycin	0	Italy	[54]
Polypeptides	Bacitracin	100	Turkey	[56]

Listeria monocytogenes resistance to antibiotics

Listeria monocytogenes is a Gram-positive bacterium responsible for a zoonotic disease called listeriosis [59,60]. This bacterium is naturally present in the environment and in some foods consumed by humans. Ingestion of food contaminated with *Listeria monocytogenes* leads to septicemia, severe gastroenteritis, and central nervous system infections, particularly in the elderly and immunocompromised individuals. In pregnant women, *Listeria* infection can result in miscarriages, premature births, and perinatal infections [61,62].

The main source of *Listeria monocytogenes* contamination of food before distribution to consumers appears to be the production environment [63,64]. *Listeria monocytogenes* can survive for a long time, sometimes from one to three years, or even longer, or even indefinitely. Furthermore, some *Listeria monocytogenes* clones may be better adapted to raw meat and to the environments and finished products in the meat industry [41,65].

This pathogenic bacterium possesses an extraordinary capacity to adapt to both environmental stresses, allowing it, for example, to survive and multiply in soil, and to the various treatments it encounters in the food chain (salt addition, freezing, etc.). This adaptability results from an arsenal of genes it possesses, the expression of which it finely regulates through various mechanisms that allow it to detect its entry into an organism and thus express genes critical for its virulence [66,67].

The level of resistance to antibiotics exhibited by *Listeria monocytogenes* isolates from poultry is detailed in Table 4.

Table 4. Antibiotic resistance of *Listeria monocytogenes* in poultry meat samples taken from butcher shops

Groups	Antibiotics	Prevalence (%)	Country	Reference
Beta-lactams	Oxacillin	82.9	Japan	[68]
	Ampicillin	27	Turkey	[69]
		3.63	Turkey	[70]
		0	Japan	[68]
	Penicilin G	12.5	Turkey	[69]
		18.18	Turkey	[70]
	Amoxicillin/ Clavunate	9.3	Turkey	[69]
		1.81	Turkey	[70]
	Cefoxitin	100	Japan	[68]
Phenicol	Chloramphenicol	3.1	Turkey	[69]
		14.54	Turkey	[70]
Sulfonamides	Sulfamethoxazole/ Trimethoprim	13.5	Turkey	[69]
		45.45	Turkey	[70]
Tetracyclines	Tetracycline	14.5	Turkey	[69]
		3.63	Turkey	[70]
	Oxytetracycline	5.2	Turkey	[69]
		1.81	Turkey	[70]
Macrolides	Erythromycin	4.1	Turkey	[69]
		1.81	Turkey	[70]
Glycopeptides	Vancomycin	7.2	Turkey	[69]
Carbapinem	Merpenem	23.9	Turkey	[69]
		14.54	Turkey	[70]
Cephalosporin	Cefoxitin	100	Japan	[68]

Practices promoting the emergence of AMR in poultry farming

Increasing the size of animal groups or raising animals at high densities increases the risk of disease emergence and therefore antibiotic consumption [12,71–73].

The increased prevalence and spread of resistance is a predictable outcome of the growing use of antibiotic therapy [74]. The use of antibiotics in animal husbandry to increase productivity also represents a major challenge [69,75–79]. In the case of prophylaxis, the goal is to protect a group against infection before it occurs within the group, and in the case of metaphylaxis, to protect a group against infection after it occurs within the group [71,80].

The uncontrolled use of antibiotics can lead to the selection of resistant pathogenic bacteria [12]. The types of antibiotic treatments can influence the risk of developing antibiotic resistance [71,72].

The most significant consequence of using low-dose antibiotics in poultry is the development of multidrug-resistant strains of bacterial pathogens [23]. Antibiotics are a powerful factor in the selection of AMR in bacteria [81]. During preventive treatment, the risk associated with the selection pressure exerted on commensal bacteria is present in all treated animals, whereas the therapeutic benefit depends on the actual presence of the pathogenic bacterium, which is only suspected.

There are significant risks associated with the use of antimicrobials in poultry as growth promoters.

More than 110 assessed countries, mostly developing and emerging economies, still lack rigorous and relevant legislation regarding the appropriate conditions for the import, manufacture, distribution, and use of veterinary products, including antimicrobials.

Legislation is sometimes completely absent. When it exists, it is very often not enforced due to a lack of public resources for monitoring [73].

In Africa, 16 countries authorize antibiotics as growth promoters, primarily due to a lack of legislation, and tetracyclines are the most commonly used. In these countries, antimicrobials are most often directly or indirectly accessible to everyone without restrictions. Even more seriously, these products, circulating like ordinary goods, are most often adulterated (lower dosage than indicated on the bottle, different active ingredient, or a complete placebo). Thousands of tons of adulterated antimicrobial products intended for animals are in circulation worldwide [73].

Feed additives containing antibiotics for animal have been banned in Europe since January 1, 2006 [12], but are still permitted for preventive and curative purposes, particularly collectively for groups of animals, due to the close quarters in industrial farming, which make individual treatment impossible [82].

The cessation of the use of growth promoters in Europe in 2006 led to a decrease in resistance of bacteria isolated from animals, food, and humans [83].

In parts of the world such as the USA and Europe, reduced antimicrobial use has already led to measurable improvements in resistance levels and treatment out-comes. Despite ongoing efforts, antimicrobial resistance (AMR) continues to rise in many countries, underscoring the urgent need to prioritize the implementation of antibiotic reduction strategies.

Transmission to humans via poultry consumption

Concern about AMR in bacteria and the resulting difficulty in treating certain human infections has led to a surge in research in recent years focusing on resistance in livestock, food, the environment, and humans [84,85]. Similarly, investigations have focused on the mechanisms of transfer of genetic traits encoding resistance between bacteria, as well as the risk factors and/or parameters that promote the spread of resistance [86].

The gut microbiota of animals can constitute a reservoir of antibiotic-resistant bacteria capable of infecting or colonizing humans through the food chain [87].

The risk of transfer of genetic elements encoding resistance between bacteria in mixed populations can open up diverse, numerous, and complex pathways for transmission [88]. Currently, considerable evidence suggests that direct transfer of resistance traits to humans, via the food chain and animal products, is one of the pathways for resistance spread.

According to Ajiboye et al. [89], multidrug-resistant (MDR) bacteria can be transmitted between animals and humans. There is a risk of transfer to humans of resistant bacteria present in animals, which generally occurs through food [7]. These strains are frequently found in animals intended for human consumption, including poultry [90,91].

McEwen and Fedorka-Cray stated that some human problems linked with antimicrobial-resistant (AMR) bacteria (Salmonella, Campylobacter, Escherichia coli, Enterococcus) originate from the animal world [8].

Currently, considerable evidence indicates that the direct transfer of resistance traits to humans, via the food chain and animal products, constitutes one of the pathways for the spread of resistance [92].

The use of antimicrobial agents in humans, as well as in animals raised for human consumption, has major consequences for human and animal health, as it can promote the development of resistant bacteria (pathogenic and/or commensal bacteria carrying genes encoding resistance) [93].

Bacterial contamination of chicken carcasses generally occurs during slaughter and processing. These microorganisms can survive in the product sold to the consumer [87].

The development of resistance in animal bacteria that can lead to foodborne (*Salmonella*, *Campylobacter*) or opportunistic (*E. coli, Enterococcus* sp., *Staphylococcus aureus*) infections must be monitored within the context of a comprehensive public health approach [12]. Bacteria can be transferred between animals and humans via water and food, direct contact, or the environment. Antibiotic resistance, on the other hand, is exchanged via the transfer of genetic material between bacteria within a single compartment, but also between bacteria in different compartments [74].

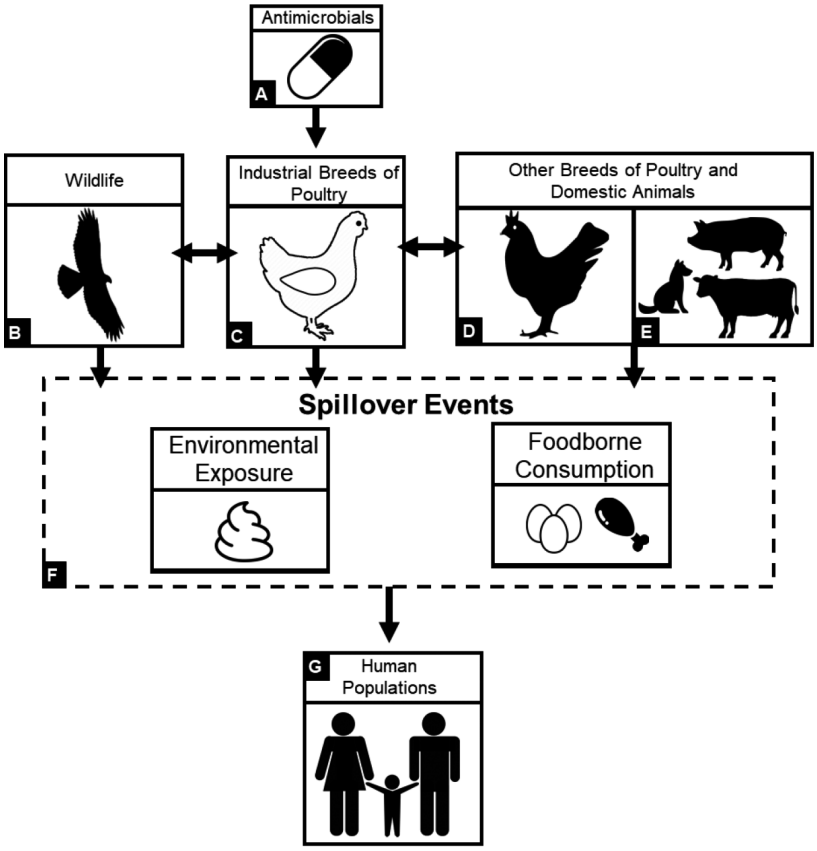

Figure 2. Conceptual graph illustrating AMR associated with intensive poultry production [9]

Action plan to combat AMR

In May 2015, the WHO, FAO and OIE adopted a global action plan to combat antimicrobial resistance within the "One Health" framework. It is summarized in five key areas:

- raising awareness among healthcare workers and the public;
- strengthening surveillance and research;
- implementing sanitation, hygiene, and infection prevention measures;
- optimizing the use of antimicrobials in human and animal health;
- supporting sustainable investments in the development of new treatments, diagnostics, and vaccines.

Antimicrobial resistance, considered by the World Health Organization to be critically important in human medicine, particularly with fluoroquinolones, third- and fourth-generation cephalosporins, and macrolides, is evolving in a particularly worrying manner [80,93–95].

The WHO encourages the agriculture, food production, animal health, and public health sectors to cooperate in order to eliminate the burden of AMR resulting from the misuse of these agents in livestock intended for human consumption [93].

Therefore, reducing this use is one important lever for action, but it should not be the only one to control the risk associated with AMR in animals [71,96]. Concerted efforts must be made to reduce the inappropriate use of these agents (for example, as growth promoters) and to limit the spread of resistant bacteria [93].

The risk of developing antibiotic resistance can only be reduced through appropriate regulations and policies [97].

Reducing antibiotic use is one way to control resistance to these agents. However, this objective requires the implementation of a wide range of appropriate measures [81,98–100].

The USA, Brazil, Europe and China together produce over 60 % of poultry meat. Surveillance of antimicrobial consumption is well established in the USA and some European countries. Data from the registration perspective on approved antimicrobials is available in all large meat producing countries. Resulted evaluation of data shows that third generation cephalosporins can be used in the USA and Brazil; glycopeptides are not approved for use in poultry production in any of the regions and macrolides and ketolides can be used everywhere except China, while quinolones can be used everywhere except the USA [101].

One possibility is the regulatory restrictive use or ban of some antimicrobials on national level, which leads to reduced use of antimicrobials. Another possibility is voluntary reduction due to communication, education, and consumer demands. This reduction happens on the local level of the individual farm, where the change of antimicrobial use is essential to observe the change in the national or global level. In our communication with farmers, we have observed that the first step to the voluntary reduction of antimicrobials on the farm is of the use resistance prevalence in poultry production.

It is widely understood that antimicrobials are a necessary tool in veterinary medicine to treat sick poultry. Antibiotics are precious molecules, whose effectiveness must be reserved for medical treatment by prudent and dedicated use. For this reason, all other possibilities to prevent animals from diseases and performance losses must be exploited, such as farm management, feed additives, biosecurity and vaccination. Feed additives are considered enablers of AGP-free feeding systems [102–104]

Today's AGP-free feeding system is built rather on replacement with feed additives, which are known to close the performance gap and even outperform AGPs. Most of solutions have the potential to perform on the same level or even better than antibiotic growth promoters. Furthermore, data-based early warning systems, such as BiocheckUGent (http://www.biocheck.UGent.be), provide producers with valuable insights into potential animal health and nutrition issues that can be addressed in a preventive, profitable way. It is important to understand the global problem of AMR and respond locally with targeted solutions that support farmers in raising healthy animals [105,106].

Conclusion

This study, while modest, reveals that antimicrobial resistance (AMR) in poultry meat is a reality present on every continent. The prevalence of antibiotic resistance in marketed products is directly linked to the regulatory framework in place in each country. The prevalence rate of AMR against different pathogens (*Salmonella*, *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes*) varies from one country to another.

Reducing the risk of antimicrobial resistance (AMR) is an urgent priority as producers around the globe strive to feed a growing population in a more sustainable way and safeguard the animal protein industries.

The fight against AMR is a shared responsibility across all sectors, transcending borders and generations. Agriculture has a crucial role to play, and the progress made so far proves that change is possible. Through continuous innovation, training, and collaboration, the animal industry can protect public health and food security, thus ensuring a sustainable future for all.

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EVALUATION OF THE IMMUNOMODULATING POTENTIAL OF FERMENTED RAW MEAT

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Keywords: *meat, beef fermentation, consortium, probiotic microorganisms, sensitization, food allergy*

Abstract

Food allergy, which manifests itself as an oversensitivity of the body's immune system to certain kinds of foods, affects a significant share of the world's population and represents a serious issue. The aim of this study was to evaluate the allergenic potential of beef exposed to a probiotic microbial consortium. The developed consortium, which consisted of strains: Lactobacillus plantarum 7K, Lactobacillus paracasei k-406, Pediococcus acidilactici PDA-27 and Staphylococcus carnosus SPHC-108, exhibited high proteolytic activity against beef proteins (after three days of fermentation, the degree of hydrolysis reached up 21.5 %). The allergenic potential of fermented beef and unfermented beef was studied in the experimental animals (rats) that were exposed to simulation of artificially induced systemic anaphylactic reaction to ovalbumin. It was found that, against the background of ovalbumin sensitization, the inclusion of fermented meat into the rats' diet reduced the severity of the anaphylactic reaction by 43.5 % compared to the corresponding parameters among the rats that were fed with unfermented meat. The concentration of specific antibodies (IgG) to ovalbumin in the blood serum of the experimental animals, that were fed with fermented meat, was also significantly lower ($p \leq 0.05$). Animals that received the fermented food along with the sensitization background featured greater body weight gain at the end of the experiment. The fecal counts of lactobacteria and bifidobacteria in the animals in this group fed with fermented meat along with ovalbumin sensitization and along with anaphylactic reaction development increased fourfold in comparison with to the group of rats fed with unfermented meat. Histological studies showed that introducing fermented meat into the diet along with food sensitization did not cause significant morphofunctional changes in the mesenteric lymph node structures, whereas the animals fed with unfermented meat demonstrated the signs of decompensation and immune mechanisms stress. These results demonstrate the potential of using meat fermented with probiotic microorganisms consortium as promising approach to reducing food allergies risk.

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Introduction

Food allergies are a serious issue that affects a significant share of the world's population today. Approximately 2.5 % of the general population suffers from the food allergies: however, in industrialized countries this values reaches up to 8 % among children and 10 % among adults. The World Allergy Organization (WAO) estimates that 220–250 million people suffer from food allergies. WHO experts warn that, if current trends persist, by 2030 allergic diseases of one or another type would be able affect up to 50 % of the world's population [1,2].

Food allergy is characterized by a condition of increased body sensitivity to certain kinds of food, which develops due to immune response mechanisms. Experts note the importance of the influence of a number of factors on the food allergies onset and development, among which the condition of the intestinal microbiota is of particular importance, because interaction with a variety of intestine microorganisms plays a key role in the development of immunity mechanism [3,4]. The pathogenetic process of food allergy development is caused by an imbalance of cellular reactions of the Th1/Th2 type. Under normal conditions, the penetration of a food protein containing antigens triggers the activation of T- and B-cells of the immune system, but the absence of allergy symptoms is explained by the body's formation of natural tolerance to such substances. When a food allergy develops, this defense mechanism fails, mainly due to the hyperreactivity of Th2 cells, provoking excessive production of IgE antibodies directed specifically against food components [5]. The body's immune system needs to maintain an optimal balance of Th1/Th2-cell activity. It is the intestinal microbiota that regulates this balance by suppressing excessive reactivity of Th2 cell and this way promoting an adequate Th1 response, or by stimulating regulatory cells (Treg) [6–8]. The current scientific concepts and clinical observations confirm that changes in the composition of the intestinal microbiota under the influence of various factors significantly affect the development of the immune response and thus contribute to the development of allergic conditions.

Given the extensive data on the role of the intestinal microbiota in the pathogenesis of food allergies, some researchers propose using probiotics as a promising method for preventing and healing allergic pathology, particularly in children. Studies of correlation between microbiome structure and immunological reactions related to food allergies have shown that microbiome structure abnormalities are associated with Th1/Th2 cell responses imbalance, which is accompanied by an increase in allergic symptoms [9]. These findings support the hypothesis of a strong correlation between the characteristics of the intestinal microbiome and the functioning of key immune defense elements involved in allergic diseases development.

A number of studies demonstrate the efficiency of certain types of probiotics in the therapy and prevention of allergic disorders in children of different ages [10]. Thus, integrated therapy that involves the probiotic Bifiform Kids, combined with B vitamin complexes (vitamins B1 and B6) for 21 days, reduced the severity of gastrointestinal and dermatological manifestations of food allergies in patients with mild forms of the disease. Based on these results, the authors recommend including a combination of strains *Lactobacillus rhamnosus GG* and *Bifidobacterium animalis spp. lactis BB-12* in therapeutic protocols for the children with gastrointestinal and skin symptoms of food allergy [11].

The capability of probiotic bacteria to stimulate anti-allergic processes has also been proven in experiments *in vivo* with *Lactobacillus gasseri* (LK001), *Lactobacillus salivarius* (LK002), *Lactobacillus johnsonii* (LK003), *Lactobacillus paracasei* (LK004), *Lactobacillus reuteri* (LK005) and *Bifidobacterium animals* (LK011) [12]. Research by M. Mennini et al. (2021) was conducted to evaluate the effect of *Lactobacillus strain rhamnosus GG* on the state of intestinal microbiota and the intensity of digestive disorders in children with milk protein intolerance, this research revealed an increase in the level of tolerance to this food products, including whole cow protein, when the recipient got *Lactobacillus-based rhamnosus GG* preparations [13].

In the study of Fedotova et al. [14] there is an analysis of the available experimental and clinical data on the modern methods of restoring the compositional formulation and functional activity of intestinal microflora and their potential impact on the therapy and prevention of allergic food intolerances. The authors noted that therapy with the preparations including bifidobacilli, lactobacilli and lactic acid microorganisms, used along with a basic diet and medications support, provided a positive effect on the dynamics of the disease management, contributed to the improvement of the gastrointestinal tract condition. Individual experiments demonstrated the efficiency of using the synbiotic complexes (for example, the combination of *Bifidobacterium breve M-16V, Lactobacillus rhamnosus GG* together with oligosaccharides) as a method of primary prevention of food allergy in the individuals with a high risk of developing allergic pathologies in the future [14]. However, according to the authors, there are no sufficient proofs that the inclusion of prebiotics, probiotics and synbiotics into the therapy protocols is capable of significantly increasing the efficiency of standard methods of curing food allergy as its is prescribed by the current national clinical regulations. Perhaps, the evaluation of the complex therapy results is complicated by the impossibility of determining the share of the contribution of each individual element of therapy to the overall final effect. Thus, the question of the real therapeutic value of the medications that particularly normalize the intestinal flora still remains unsolved [14]. Current international WHO recommendations for prevention of allergic diseases, based on a systematic analysis of a big array of scientific studies, declare that the available data are insufficient to recommend using prebiotics, probiotics and synbiotics for prevention and therapy of food allergies conditions [15,16]. A similar opinion is declared in the Russian clinical guidelines drawn up in accordance with the principles of proofs-based medicine [17].

Current practice shows that the recommended elimination approach, which is the only effective one, to therapy and prevention of the food allergies does not provide the necessary comfort and does not improve the quality of life for the patients. People who suffer from food allergies encounter constant anxiety and stress caused by the fear of accidental contact with the allergen, which anxiety leads to limitations in social interactions: difficulties visiting public places, restaurants, participating in family celebrations, and school events, which becomes a source of additional anxiety and thus requires special precaution measures in the patient's behavior [18]. This consideration underlines the need to develop new approaches and technologies to reduce the allergenic capacity of the food products.

One of the promising methods for reducing allergen capacity, actively used by the specialists, is enzymatic hydrolysis, which ensures the destruction of conformational and linear epitopes, ensures rupture of peptide chains, formation of short-chain peptides and free amino acids, as well as the modification of the structural features of allergens, including a decrease in their ability to bind to IgE-antibodies [19,20]. The obtained short peptides and individual amino acids lose the ability to initiate immune system responses through their interaction with antigen presenting T-cells. According to various sources, the least molecular weight of peptides, at the threshold of their reaching the allergen capacity of the product, decreases down, it ranges within the values of approximately 3.5–10 kDa [21–23]. Enzymes of animal, plant and microbial origin are widely used for enzymatic hydrolysis purposes: pepsin, trypsin, papain, alcalase, etc. [24]. However, the disadvantage of this method is, firstly, the possibility of new allergenic epitopes formation due to protein structure changes, and secondly, enzymatic processing may not affect or partially affect the target epitopes which are capable of binding to IgE

antibodies. Along with enzyme processing, a new promising and innovative direction has emerged — using the enzymatic activity of microorganisms which can reduce the allergenic potential of raw materials. One example of that is using probiotics, such as lactic acid bacteria, for the fermentation of soy milk, for example. The extract of the resulting fermented product reduced the manifestation of allergic symptoms, including hay fever caused by Japanese cedar pollen [25,26]. It is worth noting the emergence of data that non-living microorganisms and bacterial metabolites of their functioning are biologically active as postbiotic materials [27]. In particular, it was reported that introduction of soy milk extract obtained by fermentation with several strains of lactic acid bacteria caused changes in the intestinal microflora, improved the intestinal condition, etc. [28].

Using of probiotic microorganisms for fermented products production is widespread. It is well known that the allergenic potential of food varies depending on the methods of its production. The activity of probiotic bacteria results in the formation of secondary metabolic products, including short-chain fatty acids like butyrate, propionate, acetate, and others, which participate in the regulation of the human immune response and provide protection against the allergic reactions development [29]. In particular, it has been shown that butyrate stimulates the production of IL-10 and IFN-γ and reduces the rate of DNA methylation in these cytokines genes, as well as it promotes the differentiation of B-cells and increases the production of secretory IgA in the intestinal mucosa [30–32]. The main mechanism of the immune regulatory action of short-chain fatty acids, according to the researchers' data, is the increase of T-regs production and activity, which leads to the suppression of allergic inflammation [32–34]. Based on the above, it follows that postbiotics, which represent promising and potentially useful components in the therapy of allergic conditions, also provide a significant impact on the regulation and restoration of immune responses controlled by Th1/Th2 cells, peculiar for probiotic microorganisms. Furthermore, the introduction of postbiotics as functionally active ingredients in food products opens up new opportunities for expanding the range of fortified food products.

Beef and its derivatives are an important source of complete protein, essential amino acids, heme iron, and B group vitamins, all essential for a balanced diet. However, meat proteins are serum proteins: bovine serum albumin (BSA, Bos d 6) and immunoglobulins (Bos d 7), muscle proteins: actin, myosin, and tropomyosin, as well as the carbohydrate component (α - Gal) are able to cause allergic reactions. It has been noted that beef allergy or intolerance rarely occurs in isolation, and due to the similarity of proteins, cross-reactions with cow's milk, meat of other mammals, gelatin, etc. are also possible [35–38].

The purpose of our research is to evaluate the allergenic potential of beef exposed to a consortium of probiotic microorganisms effect.

Objects and methods

Methods for determining physical and chemical properties and process parameters

For the study the samples of spinal muscle tissue (*Longissimus dorsi)* were taken from beef cuts obtained from 18-month-old cattle raised at Ulekchin Agricultural Production Cooperative (Ulekchin village, Zakamensky area, Republic of Buryatia), chilled for 18–20 hours at 2–4 °C. The meat was pre-trimmed to remove fat and connective tissue. The beef muscle tissue was then minced into 120–130 g pieces by slicing the slightly chilled meat across the fibers with a sharp knife into slices approximately 2 cm thick. The test meat samples were mixed with a microbial consortium at a rate of 1 unit of enzyme activity per 100 kg of raw material. The processed meat then was massaged using a GRZK20 vacuum massager (Hualian, China) at a speed of 9 rpm, a vacuum level of –0.08 MPa, and was held still for 1–2 hours at a temperature of 18–20 °C. The processed (fermented) raw materials were packaged in film, stored at 2–4 °C for seven days, and then tested. Unprocessed (non-fermented) meat served as a control sample.

The degree of hydrolysis (*DH*) was determined with the equation 1:

$$DH = \frac{N_a}{N_g} \times 100\%, \quad (1)$$

where N_a — is amino nitrogen content, %; N_g — is total nitrogen content, %.

The protein content (*P*) was determined using equation 2:

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

N_a and N_g were determined using the Kjeldahl method (GOST 25011-2017[1]) with the help of semi-automatic device KDN-812 (Shanghai Xianjian, China).

A consortium of microorganisms was developed based on four strains: *Lactobacillus plantarum 7K, Lactobacillus paracasei k-406, Pediococcus acidilactici PDA-27 and Staphylococcus carnosus SPHC-108,* for which a priority certificate for the invention and notification of a positive result of the formal expert examination were received[2]. The bacterial strains were obtained from the National Bioresource Center "All-Russian Collection of Industrial Microorganisms" of the National Research Center "Kurchatov Institute".

The activity of the microorganisms consortium was determined by the number of viable cells based on their content in one unit of enzyme activity (1 EA), which is equivalent to 10^9 CFU/g (cm^3)[3].

[1] GOST 25011-2017 "Meat and meat products. Protein determination methods". Retrieved from https://docs.cntd.ru/document/1200146783. Accessed January 18, 2026. (In Russian)

[2] Application No. 2025129394/10(069370) for the invention "Consortium of microorganisms with high proteolytic activity for the preparation of starters and fermented products" Dated 10.30.25. (In Russian)

[3] GOST 34272-2017 "Bacterial starter cultures for the production of dairy products. General specifications". Retrieved from https://docs.cntd.ru/document/1200157895. Accessed January 18, 2026. (In Russian)

The proteolytic activity of the microorganisms' consortium was determined according to GOST 34430-2018[4].

Methods of biological research

The allergen capacity of unfermented and fermented beef was assessed in an experiment on 21 outbred white rats (males) aged 3 months, obtained from the vivarium of Buryat State Agricultural Academy, with their initial body weight of 220–240 g.

Before the experiment, the animals underwent a week-long adaptation to laboratory conditions. Throughout the experiment, they were housed in the cages 2–3 animals per one cage under the following conditions: temperature (22 ± 3 °C), humidity (50–60 %), and a 12/12 light/darkness schedule.

After adaptation to the standard vivarium diet (SVD — holistic feed mixture from Aleysk Cereal Product JSC), the rats were divided into 3 groups (7 animals in each group).

The model of systemic anaphylaxis was reproduced according to the research [39] with some modifications. On the 1st, 3rd and 5th day of the experiment, the rats of all groups were intraperitoneally sensitized with hen egg ovalbumin (OVA) in isotonic 0.15 M NaCl solution (100 μg per rat) with Freund's complete adjuvant (1:1) in a volume of 0.2 ml each. To induce a secondary immune response, on the 21st day of the experiment, the rats were injected with another 10 μg of OVA under the same conditions. Rats in the group I (control group) received only CVD for 28 days, the rats in the group II received CVD + unfermented beef (7 g), and the rats in the group III received CVD + fermented beef (7 g). At the end of feeding (on the day 29th), 0.2 ml of blood was collected from the tail vein of the animals from all three groups to analyze the antibody level, after which a resolving dose of OVA was administered intravenously — 30 mg/kg of body weight in 0.5 ml of isotonic 0.15 M NaCl solution. Over the next 24 hours, the animals were monitored for the development of symptoms of anaphylactic shock, and their condition was scored according to the scale:

+ (1) — chills, shortness of breath;
++ (2) — weakness, ataxia, cyanosis of the extremities;
+++ (3) — convulsions, paralysis;
++++ (4) — fatal outcome.

The anaphylactic shock reaction severity was assessed in score points. The anaphylactic index (AI) was calculated as the arithmetic mean of the score points for the reaction severity in the group according to [40].

The intensity of the humoral immune response was assessed by the concentration of circulating specific IgG antibodies with the help of indirect enzyme-linked immunosorbent assay on polystyrene (ELISA) [41].

The animals' condition was monitored in general daily throughout the experiment. Behavior, coat condition, food intake, and water consumption were observed and recorded. Animal weights were also determined at the beginning and at the end of the experiment (on the 29th day).

Also on the 29th day fecal samples were collected from the rats' colons into sterile vials. The intestinal contents were re-suspended in sterile isotonic sodium chloride solution at a ratio of 1:10, followed by plating of 1 ml of the sample using serial dilutions of $1\times10^{-1}\times10^{-7}$ using the submersible method on a semi-solid thioglycollate medium in order to detect content of lactobacteria and bifidobacteria[5].

All manipulations with the animals were conducted in compliance with international moral and ethical standards in accordance with the requirements of the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, 1986)[6] and in accordance with the WMA Declaration of Helsinki[7], as well as Federal Law No. 498-FZ[8]. The study protocol was approved at the Ethics committee meeting.

Methods of morphological assessment

At the end of the experiment, the animals were euthanized with chloroform, and the bean-shaped cranial mesenteric lymph nodes (CMLNs) were extracted for histological examination. After washing the samples to remove excess formalin, standard alcohol processing was performed. Paraffin blocks and sections were prepared using standard histological methods. Staining was performed with Ehrlich hematoxylin and eosin, and azure eosin. The size of the mesenteric nodules was determined in 10 fields of microscope view for the animals of each group. Measurements of the specimens' structures thickness — the relative area of the *cortex* and *medulla* to the section area — and other cellular inclusions were performed using a Micromed 3 U 3 microscope and a video eyepiece of the Toup microscope Cam 5/1 at magnification A — ×100 (PL magnification 10×/0.25); B — ×400 (PL magnification 40×/0.65).

Statistical data processing

The obtained digital histological data were subjected to statistical processing with mean values (M) calculation, calculation of standard error of the mean (m) and parametric test (Student's t-test) using the Micromed Excel software. The software Statistica and Microsoft Excel were

[4] GOST 34430-2018 "Enzyme preparations for food industry. Method for the determination of proteolitic activity". Retrieved from https://docs.cntd.ru/document/1200160228. Accessed January 18, 2026 (In Russian)

[5] GOST R 56139-2014 "Functional foods and foods for special dietary uses. Methods for detection and enumeration of probiotic microorganisms" Retrieved from https://docs.cntd.ru/document/1200115455. Accessed January 18, 2026. (In Russian)

[6] ETS No. 123, Strasbourg, 1986) (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Retrieved from https://rm.coe.int/168007a67b. Accessed January 18, 2026.

[7] WMA Declaration of Helsinki — ethical principles for medical research involving human subjects Retrieved from https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/ Accessed January 18, 2026.

[8] Federal Law of the Russian Federation dated December 27, 2018 No. 498FZ "On the responsible treatment of animals and on amendments to certain legislative acts of the Russian Federation." Retrieved from https://docs.cntd.ru/document/552045936. Accessed May 17, 2025. (In Russian).

used to analyze biochemical parameters and biological test results. The significance of biological test parameters differences was determined using the nonparametric Mann–Whitney rank test. A probability of 0.05 was chosen as the level of significance.

Results and discussion

To produce fermented meat raw materials with reduced allergenic potential, the selection of effective probiotic cultures is critically important. Therefore, when developing multi-strain probiotic consortia (for fermentation starters and functional products with certain predetermined properties), it is necessary to use biocompatible strains with both probiotic properties and high synergistic proteolytic activity [42].

We have developed a consortium of the microorganisms that feature high biochemical and proteolytic activity, suitable for the production of fermented food products. The above-specified microorganism strains *(Lactobacillus plantarum 7K, Lactobacillus paracasei κ-406, Pediococcus acidilactici PDA-27 u Staphylococcus carnosus SPHC-108)* were cultured in a milk medium at 37 °C in the optimal quantitative ratio of 3:2:2:1, respectively. Analysis of the degree of proteolysis demonstrated a significant superiority of the microbial consortium over its monoculture (monostrain) analogues. In particular, in terms of the ability to peptonize protein, the bacterial consortium showed higher proteolysis rates (the clearing zone was equal to 27–29 mm). Monocultures of the studied strains demonstrated proteolytic activity with a clearing zone diameter in the range of 15–25 mm. The maximum level of protein hydrolysis (78 %) was registered using this consortium after 12 hours of fermentation, which fact proves a synergistic effect of the proteolytic action of the consortium compared to the relevant parameters of monocultures.

Further, the use of the selected consortium as sources of proteases for the hydrolytic cleavage of beef muscle proteins was the subject of interest. Figure 1 shows the dynamics of beef protein hydrolysis (control sample — unfermented beef; experimental sample — fermented beef).

As the data presented shows, the proteolytic process intensified during the post-slaughter period: in the control sample the degree of hydrolysis in the initial period was low, accounting to 6.23 %. During further studies, the degree of protein hydrolysis increased, reaching 8.85 % on the third day.

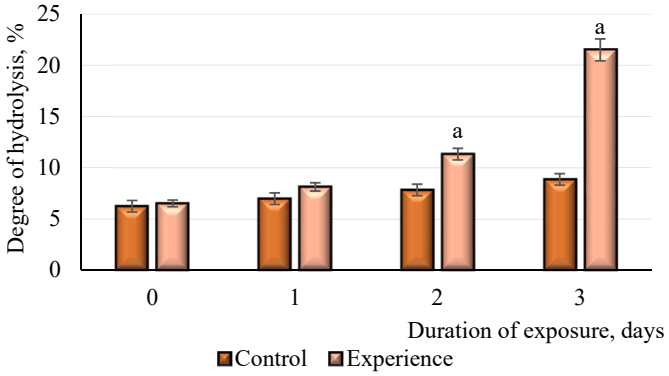

Figure 1. Dynamics of hydrolysis of beef proteins exposed to the effect of the developed consortium of microorganisms (a-p ≤ 0.01 in comparison with the control sample, according to Student's t-test)

Protein proteolysis processes were more active in the experimental sample. After two days, the degree of hydrolysis was 11.32 %. The significant increase in the analyzed parameter was noted after three days, reaching up to 21.5 %, which is 3.3 times higher than that value of the original raw material ($p \leq 0.01$).

Similar data were obtained by the group of authors Bazhenova et al. in the study [43], where a consortium of probiotic microorganisms (including *Lactobacillus paracasei)* was used for horse meat fermentation. Its notable proteolytic activity was observed compared to the monoculture effect: the degree of hydrolysis after three days increased fourfold compared to the original raw material. The authors came to the conclusion that proteolytic modification of proteins affected with the consortium led to a decrease in the antigenic epitopes content level and decreasing the sensitizing activity of the meat raw material.

Yang et al. [44] showed that co-fermentation with the combination of *Staphylococcus cohnii, S. saprophyticus* and *Lactobacillus plantarum* enhanced proteolytic processes in a meat product — Chinese bacon — and provided a positive effect on the quality of the food product.

This way the developed consortium of microorganisms exhibited its proteolytic activity against beef muscle tissue proteins, which opens up the opportunities for its using in the production of a wide assortment of food products.

As it has been noted above in the analysis of references, the studies including the most recent, demonstrate that enzymatic hydrolysis of food proteins is an effective method for reducing the allergen capacity of the food product [19,20,41]. To define the presence or absence of these anti-allergenic properties, the severity of the systemic anaphylaxis reaction and the level of the humoral immune response were assessed in rats sensitized with a model allergen (OVA) against the background of getting a standard vivarium diet and a diet supplement including unfermented and fermented beef.

At the beginning of the experiment, the rats were kept in normal physiological condition. They were active and alert; their muscles were toned; their fur was tightly attached to their bodies, smooth, clean, and shiny; their skin was elastic; visible mucous membranes were pale pink; no inflammatory reactions were observed. Their eyes were bright red. Defecation and urination were within norm.

During the experiment, no significant changes in the animals' clinical condition were observed. The rats actively consumed the food, including unfermented and fermented beef.

The results of changes in the rats' weight at the beginning and end of the experiment are presented in the Table 1.

Table 1. Body weight of the rats in a model experiment sensitized with OVA ($M \pm m$)

Groups of animals sensitized with OVA on the model of anaphylactic reaction of the body, which received	Body weight at the beginning of the experiment, g	Body weight at the end of the experiment, g	Body weight gain, %
I. SVD (control group)	228.0 ± 12.0	243.3 ± 11.8	6.7
II. SVD+ unfermented beef		249.9 ± 12.0	9.6
III. SVD+ fermented beef		271.8 ± 13.6[a, b]	19.2

Note: [a, b] — indicators are reliable in regards to the groups I and II ($p \leq 0.05$) according to the Mann-Whitney test.

As shown in Table 1, the animals in the study groups showed normal growth rate. Rats in the experimental group III demonstrated greater weight gain at the end of the experiment compared to the groups I and II. The average weight gain in the group that received fermented meat was 2.9 and 2.0 times greater respectively, than in the groups I and II ($p \leq 0.05$). The significant weight gain in the group III may be related to its higher bioavailability and digestibility; however, further additional studies of direct digestibility parameters are required to make a final conclusion.

Table 2 presents data on the assessment of anaphylactic reaction severity in the animals. The data in Table 2 show that the anaphylaxis reaction severity in the groups I and II did not differ significantly from each other, although the AI in the group II was 4.5 % higher than in the group I of the animals ($p > 0.05$). In the group III, the anaphylaxis reaction severity accounted to 40.9 % and 43.5 % lower, respectively, in comparison with the groups I and II. In the group III no severe reactions (+++ and +++++) were observed. There were no fatal cases in any groups.

Table 2. Results of assessing the systemic anaphylaxis reaction severity in the rats during the experiment (M)

Groups of animals sensitized with OVA on the model of anaphylactic reaction of the body, which group received	Anaphylactic index (AI)	Severe reactions, %	Mortality rate, %
I. SVD (control group)	2.2	28.6	0.0
II. SVD+ unfermented beef meat	2.3	28.6	0.0
III. SVD+ fermented beef meat	1.3[a, b]	0.0	0.0

Note: [a, b] — indicators are reliable in regards to the groups I and II ($p \leq 0.05$) according to the Mann-Whitney test.

Figure 2 presents the data on the assessment of the severity of the anaphylactic shock reaction in three groups of experimental rats. As it follows from the Figure 2, adding of unfermented beef into the rats' diet (group II) caused a redistribution of the anaphylactic shock reaction severity: there was no mild manifestation (+), and the severity of the reaction (++) was 1.3 times higher than in Group I. The degree of the reaction (+++) was similar to that in the group I. When fermented meat was introduced into the rats' diet (group III), the severity of the anaphylactic shock reaction became the least, since 71.4 % and 28.6 % accounted for the lowest reaction score points (+ and ++), while the score points (+++) were absent.

Figure 3 represents the serum antibody concentrations to ovalbumin in three experimental groups of rats. As can be seen from this figure, humoral immunity in the group II,

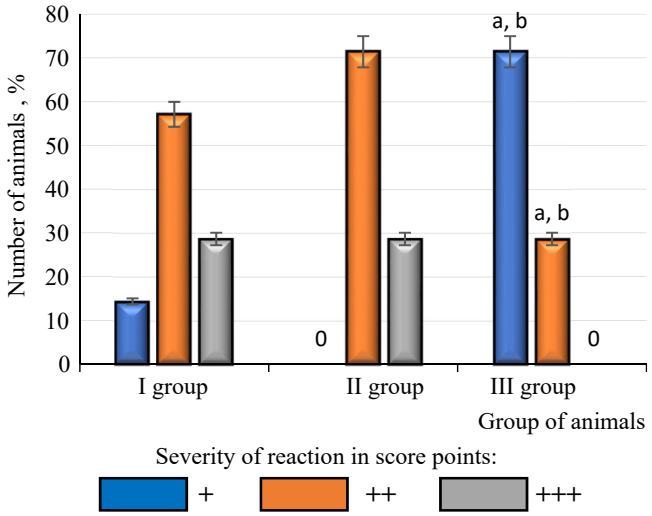

Figure 2. Distribution of anaphylactic shock reaction severity in the rats experimental groups ([a, b] — indicators are reliable in regards to the groups I and II, $p \leq 0.05$) according to the Mann-Whitney test

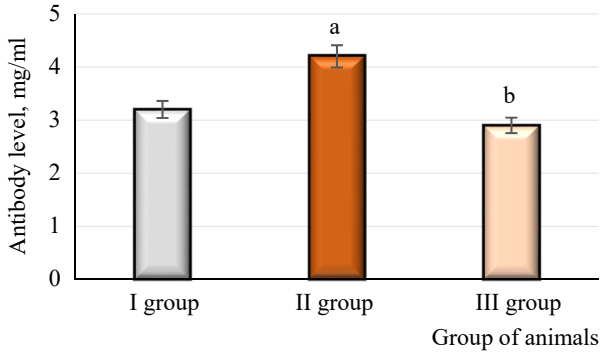

Figure 3. The intensity of the humoral immune response in rats of different experimental groups ([a, b]– indicators are reliable in regards, respectively, to the group I or II, $p \leq 0.05$) according to the Mann-Whitney test.

which consumed unfermented beef, was significantly higher than in the group I, which received SVD only. Humoral immunity in the group III, which received fermented beef, was significantly lower than in the group II (by 1.4 times) and even lower than in the group I ($p > 0.05$). This proves a significant increase in the level of sensitization of animals to the model allergen (OVA) upon consumption of unfermented beef and a decrease in sensitization as a result of fermented beef consumption.

The similar studies were implemented by Simonenko et al. [45] conducted a study on enzymatic hydrolysates of mare's milk, which demonstrated their effect on anaphylaxis in OVA sensitized rats. The parameters like anaphylactic shock severity and the level of specific IgG antibodies were recorded. The authors found that consumption of

hydrolysates did not affect the reaction severity, but led to a 1.9-fold decrease in the level of IgG antibodies to OVA ($p < 0.001$).

Thus, studies conducted on a model of systemic anaphylaxis to OVA revealed that rats that consumed fermented beef in their diet showed higher weight gain at the end of the experiment, a less severe anaphylactic shock reaction, and lower levels of antibodies to OVA compared to the rats fed with standard diet and the rats fed with unfermented beef after sensitization. Apparently, the degree of consortium-fermented beef hydrolysis achieved in the experiment reduced the immunomodulatory effect after ovalbumin sensitization.

The obtained results are consistent with the chosen approach and the efficiency of hypoallergenic meat products for children produced by domestic manufacturers (trademarks "Tema", "Agusha", etc.) and foreign (companies "Nestle", "Gerber", etc.), which use raw materials with low sensitizing activity and which use hydrolysis as a method of biological transformation of allergenic proteins [46,47].

The immunomodulatory effect observed in the group II of the rats that were fed with unfermented meat is likely related to the presence of common allergenic epitopes that give cross-reactions with ovalbumin sensitization. Meat allergies are primarily associated with beef consumption and are more common among the people who suffer from milk and egg allergies. However, other cases are also possible. Research of Revyakina [48] provides examples of anaphylaxis in patients with an allergy to cats when those patients consumed pork (cat-pork syndrome), when consuming meat with an allergy to antibiotics and to other medications.

An important condition for reducing the allergen capacity of the meat products with the help of fermentation starter cultures is their viability during their passing through the gastrointestinal tract (GIT), which directly determines the proteolytic activity and probiotic properties of microorganisms.

Figure 4 shows the results of the bifidobacilli and lactobacilli content analysis of the gastrointestinal tract, determined in the feces of the three studied groups of the rats, on which the model of systemic anaphylaxis to the model allergen OVA was simulated.

As it is shown in Figure 4, adding unfermented beef into the diet during OVA sensitization increased the levels of lacto- and bifidobacteria content by an order of magnitude. Furthermore, introducing fermented meat into the diet during the experimental model of systemic anaphylaxis increased this indicator by 57.1 and 4.0 times, respectively, compared to the groups I and II of the experimental rats ($p \leq 0.01$), thus reaching an average value of 4×10^6 CFU/g. This may indicate that during sensitization of the experimental animals, introducing into their diet the meat fermented by microorganisms consortium increases the amount of beneficial intestinal microflora, which is likely responsible for the attenuated allergenic potential of

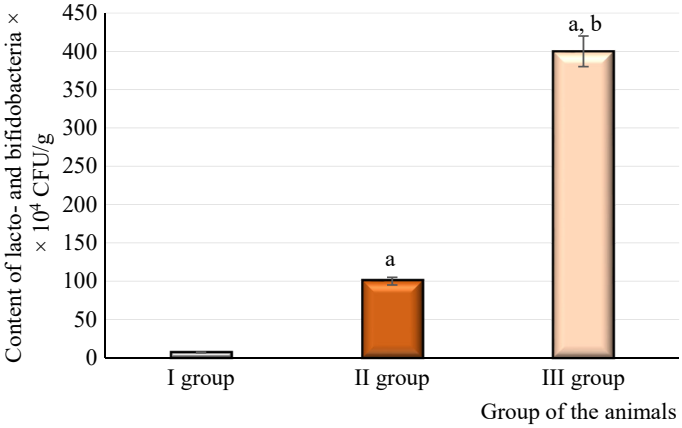

Figure 4. The content of lacto- and bifidobacteria in the excrement of experimental groups of rats ([a, b] — indicators are reliable in regards to, respectively, the group I or II, $p \leq 0.01$) according to the Mann-Whitney test

fermented beef compared to unfermented beef. Whether this is due to the replenishment of beneficial microorganisms that restore the balance of Th1/Th2 cells activity, or due to the biological activity of probiotic metabolites — it still remains unsolved. Further research is needed to assess the impact of the studied consortium on the microbiota composition.

For manifestation of probiotic effect it is important that beneficial microorganisms reach the intestine and colonize it in sufficient quantities. Research shows that for probiotics to adhere to intestinal cells, their numbers in the human body must reach at least 10^6–10^7 CFU/g (CFU/ml). At these concentrations, beneficial microorganisms exert a probiotic effect by modulating the activity of the intestinal microbiota, inhibiting pathogens, alleviating gastrointestinal disorders, and promoting the activation of the immune response [49].

According to modern scientific data, the survival of probiotics in the gastrointestinal tract depends on the type of carrier product (food matrix), the biological characteristics of the strains, and the use of protective technologies (in particular, encapsulation). The review [50] summarizes the main process approaches that ensure the safety of probiotics from production and storage to passage through the gastrointestinal tract. According to Rubio et al. [51], *Lactobacillus strain rhamnosus CTC1679,* used as a starter culture for fermented meat products, successfully survived the harsh gastrointestinal environment and maintained viability in a human clinical trial.

Research results confirm the efficiency of probiotic therapy in experimental models. For example, in the work of Kim et al. al. [52] mice with OVA-induced allergy were given *Lactobacillus acidophilus* and *Bifidobacterium lactis.* Mice given probiotics had suppressed production of OVA-specific antibodies (IgE, IgG1, IgA) in serum and feces, indicating their antiallergenic potential.

Authors Lu et al. [53] studied the effect of three probiotic strains (*L. plantarum, L. reuteri, B. longum)* on mice allergic to OVA. Administration of probiotics not

only alleviated allergy symptoms and reduced the level of OVA-specific IgE, but also changed the composition of the intestinal microbiota. For example, the *B. longum strain* increased the content of bifidobacteria and bacteria producing short-chain fatty acids.

The data obtained are also consistent with modern studies on the efficiency of postbiotics. Unlike probiotics, postbiotics are naturally formed in fermented foods (yogurts, tea fungus kombucha, kefir, sour cabbage, etc.), they are stable over a wide range of temperatures and pH, and their adequate amount can be controlled under production and storage conditions where survival is not the major determining factor [54]. The efficiency of postbiotics in the management of clinical manifestations of food allergies has been noted in a number of studies; in particular, oral administration of bacterial lysate is indicated by the group of authors Bodemer et al. as the best outcome in the therapy of atopic dermatitis in children [55], way of respiratory diseases prevention and therapy [56].

Histological studies of the cranial lymph nodes of three studied groups of rats were conducted. The mucosa is the site of initial contact with the allergen, Peyer's patches provide the primary inductive response, and the mesenteric lymph nodes act as a "decision center" for determining whether to induce food tolerance or initiate food allergic response [57,58].

In a model of OVA systemic anaphylaxis in experimental animals, adding of fermented and non-fermented meat to the rats' diet contributed to antigenic stimulation and structural and functional rearrangement of the mesenteric lymph nodes.

Morphometric parameters of the mesenteric lymph nodes of the studied groups of animals are presented below in the Table 3.

In experimental animals, the cranial mesenteric lymph nodes, which collect lymph from the small intestine and the initial part of the large intestine, are small in dimensions and feature variable shape. They are arranged in a chain of 5–9 nodules along the cranial mesenteric artery and its branches. The mesenteric lymph node is covered by a convoluted connective tissue capsule with an average dimensions of 7.5 to 14.5 μm. In the mesenteric lymph nodes, the border between the cortex and medulla is well differentiated. Figure 5 shows the lymph nodes of the rats in the group I, which contained both primary lymphoid nodules and secondary ones with formed germinal centers that show distinct dark and light zones (Figure 5a and Figure 5b), which indicates the reaction of the body's immune system to OVA sensitization [59].

In the animals of the group II, against the background of OVA systemic anaphylaxis and the unfermented beef inclusion into the diet, the significant structural and morpho-functional transformations were detected in the cranial mesenteric lymph nodes (Table 3). In the lymph node preparations of this group of animals, the formation of a finely looped cellular structure in the parenchyma of the cortex and a large-loop cellular structure in the medullary zone was noted (refer to the Figure 6b). Microscopy of the preparations revealed a slight increase in the dimensions of the primary and secondary lymphoid nodules in the cortex against the background of thinning of their capsule down to 7.5 μm. In contrast to the group I, large number of swollen sinuses of the node filled with lymphoid cells were observed (Figure 6a and Figure 6b). In the preparations obtained from this group of animals, a significant reduction in capsule thickness was achieved by increasing the area of marginal sinus by 2.2 times. A decrease in the quantity of primary and secondary lymphoid nodules, which are the lymph nodes dynamic structures,

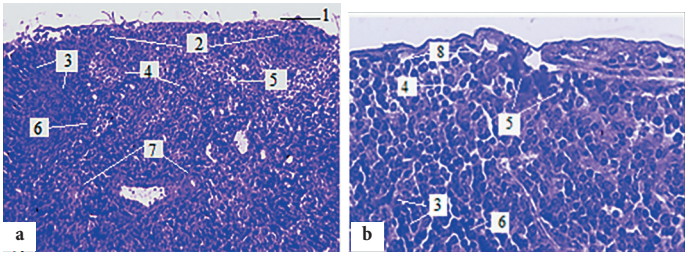

Figure 5. Micrographs of the mesenteric lymph node in the group I: a) cortex and medulla: 1 — capsule; 2 — cortex; 3 — primary lymphoid nodules clusters; 4 — secondary lymphoid nodules with a dark center; 5 — secondary lymphoid nodules with a light center; 6 — paracortical zone; 7 — medulla; 8 — cortical sinus. b) cortex. Stained with hematoxylin and eosin. A — zoom (PL 10×/0.25); B — zoom (PL 40×/0.65)

Table 3. Morphometric parameters of the cranial mesenteric lymph nodes structures in rats in the experiment ($M \pm m$)

Structure	Group I	Group II	Group III
Capsule thickness, μm	13.5 ± 1.5	7.5 ± 2.0[a]	14.5 ± 2.5[b]
Marginal sinus area, μm^2	670.5 ± 25.5	1505.0 ± 105.0[a]	755.5 ± 25.0[b]
Area of the cerebral sinus, μm^2	3475.0 ± 350.0	1300.0 ± 250.0[a]	4252.5 ± 450.0[b]
The number of primary lymphoid nodules on the section	12.5 ± 1.0	5.0 ± 1.5[a]	13.5 ± 2.0[b]
The number of lymphoid nodules with germinal centers on the section	9.0 ± 2.5	6.5 ± 3.0	12.5 ± 2.0[b]
Thickness of a lymphoid nodule with a light germinal center, μm	13.0 ± 4.5	11.4 ± 5.0	14.0 ± 6.5
Thickness of a lymphoid nodule with a dark germinal center, μm	9.0 ± 4.0	11.5 ± 4.0	8.0 ± 3.5
Paracortical zone thickness, μm	155.0 ± 25.0	95.0 ± 30.0	165.0 ± 35.0
Paracortical zone, % of the slice area	18.0 ± 4.0	14.0 ± 2.0	19.0 ± 3.5
Medulla, % of section area	30.0 ± 4.0	43.0 ± 5.5	28.5 ± 4.5
Cortex, % of section area	51.5 ± 10.5	42.5 ± 3.5	52.5 ± 6.0

Note: [a, b] indicators are reliable in regards to, respectively, the group I or the group II ($p \leq 0.05$) according to Student's t-test.

and form and disappear under the effect of various factors, was observed. A slight decrease in the area (as a percentage of the lymph node cross-sectional area) of the paracortical and cortical zones was noted, while an increase in the area of the medullary zone relative to the lymph node cross-sectional area was observed.

In some sections with expanded medulla, compression and displacement of the cortex to the periphery of the node were observed, with a significant reduction in the paracortical zone to 95 μm (Table 3 and Figure 6a). The table shows that, together with a slight increase in the primary and secondary nodules dimensions, there was a decrease in the thickness and quantity of lymphoid nodules with a light proliferation center, compared to the corresponding values in the group I (Table 3). According to references data, a significant increase in the sinuses of the mesenteric lymph nodes indicates increasing of drainage function of lymph through them [60].

Based on the obtained results, it can be assumed that the inclusion of beef meat provided a negative effect on the anaphylaxis reaction course, as evidenced by structural and functional changes associated with a decrease in barrier function, the development of hyperplasia and suppression of the lymphoid structure.

In most of the CMLN preparations of group III animals (inclusion of fermented beef meat in the diet against the background of the anaphylactic shock model), all the studied quantitative and dimensional parameters and the morphological picture practically corresponded to those in the group of animals on SVD and sensitized with OVA (group I), or changed insignificantly and insignificantly ($p > 0.05$) (Table 3 and Figure 7).

The experimental data obtained above in the systemic anaphylaxis model to OVA allow us to draw the general conclusion that introducing unfermented beef into the diet of sensitized animals (the group II) resulted in significant structural changes in the mesenteric lymph nodes, manifested by functional decompensation of the lymph nodes in response to dietary changes. Capsule thinning and sinus swelling indicate signs of strain on the filtration mechanisms and immune control of lymph flowing from the intestine. This condition can be classified as adaptive hyperplasia of lymphoid tissue with a predominant sinus component, demonstrating the active participation of lymph nodes in the immune response and lymph filtration during dietary changes.

The morphological pattern of CMLN in a model of OVA systemic anaphylaxis with the fermented beef inclusion into the diet (the group III) did not negatively affect the sensitization process compared to unfermented meat. The correspondence of most measured parameters to values close to those in control animals (the group I) indicates that fermented meat did not cause significant changes in the anaphylaxis reactions course or in lymph node structure that would indicate the allergenic effect severity.

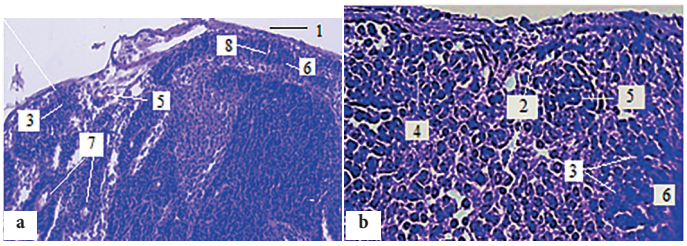

Figure 6. Micrographs of the mesenteric lymph node in group II: a) cortex and medulla: 1 — capsule; 2 — cortex; 3 — primary lymphoid nodules clusters; 4 — secondary lymphoid nodules with a dark center; 5 — secondary lymphoid nodules with a light center; 6 — paracortical zone; 7 — medulla; 8 — cortical sinus. b) — cortex. Stained with hematoxylin and eosin. A — zoom (PL 10×/0.25); B — zoom (PL 40×/0.65)

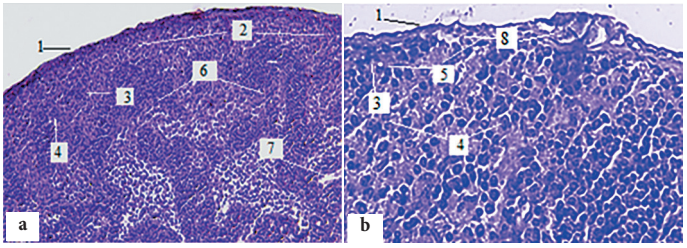

Figure 7. Micrographs of the mesenteric lymph node in group III: a) cortex and medulla: 1 — capsule; 2 — cortex; 3 — clusters of primary lymphoid nodules; 4 — secondary lymphoid nodules with a dark center; 5 — secondary lymphoid nodules with a light center; 6 — paracortical zone; 7 — medulla; 8 — cortical sinus; b) — cortex. Stained with hematoxylin and eosin. A — zoom (PL 10×/0.25); B — zoom (PL 40×/0.65)

Limitations of the study

When interpreting the obtained results, several methodological limitations should be considered. First, the model of ovalbumin-induced systemic anaphylaxis used does not fully reproduce the physiology of food allergy in humans, which limits the direct extrapolation of the findings. Second, although IgG was chosen as a marker of the humoral response in this study, the lack of a parallel determination of IgE serves as methodological limitation, because this isotype is considered to be the main mediator of class I allergy. The obtained data on IgG only indirectly proves an immunomodulatory effect but do not allow differentiation between IgE- and non- IgE -mediated pathways. Third, the relatively small quantity of the rats in each group requires confirmation of the identified patterns in more representative samples array. Further studies with a larger quantity of the animals, measurement of IgE content level, and using allergen-specific test systems are required to verify the obtained data.

Conclusion

The immunomodulatory potential of beef meat exposed to an effect of probiotic microorganisms consortium was evaluated.

The consortium was represented by four strains: *Lactobacillus plantarum 7K, Lactobacillus paracasei κ-406, Pediococcus acidilactici PDA-27 u Staphylococcus carnosus SPHC-108* exhibited high proteolytic activity during beef

muscle proteins hydrolysis. After two days of meat fermentation, the degree of hydrolysis was 11.32 %, and after three days it was 21.5 %, which is 3.3 times higher than the hydrolysis rate of the control sample.

The allergenic potential of fermented and unfermented beef was studied using a model of systemic anaphylactic reaction in the experimental animals. Fermented beef was found capable to reduce the severity of the systemic anaphylactic reaction to a model allergen (OVA) in the sensitized animals. Sensitized animals that received fermented meat featured a significantly lower anaphylactic index (1.3 points) compared to the animals in the control group (2.2 points) who consumed SVD, and the rats that consumed SVD and unfermented meat (2.3 points). The anaphylaxis reaction severity decreased by 40.9 % and 43.5 %, respectively. The concentration of specific antibodies (IgG) to ovalbumin in the blood serum of the rats that consumed fermented meat was significantly lower ($p \leq 0.05$), than in the group of the rats that consumed non-fermented meat, and which level approached to the level in the control group.

The animals that were fed with fermented meat during sensitization showed greater weight gain at the end of the experiment. The average weight gain in the group of rats that ate fermented meat was 19.2 %, which was 2.9 times higher than in the control group and 2.0 times higher than in the group of rats that consumed unfermented meat. These results indicate the potential for improved digestibility and absorption of fermented meat, but further studies of direct metabolic parameters are required to draw final conclusion.

Changes in the intestinal microbiota of sensitized animals were also noted. The levels of lacto- and bifidobacteria in the feces of the animals that were fed with fermented meat increased by 57.1 and 4.0 times, respectively, in comparison with the control group and the group of rats that consumed unfermented meat. The average value was approximately 4×10^6 CFU/g. Thus, a correlation was observed between increased levels of lacto- and bifidobacteria and less severe anaphylaxis, which requires further study of the mechanisms of their protective effect.

Histological studies of mesenteric lymph nodes of the rats showed that introducing fermented beef into the diet during OVA sensitization did not cause significant changes in lymphoid tissue, and the nodes were virtually identical to those in the rats' control group, which were fed with standard vivarium diet. On contrary the rats fed with unfermented meat during sensitization showed significant changes, thus evidencing increased antigen load and stressed immune mechanisms.

Thus, data obtained in the systemic anaphylaxis model showed that using fermented meat in the diet after its exposure to the developed consortium of microorganisms, reduced the severity of the systemic anaphylactic reaction to the model allergen in the sensitized animals, contributed to greater weight gain and increased the population of beneficial intestinal microflora.

The results confirm that using the probiotic microorganisms for meat fermentation is a promising approach for reducing food allergies risk and developing hypoallergenic food products. The mechanisms of interaction between intestines microbiota, allergy, and fermentation process technologies are currently being intensively studied, and this study contributes to this field, opening up the opportunities for development of effective allergy prevention and therapy strategies, as well as improving the quality of life of people who suffer from food allergies.

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