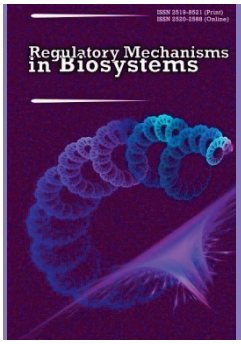

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Influence of *Lactobacillus curvatus* and *Lactococcus lactis* subsp. *lactis* on the shelf life of sausages in vacuum packaging

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Boiled meat products, including sausages, are perishable items, making them potentially hazardous and requiring the use of preservatives. Promising preservatives for sausages include starter cultures of lactic acid microorganisms, which act as antagonists to pathogenic and opportunistic bacteria. Therefore, this study aimed to determine the types of microbiota causing spoilage of sausages during storage in vacuum packaging and the effectiveness of their treatment with a starter culture of *Lactobacillus curvatus* or a mixture of starter cultures of *Lactobacillus curvatus* + *Lactococcus lactis* subsp. *lactis*. On the first day of storage, the dominant microorganisms in the sausages were *Klebsiella variicola* and *Bacillus amyloliquefaciens plantarum*. By the 21st day of storage, signs of sausage spoilage were observed, including the appearance of cloudy juice and the separation of the vacuum packaging. The main microorganisms causing sausage spoilage were *Moellerella wisconsensis*, *Proteus mirabilis*, and *Bacillus cereus*. In the control sausages on the 18th day, the number of mesophilic aerobic and facultative anaerobic microorganisms approached the allowable limit, and they were not suitable for further storage. Treatment of sausages with a starter culture or a mixture of *Lactobacillus curvatus* + *Lactococcus lactis* subsp. *lactis* led to an increase in the number of mesophilic aerobic and facultative anaerobic as well as lactic acid microorganisms practically throughout the entire storage period. However, signs of spoilage in both treatment groups appeared on the 36th day. Both treatments with starter cultures extended the shelf life of sausages in chilled conditions by 12 days. The use of a mixture of starter cultures for treating sausages during storage in vacuum packaging was not advisable. The obtained results can be used to extend the shelf life of boiled meat products, taking into account the species composition of spoilage microbiota.

Keywords: boiled meat products; casing; vacuum packaging; spoilage; lactic acid bacteria.

Introduction

Sausages are highly popular among consumers and are one of the oldest processed food products known to mankind. However, boiled meat products are characterized by a short shelf life due to contamination by microorganisms that cause spoilage. Microorganisms enter the meat from the hands of workers, utensils, tables, protective clothing, and the air in production facilities (Rocha et al., 2024). Furthermore, there is a redistribution of microorganisms from the surface to the inner parts of the muscle tissue during the cutting, deboning, and trimming processes, leading to the accumulation of blood and muscle juice, which provide a favorable environment for microbial growth. During the preparation of sausage mince, meat from different animal species is ground in meat grinders, resulting in further contamination by microorganisms from equipment, air, and workers' hands.

The qualitative composition of the microbiota found in the mince and finished meat products is highly diverse and consists of saprophytic and opportunistic pathogenic microorganisms capable of causing spoilage during sausage storage, manifested by the appearance of cloudy juice, slime, and a specific odor. Concerns are also raised about pathogenic microorganisms such as *Salmonella* spp., *Escherichia* spp., and *Listeria* spp., which do not cause unpleasant odor, taste, or changes in the appearance of sausages but possess resistance to antimicrobial agents (Bayer et al., 2017; Shevchenko et al., 2019; Popov et al., 2021; Melnychuk, 2024) and can cause human illness. To prevent their multiplication and extend the shelf life of boiled meat products, including sausages, various methods are used, including chemical methods such as kitchen salt; physical methods such as heat treatment and packaging; and biological methods, such as the use of extracts of essential oils (Ajourloo et al., 2021; Aminzare et al., 2022), as well as microorganisms that exhibit antagonistic action against pathogenic bacteria (Matthews et al., 2017; Yu et al., 2021; Barcenilla et al., 2022).

Considering the undesirable effects of chemical preservatives on consumers, including toxicity and carcinogenicity (Mologni et al., 2019), as well as changes in the organoleptic properties of food products (Aaliya et al., 2021), promising preservatives for boiled meat products, including sausages, include the use of lactic acid bacteria cultures, particularly *Lactobacillus curvatus*, whose metabolites exhibit antimicrobial properties (Rahman et al., 2022). Lactic acid bacteria have been extensively studied, and it has been found that many of their metabolites demonstrate bactericidal or bacteriostatic properties (Darbandi et al., 2022), including reuterins, bacteriocins, organic acids, amino acids, mono-hydroxy fatty acids, exopolysaccharides, and others (Cheng et al., 2024).

Bacteriocins of lactic acid bacteria constitute a wide and versatile group of bioactive bacterial peptides or proteins with high antimicrobial potential against other bacteria (Simons et al., 2020). They exert their antibacterial activity through four different mechanisms: by forming pores in the cell membrane, inhibiting the biosynthesis of cell wall components, affecting the activity of autolytic enzymes, and suppressing the formation of bacterial spores. The most significant role of bacteriocins lies in their use as natural preservatives in food products, as well as an alternative to harmful and potentially carcinogenic synthetic additives. Additionally, lactic acid microorganisms have many other beneficial functions in consumers' bodies, including probiotics (Wang et al., 2021) and antimutagenic (Kim et al., 2019) effects. Their presence in food products influences

their rheological and sensory characteristics. Various lactic acid and other beneficial food microorganisms, such as yeasts, propionic acid bacteria, etc., are used in the food industry to create well-adapted compositions artificially added to food products, including meat products, for fermentation, quality improvement, flavor and aroma enhancement, or increased shelf life. One of the important conditions for extending the shelf life of boiled meat products, including sausages, is to ensure anaerobic conditions. This is achieved through the use of vacuum packaging, modified gas atmospheres (Czerwiński et al., 2021), and various oxygen scavengers aimed at reducing the intensity of oxidative processes in food products (Kawecki et al., 2021; Gupta, 2023). Under such conditions, lactic acid microorganisms capable of providing conservation of boiled meat products, including *Lactococcus lactis* and *Lactobacillus curvatus*, can proliferate, offering advantages over any chemical preservatives (Ramaroson et al., 2018; Chen et al., 2020). *Lactobacillus curvatus* exhibits pronounced resistance to the acidic pH of gastric juice, lysozyme, and bile components, with high-intensity bacteriocin synthesis demonstrating strong antilisterial activity and the ability to inhibit some spoilage bacteria of meat products. As a resident component of the microbiota in meat products, it also plays a role in their maturation and the formation of desired flavor. Therefore, *Lactococcus lactis* and *L. curvatus* are often used in the food industry as starter cultures for fermented sausages and as biological protection cultures for meat products (Chen et al., 2020; Chaillou et al., 2022). Moreover, bacteriocins of lactic acid microorganisms can be used in various forms, including purified or partially purified fractions loaded into active films/coatings or incorporated into encapsulated systems designed for the storage of meat products (Smaoui et al., 2023).

Most research on the effectiveness of using lactic acid cultures and their compositions is dedicated to fermented meat products, including dried sausages. There is little research on the use of preservatives in the storage of boiled meat products such as sausages, and this does not provide answers regarding their impact on the shelf life as it depends on a significant number of factors unique to each meat processing plant, including the sanitary condition of the technological equipment, hygiene of workers and animal slaughter, the combination of primary and auxiliary raw materials, the species composition of food additives, the characteristics of sausage casing and packaging, and their microbial contamination.

Therefore, the research goal is to determine the species composition of microbiota causing sausage spoilage during storage in vacuum packaging under refrigerated conditions and, based on the obtained data, select effective strains of starter lactic acid microorganisms capable of suppressing the growth of spoilage bacteria and extending the shelf life of sausages.

Materials and methods

For the research, a batch of "Juicy" sausages of the first grade was produced at a local meat processing plant in the Zakarpattia region. The following recipe was used: main ingredients, kg/100 kg: fatty pork – 20, semi-fat pork – 34, poultry meat – 30, pork skin emulsion – 10, dry milk – 3, potato starch – 3, water (ice) – 25; auxiliary ingredients, kg/100 kg: table salt – 2.3, sodium nitrite – 0.0075, food additive Emulin (guar gum thickener, mixture of milk proteins, sodium tripolyphosphate stabilizer, table salt) – 2, food additive Parovka combo (spices: white pepper, mustard, celery), spice extracts (cardamom, paprika), dextrose, yeast extract – 1, food additive Ham flavor (black pepper extracts, garlic, smoked pepper, and lovage, maltodextrin, soy protein hydrolyzate, yeast hydrolyzate) – 0.2, food additive Milk fortified (spices and spice extracts: cardamom, nutmeg, garlic, meat flavor and aroma, blood hemoglobin (color fixative), milk proteins, carriers (dextrose, rice flour, table salt) – 1.

The research was conducted in two stages: In the first stage, the main types of microorganisms causing spoilage of "Juicy" sausages during storage in vacuum packaging in a refrigerator at a temperature of 4 ± 1 °C were determined. For the study, 10 vacuum packs of "Juicy" sausages weighing 200 g each were used. For microbiological analysis, 5 packs of sausages were used for the first day of storage and 5 packs for the appearance of signs of spoilage (appearance of cloudy juice and delamination of the vacuum packaging) on the 21st day of storage.

In the second stage, the effectiveness of using starter lactic acid cultures to suppress microorganisms causing spoilage of "Juicy" sausages during storage in vacuum packaging in a refrigerator at a temperature of 4 ± 1 °C was investigated. For this purpose, 60 vacuum packs of sausages weighing 200 g each were prepared, which were divided into 3 variants: control and two experimental ones. Sausages of the control variant were not subjected to treatment, while sausages of the experimental variants were treated by spraying a suspension of starter cultures according to the scheme shown in Table 1.

Table 1
Study scheme to determine the impact of starter cultures of lactic acid microorganisms on the quality and safety of "Juicy" sausages during storage in vacuum packaging in a chilled state (n = 20)

Variant	Experimental conditions
Control	"Juicy" sausages of the first grade in vacuum packaging
Experimental 1	"Juicy" sausages of the first grade treated with starter culture SafePro BLC-48 (*Lactobacillus curvatus*) at a rate of 5 x 10^6 CFU/cm^2 of surface
Experimental 2	"Juicy" sausages of the first grade treated with a mixture of starter cultures SafePro BLC-48 (*Lactobacillus curvatus*) + Bactoferm Rubis (*Lactococcus lactis subsp. Lactis*) at a rate of 5 x 10^6 CFU/cm^2 of surface

For the research, lactic acid microorganisms SafePro BLC-48 (*Lactobacillus curvatus*) + Bactoferm Rubis (*Lactococcus lactis* subsp. *lactis*) from Chr. Hansen (LLC "Chr. Hansen, Kyiv, Ukraine") was used. Polyamide casing Evro-Bar (Company "ATRIM PACK", Kharkiv, Ukraine) and vacuum packaging Amilen PA/PE (LLC "Kozak+", Vinnytsia, Ukraine) were used for making sausages. All sausage variants were stored in the refrigerator until signs of spoilage appeared. The results of the study were recorded on days 1, 12, 18, 25, 30, and 36 of sausage storage.

Microbiological studies were conducted at the Zakarpattia Regional State Laboratory of the State Service of Ukraine for Food Safety and Consumer Protection in Uzhhorod and LLC "Expert Center "Biolights" in Ternopil. For each study, 5 samples of casing and vacuum packaging for sausages, fresh minced meat, and ready-made sausages were taken.

To determine the quantity of microorganisms, fungi, and mold, average samples of sausages were taken, and washings from sausage casings of 5 vacuum packages of each variant were made. For this, sequential tenfold dilutions were prepared in a sterile physiological solution. The quantity of microorganisms was determined in colony-forming units (CFU), and the results were expressed in lg CFU/g for minced meat and in lg CFU/cm^2 of casing surface for sausages. The genus and species identification of isolated microorganisms were performed according to current methods.

A mass spectrometer Bruker Daltonics, Maldi Tof Microflex (Bruker Daltonics, Germany) was used for microbiological studies. Plate count agar M091 (HiMedia, India) medium was used to determine the quantity of mesophilic aerobic and facultative anaerobic microorganisms (MAFAM). Lactobacillus deMan, Rogosa, Sharpe Agar M641 (HiMedia, India) was used for the isolation and quantitative determination of bacteria of the genus *Lactobacillus*, Baird Parker Agar M043 (HiMedia, India) for the isolation of pathogenic and non-pathogenic staphylococci, Bismuth Sulphite Agar M027 (HiMedia, India) and Xylose Lysine Deoxycholate Agar M031 (HiMedia, India) for *Salmonella*, Agar Palcam (HiMedia, India) and Agar Oxford (HiMedia, India) for *L. monocytogenes*, *Bacillus cereus* (Selective agar) M833 (HiMedia, India) for *Bacillus cereus*, Endo Agar (HiMedia, India) for Enterobacteria, and Sabouraud Agar (HiMedia, India) for the isolation of yeast and mold.

The statistical analysis of the obtained results was performed using the ANOVA program, and the data in the tables are presented as x ± SD (mean ± standard deviation). The difference between groups was considered significant using the Tukey test at $P < 0.05$ (taking into account the Bonferroni correction).

Results

The reasons for the spoilage of sausages during storage in vacuum packaging were investigated. The level of contamination of the fresh sausage meat with mesophilic aerobic and facultative anaerobic microorganisms (MAFAM) for "Juicy" sausages was 3.23 ± 0.16 lg CFU/g, and after thermal processing during boiling, it decreased to 1.21 ± 0.14 lg CFU/g, a decrease of 2.02 lg CFU/g ($P < 0.001$). The microbiological

indicators of the polyamide single-layer casing Evro-Bar and the vacuum multilayer packaging Amilen PA/PE for sausages in terms of the quantity of MAFAM, lactic acid microorganisms, yeast and mold fungi complied with current requirements (Table 2).

Among the microorganisms that formed the basis of MAFAM, *Klebsiella variicola*, and *Bacillus amyloliquefaciens plantarum* were found in sausages stored in vacuum packaging in a refrigerated state on the first day (Table 3).

Storage of sausages in vacuum packaging in the refrigerator conditions led to signs of spoilage by the 21st day, such as the appearance of juice, its clouding, and the delamination of the vacuum packaging. This was accompanied by an increase in the quantity of MAFAM to 4.89 lg CFU/g compared to the initial data of sausages on the first day of storage. The main microorganisms found in the spoiled sausages were *Moellerella wisconsensis*, *Bacillus cereus*, and *Proteus mirabilis*.

Table 2
Microbiological indicators of the casing and packaging for "Juicy" sausages (x ± SD, n = 5, lg CFU/cm^2)

Casing	Microbiological indicators		
	MAFAM	lactic acid bacteria	yeasts, molds
Evro-Bar single-layer polyamide casing	1.20 ± 0.14	< 1	< 1
Amilen PA/PE multi-layer vacuum packages	< 1	< 1	< 1

Table 3
Microbiological indicators of "Juicy" sausages stored in vacuum packaging in a chilled state (x ± SD, n = 5, lg CFU/g)

Research period, days	MAFAM	Microbiological screening on MALDI-TOF
1	2.34 ± 0.28	*Klebsiella variicola, Bacillus amyloliquefaciens plantarum*
21	7.23 ± 0.98***	*Moellerella wisconsensis, Bacillus cereus, Proteus mirabilis*

Note: *** – P < 0.001 compared to data from day 1 of storage.

The impact of starter cultures *Lactobacillus curvatus* and *Lactococcus lactis* subsp. *lactis* on the microbiological indicators of sausages during storage in vacuum packaging was examined. Treating "Juicy" sausages with the SafePro BLC-48 (*Lactobacillus curvatus*) starter culture before vacuum packaging for the first day of storage led to an increase in the MAFAM count by 1.19 lg CFU/g, while treating them with a mixture of SafePro BLC-48 (*Lactobacillus curvatus*) + Bactoferm Rubis (*Lactococcus lactis* subsp. *lactis*) led to an increase by 1.46 lg CFU/g compared to the control. Storage of sausages up to the 12th day further increased the MAFAM count in the case of treatment with a single lactic acid microorganism starter culture by 0.98 lg CFU/g, whereas using a mixture of starter cultures led to an increase of 1.24 lg CFU/g compared to the control (Table 4). In the "Juicy" sausages of the control group, the MAFAM count approached 3 lg CFU/g by the 18th day, making further storage impractical. Treating "Juicy" sausages with the SafePro BLC-48 starter culture led to an increase in the MAFAM count by 0.72 lg CFU/g on the 18th day of storage while using a mixture of starter cultures (SafePro BLC-48 + Bactoferm Rubis) increased it by 1.67 lg CFU/g compared to the control. Additionally, sausages treated with the mixture of lactic acid starter cultures had a 0.95 lg CFU/g higher MAFAM count compared to those treated with a single starter culture.

Storage of sausages from both treatment groups up to the 30th day was characterized by an increase in the MAFAM count. On the 25th day of storage, a higher rate of increase in the MAFAM count was observed in sausages treated with the mixture of starter cultures (SafePro BLC-48 + Bactoferm Rubis) compared to those treated with only SafePro BLC-48 by 0.94 lg CFU/g, and on the 30th day, by 1.11 lg CFU/g (Table 4).

By the 36th day of storage, both treatment groups of sausages showed signs of spoilage, including the appearance and clouding of juice and delamination of the vacuum packaging.

By the first day of storage, the sausages in the control group did not differ from those processed with the SafePro BLC-48 starter culture. For sausages treated with a mixture of starter cultures, there was an increase in the quantity of lactic acid microorganisms by 0.76 lg CFU/g compared to the control variant (Table 5). On the 12th day of storage, in sausages processed with the SafePro BLC-48 starter culture, the quantity of lactic acid microorganisms exceeded the analogous indicator in the control variant by 0.36 lg CFU/g, while in sausages treated with a mixture of starter cultures SafePro BLC-48 + Bactoferm Rubis, it exceeded it by 1.03 lg CFU/g. By the 18th day of storage, the quantity of lactic acid microorganisms in sausages processed with a single starter culture predominated over the control by 0.43 lg CFU/g, and in sausages treated with a mixture of starter cultures, it predominated by 1.39 lg CFU/g. Further storage of sausages in both treatment variants led to an increase in the quantity of lactic acid microorganisms. Moreover, on the 25th and 30th days of storage, sausages treated with a mixture of starter cultures showed a higher quantity of lactic acid bacteria by 1.51 lg CFU/g and 1.22 lg CFU/g, respectively, compared to sausages treated with a single starter culture.

Table 4
Number of MAFAM in "Juicy" sausages processed with starter cultures of lactic acid microorganisms and stored in vacuum packaging in a chilled state (x ± SD, n = 5, lg CFU/g)

Research period, days	Control variant	SafePro BLC-48	SafePro BLC-48 + Bactoferm Rubis
1	2.02 ± 0.12^{a}	3.21 ± 0.23^{b}	3.48 ± 0.25^{b}
12	2.31 ± 0.20^{a}	3.29 ± 0.19^{b}	3.55 ± 0.32^{b}
18	2.92 ± 0.24^{a}	3.64 ± 0.27^{b}	4.59 ± 0.21^{c}
25	–	4.09 ± 0.30^{b}	5.03 ± 0.37^{c}
30	–	5.98 ± 0.23^{b}	7.09 ± 0.56^{c}

Note: on day 21, the sausages spoiled and were not subjected to examination; different letters of the superscripts indicate values that likely differed within the same row of the table (P < 0.05) according to the results of comparison using the Tukey test with Bonferroni correction.

Table 5
Quantity of lactic acid microorganisms in "Juicy" sausages treated with lactic acid microorganism cultures and stored in vacuum packaging under refrigeration (x ± SD, n = 5, lg CFU/g)

Research period, days	Control variant	SafePro BLC-48	SafePro BLC-48 + Bactoferm Rubis
1	1.19 ± 0.25^{a}	1.28 ± 0.13^{ab}	1.95 ± 0.33^{b}
12	1.26 ± 0.16^{a}	1.62 ± 0.17^{b}	2.29 ± 0.28^{b}
18	1.69 ± 0.27^{a}	2.12 ± 0.25^{b}	3.08 ± 0.19^{b}
25	–	2.26 ± 0.18^{b}	3.77 ± 0.12^{c}
30	–	3.34 ± 0.32^{b}	4.56 ± 0.27^{c}

Note: see Table 4.

Over the storage period, both for the control variant sausages on the first and 18th day, as well as for sausages in both treatment groups with lactic acid bacterial cultures on the first, 18th, 25th, and 30th days, no conditionally pathogenic or pathogenic microorganisms were detected, including *S. aureus*, *L. monocytogenes*, *Salmonella* spp., *E. coli*, coliform bacteria, as well as yeast and mold.

The quantity of MAFAM on the surface of the polyamide casing Evro-Bar on the first day after treatment with lactic acid bacterial cultures did not differ between the experimental variants of sausages (Table 6).

Table 6
Quantity of MAFAM in the Evro-Bar casing of "Juicy" sausages treated with lactic acid bacterial cultures and stored in vacuum packaging under refrigeration (x ± SD, n = 5, lg CFU/cm^2)

Research period, days	SafePro BLC-48	SafePro BLC-48 + Bactoferm Rubis
1	6.29 ± 0.45	6.93 ± 1.11
18	6.47 ± 0.32	7.09 ± 0.37
30	6.53 ± 0.19	7.44 ± 0.45

Storage of sausages up to the 18th and 30th day revealed that treating them with a mixture of starter lactic acid bacterial cultures tended to increase the quantity of MAFAM on the surface of the polyamide casing Evro-Bar compared to the corresponding indicators for treatment with only one starter culture.

Discussion

The reasons for the spoilage of sausages during storage in vacuum packaging are multifaceted. In the production of cooked meat products, including sausages, there are several stages of contamination of raw materials by microorganisms capable of causing their spoilage. A significant factor determining the microbiological indicators of finished products is the hygiene of processing slaughterhouse carcasses, the level of mincing of the main raw material, as well as the sanitary and hygienic conditions of the sausage production facility (technological equipment, air environment, tools, and workers' hands). Additionally, spices, auxiliary ingredients, sausage casings, and packaging also contain various types of aerobic and anaerobic bacteria, which under appropriate temperatures and nutrient conditions can grow and multiply.

The presence of many pathogenic bacteria in the raw materials for sausage production, including meat from animals and poultry, is well documented. Pathogenic bacteria may include *Salmonella* spp., *Campylobacter* spp., *Escherichia coli*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus*, as well as saprophytes such as *Pseudomonas*, which accelerate meat spoilage (Łaszkiewicz et al., 2021). The risk of increasing the quantity of microorganisms in the finished meat product increases with the level of mincing and the degradation of muscle fibers that occurs during the preparation of sausage meat.

As our research has shown, the level of contamination by microorganisms in fresh sausage meat, as measured by the MAFAM index, reached 3.23 lg CFU/g, which is associated with the use of a significant amount of main and auxiliary raw materials, including fatty and semi-fat pork, poultry meat, pork skin emulsion, dry milk, potato starch, water (ice), food additives containing spices and spice extracts, as well as sausage casings. This significantly increases the risk of microbial contamination of the meat mixture. Thermal processing of sausages during boiling provides a temperature inside the sausage at around 70 °C, which ensures the destruction of a significant portion of microorganisms, resulting in a substantial reduction in their quantity in the finished product.

On the first day of storage, the MAFA index in "Juicy" sausages was within the requirements of regulatory documents. The predominant microorganisms detected in the sausages on the first day of storage were *Klebsiella variicola* and *Bacillus amyloliquefaciens plantarum*.

Klebsiella variicola bacteria belong to the Enterobacteriaceae family and are widespread in plants, the human body, and animals. It was previously considered a component of the normal gut microbiome in humans and the rumen of cattle but is now being considered a pathogen capable of causing urinary tract infections in both humans and animals. *Klebsiella variicola* has also been found in pork and can cause mastitis in cattle and be excreted in milk and animal products. The contamination of sausage meat with *K. variicola* could be due to workers' hands, raw materials, and food additives, which require further investigation into the transmission chain of *K. variicola* in the conditions of this meat processing plant.

Bacillus amyloliquefaciens plantarum are widely distributed in the environment and known as colonizers of plant rhizospheres, protecting and stimulating their growth. These microorganisms are not harmful to humans and animals; instead, they play an important probiotic role in the digestive system. They also synthesize proteases capable of hydrolyzing native insoluble proteins (elastin, fibrin, collagen). Some strains of *Bacillus amyloliquefaciens* ssp. *plantarum* are major components of probiotic preparations, including veterinary products such as endospore. This partly explains the presence of these microorganisms in the raw materials used for making sausages. Storage of sausages in vacuum packaging in the refrigerator was accompanied by an increase in the MAFAM index, reaching a critical level by day 21st, characterized by signs of spoilage – juice and its cloudiness and separation of the vacuum packaging layers. Among the dominant microorganisms detected in the sausages on day 21st of storage were representatives of the Enterobacteriaceae family: *Moellerella wisconsensis*, *Proteus mirabilis*, and Bacillaceae: *Bacillus cereus*. *Proteus mirabilis* is widely distributed in fermented meat products, including sausages, and is often the cause of their spoilage. *Moellerella wisconsensis* is frequently found in cooked meat products showing signs of spoilage.

Bacillus cereus belongs to the Bacillaceae family, which is ubiquitous in the environment and can be isolated from food and food materials. It also forms heat-resistant spores, which likely survive the mild thermal processing of cooked meat products, which involves reaching a temperature of 70 °C inside the sausage core. Some strains of *B. cereus* can grow at temperatures of 8 °C and lower, conditions commonly found in household refrigerators where sausages are stored. Our research results indicate a preference for various types of microorganisms, even within the same family, in sausages depending on the duration of their storage in vacuum packaging. This is consistent with previous findings that confirm changes in the species composition of microbial populations in sausages during refrigerated storage. However, the species composition of spoilage microorganisms does not change significantly during extended storage periods, while the ongoing metabolic activity of spoilage bacteria leads to a noticeable deterioration in organoleptic characteristics, including the appearance of juice, unpleasant odor, and sliminess.

The impact of starter cultures *Lactobacillus curvatus* and *Lactococcus lactis* subsp. *lactis* on the microbiological indicators of sausages during storage in vacuum packaging is noteworthy. Extending the storage period of sausages is possible through the use of starter cultures of lactic acid microorganisms, which antagonize the microbiota responsible for their spoilage. This aligns with the results of our previous research, which demonstrated an extension of the storage period of pork halves in refrigerated conditions for up to 7 days through the use of starter cultures of lactic acid microorganisms. Treating sausages with the SafePro BLC-48 starter culture (*Lactobacillus curvatus*) or a mixture of SafePro BLC-48 (*Lactobacillus curvatus*) + Bactoferm Rubis (*Lactococcus lactis* subsp. *lactis*) before vacuum packaging led to an increase in the MAFAM index practically throughout the entire storage period compared to the control. Their numbers reached or exceeded the regulated value of 3 lg CFU/g stipulated by the current standard. However, in this case, it can be considered that the desired lactic acid microorganisms formed the basis of the MAFA in sausages: in the variant using SafePro BLC-48, *Lactobacillus curvatus*, and in the variant using the mixture SafePro BLC-48 + Bactoferm Rubis, *Lactobacillus curvatus* and *Lactococcus lactis* subsp. *lactis*.

The storage of sausages in the control group in vacuum packaging under refrigerated conditions until the 18th day showed that the level of MAFAM approached the maximum allowable value, indicating their unsuitability for further storage. Thus, it can be considered that the storage of sausages in vacuum packaging under refrigerated conditions is possible only until the 18th day when the MAFAM level does not exceed 3 lg and the organoleptic characteristics meet the requirements for high-quality products.

On the 25th and 30th days of storage, the intensity of MAFAM increase in sausages treated with the starter culture mixture SafePro BLC-48 + Bactoferm Rubis was higher than that in sausages treated with the culture SafePro BLC-48 alone. Despite this increase in MAFAM levels, no changes in organoleptic characteristics indicating spoilage were observed in sausages from both treatment groups until the 36th day. On the 36th day of storage, both treatment groups exhibited signs of spoilage, including juice turbidity and vacuum packaging delamination.

The extension of the storage period of sausages in both treatment groups with starter lactic acid cultures can be explained by the ability of *Lactobacillus curvatus* to inhibit the growth of spoilage bacteria, including Enterobacteriaceae, *Pseudomonas fragi*, and *Brochothrix thermosphacta*, which are often found in meat products during storage in vacuum packaging. It can also inhibit the growth of *Pseudomonas putida* in the later stages of storage. *L. curvatus* significantly reduces microbial diversity in meat products through the synthesis of bacteriocin, which can control fermentation processes by inhibiting the growth of specific bacteria and competing microbial communities, thereby enhancing the safety of cooked meat products, including sausages. The inhibitory properties of *L. curvatus* are closely related to the formation of organic acids lactic and acetic acid and hydrogen peroxide, as well as competition with other microorganisms for limited nutrients (Zhang et al., 2018; Laranjo et al., 2019).

The number of lactic acid bacteria in the sausages in the control variant and both variants treated with starter lactic acid cultures increased throughout the entire storage period. In the sausages of both treatment variants, the number of lactic acid bacteria exceeded the corresponding indicators in the control only starting from the 12th day of storage, which may be associated with the low permeability of the polyamide casing

Evro-Bar for microorganisms. It should be noted that in the control variant and both treatment variants, the number of lactic acid bacteria constituted the basis of the entire total viable count. Therefore, lactic acid bacteria of field strains, which were the main microbiota in sausages in the control variant, could also be the cause of their spoilage along with other bacteria, including Enterobacteriaceae.

Research by Hultman et al. (2020) has shown that psychrotrophic lactic acid bacteria form the basis of spoilage microorganisms in meat during storage in a modified atmosphere. In this study, *Leuconostoc* species were the most common active lactic acid bacteria throughout the storage period, while the activity of Streptococcaceae (mainly *Lactococcus*) increased after product spoilage. This is also confirmed by the results of studies on Cypriot sausages, which revealed gram-positive bacteria, such as genera *Latilactobacillus* (formerly known as *Lactobacillus*), *Bacillus*, and *Enterococcus* (Kamilari et al., 2021).

The number of lactic acid bacteria in sausages treated with a mixture of starter cultures SafePro BLC-48 + Bactoferm Rubis predominated over similar indicators in sausages treated with only the starter culture SafePro BLC-48, especially on the 25th and 30th day of storage. This may be due to a higher number of lactic acid bacteria that entered the sausages at the beginning of storage. These results are consistent with the number of total viable counts on the surface of the polyamide casing Evro-Bar after treatment with starter lactic acid bacteria cultures. Thus, by treating the surface of sausages with a spray of starter lactic acid bacteria cultures, an additional barrier was created for the microbiota causing spoilage. These results are also supported by studies on the number of conditionally pathogenic and pathogenic microorganisms, such as *S. aureus*, *L. monocytogenes*, *Salmonella* spp., *E. coli*, coliform bacteria, as well as yeasts and molds in sausages throughout the entire storage period.

The idea of creating a bio-protective film for food products was evaluated for boiled sausages (Hashemi et al., 2023) and meat (Yang et al., 2019). The results showed that the food film with active plantaricin improved oxygen barrier properties, reduced the number of viable *Listeria monocytogenes* by 3.6 log10 CFU/mL in liquid medium and approximately by 1.4 log10 CFU/g in meat stored at 4 °C for 8 days compared to the control. Furthermore, the count of viable aerobes and anaerobes in meat packaged in the film with active plantaricin was reduced by approximately 0.6 log10 CFU/g and 1.1 log10 CFU/g compared to meat packaged in polypropylene film during storage at 4 °C for 12 days. This study demonstrated that the active film could extend the shelf life of meat by inhibiting *L. monocytogenes* and spoilage bacteria in refrigerated meat stored at a household refrigerator temperature of 4 °C.

It is worth noting that the use of a mixture of SafePro BLC-48 (*Lactobacillus curvatus*) + Bactoferm Rubis (*Lactococcus lactis* subsp. *lactis*) in our experiment did not improve but also did not worsen the results regarding the extension of the shelf life of "Juicy" sausages compared to using only the starter culture SafePro BLC-48 (*Lactobacillus curvatus*). This does not contradict the requirements for using mixed starter cultures in the production of meat products (Hwang et al., 2023), however, in this case, it was economically unjustified.

As evident from the data provided, the main microorganisms causing spoilage of sausages, especially towards the end of the storage period, were facultative anaerobes, indicating an oxygen deficit in the vacuum packaging and the creation of unfavorable conditions for the growth and reproduction of aerobic bacteria, including a significant number of spoilage bacteria, as well as the strain of the Bactoferm Rubis (*Lactococcus lactis* subsp. *lactis*) starter culture. In such a case, the use of this bacterium as an oxygen scavenger in the environment proved to be ineffective. Similar results were obtained in a study of hop extract as an oxygen scavenger in a modified atmosphere for sausage storage. The authors noted that despite oxygen absorption, it was not possible to extend the shelf life of sausages beyond 21st days under refrigerator conditions (Carballo et al., 2020).

Conclusions

On initial storage of "Juicy" sausages in vacuum packaging, the dominant microorganisms were representatives of the families Enterobacteriaceae – *Klebsiella variicola* and Bacillaceae – *Bacillus amyloliquefaciens plantarum*. By the end of the storage period of sausages in vacuum packaging under refrigerator conditions, along with the increase in the count of the most probable number of aerobic mesophilic bacteria (MAFAM) by the 21st day, signs of spoilage appeared in the form of juice appearance and cloudiness, and delamination of the vacuum packaging. The cause of spoilage of sausages on the 21st day of storage was the representatives of the families Enterobacteriaceae: *Moellerella wisconsensis*, *Proteus mirabilis*, and Bacillaceae: *Bacillus cereus*.

Treating sausages before vacuum packaging with the starter culture SafePro BLC-48 (*Lactobacillus curvatus*) or a mixture of SafePro BLC-48 (*Lactobacillus curvatus*) + Bactoferm Rubis (*Lactococcus lactis* subsp. *lactis*) led to an increase in the count of MPNAM and lactic acid microorganisms practically throughout the entire storage period compared to untreated sausages. Storing sausages in vacuum packaging in a chilled state without treatment with starter lactic acid cultures is reasonable only up to the 18th day. Signs of spoilage in the form of juice appearance cloudiness, and delamination of the vacuum packaging occur by the 36th day of storage for sausages treated before vacuum packaging with the starter culture SafePro BLC-48 or a mixture of starter cultures SafePro BLC-48 + Bactoferm Rubis.

Treating sausages before vacuum packaging with the starter culture SafePro BLC-48 can be considered effective in suppressing the growth of microorganisms causing their spoilage and extending the shelf life in chilled conditions by 12 days. The use of a mixture of starter cultures SafePro BLC-48 + Bactoferm Rubis for treating sausages before vacuum packaging may be promising in the case of detecting aerobic microbiota causing their spoilage and requires further research.

The authors declare that they have no potential conflict of interest concerning the authorship or publication of this article.

References

Aaliya, B., Valiyapeediyekkal Sunooj, K., Navaf, M., Parambil Akhila, P., Sudheesh, C., Ahmad Mir, S., Sabu, S., Sasidharan, A., Theingi Hlaing, M., & George, J. (2021). Recent trends in bacterial decontamination of food products by hurdle technology: A synergistic approach using thermal and non-thermal processing techniques. Food Research International, 147, 110514.

Adamski, P., Byczkowska-Rostkowska, Z., Gajewska, J., Zakrzewski, A. J., & Kłębukowska, L. (2023). Prevalence and antibiotic resistance of *Bacillus* sp. isolated from raw milk. Microorganisms, 11(4), 1065.

Ajourloo, M., Khanjari, A., Misaghi, A., Akhondzadeh Basti, A., Kamkar, A., Yadegar, F., Gholami, F., Khansavar, F., & Fallah, F. (2021). Combined effects of *Ziziphora clinopodioides* essential oil and lysozyme to extend shelf life and control *Listeria monocytogenes* in Balkan-style fresh sausage. Food Science and Nutrition, 9(3), 1665–1675.

Aminzare, M., Hashemi, M., Afshari, A., Mokhtari, M. H., & Noori, S. M. A. (2022). Impact of microencapsulated *Ziziphora tenuior* essential oil and orange fiber as natural-functional additives on chemical and microbial qualities of cooked beef sausage. Food Science and Nutrition, 10(10), 3424–3435.

Barcenilla, C., Álvarez-Ordóñez, A., López, M., Alvseike, O., & Prieto, M. (2022). Microbiological safety and shelf-life of low-salt meat products – A review. Foods, 11(15), 2331.

Bayer, E. V., Novozhitskaya, Y. N., Shevchenko, L. V., & Mykhalska, V. M. (2017). Monitoring of residues of veterinary preparations in food products. Ukrainian Journal of Ecology, 7(3), 251–257.

Bin Hafeez, A., Pełka, K., Worobo, R., & Szweda, P. (2024). *In silico* safety assessment of *Bacillus* isolated from Polish bee pollen and bee bread as novel probiotic candidates. International Journal of Molecular Sciences, 25(1), 666.

Carballo, D. E., Andrés, S., Giráldez, F. J., Khanjari, A., Caro, I., Llamazares, D., Operta, S., & Mateo, J. (2020). The effects of storage and hop extract on aroma and flavour compounds in Balkan-style sausages packed under a CO_2-containing anaerobic atmosphere. Heliyon, 6(10), e05251.

Chaillou, S., Ramaroson, M., Coeuret, G., Rossero, A., Anthoine, V., Champomier-Vergès, M., Moriceau, N., Rezé, S., Martin, J. L., Guillou, S., & Zagorec, M. (2022). Combination of high-pressure treatment at 500 MPa and biopreservation with a *Lactococcus lactis* strain for lowering the bacterial growth during storage of diced cooked ham with reduced nitrite salt. Microorganisms, 10(2), 456.

Chen, Y., Yu, L., Qiao, N., Xiao, Y., Tian, F., Zhao, J., Zhang, H., Chen, W., & Zhai, Q. (2020). *Latilactobacillus curvatus*: A candidate probiotic with excellent fermentation properties and health benefits. Foods, 9(10), 1366.

Cheng, C., Jiang, L., Li, X., Song, H., & Fang, W. (2024). Can natural preservatives serve as a new line of protective technology against bacterial pathogens in meat and meat products? Food Quality and Safety, 8, fyad049.

Cocolin, L., Rantsiou, K., Iacumin, L., Urso, R., Cantoni, C., & Comi, G. (2004). Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. Applied and Environmental Microbiology, 70(4), 1883–1894.

Czerwiński, K., Rydzkowski, T., Wróblewska-Krepsztul, J., & Thakur, V. K. (2021). Towards impact of modified atmosphere packaging (MAP) on shelf-life of polymer-film-packed food products: Challenges and sustainable developments. Coatings, 11, 1504.

Darbandi, A., Asadi, A., Mahdizade Ari, M., Ohadi, E., Talebi, M., Halaj Zadeh, M., Darb Emamie, A., Ghanavati, R., & Kakanj, M. (2022). Bacteriocins: Properties and potential use as antimicrobials. Journal of Clinical Laboratory Analysis, 36(1), e24093.

Giannattasio-Ferraz, S., Ene, A., Johnson, G., Maskeri, L., Oliveira, A. P., Banerjee, S., Barbosa-Stancioli, E. F., & Putonti, C. (2022). Multidrug-resistant *Klebsiella variicola* isolated in the urine of healthy bovine heifers, a potential risk as an emerging human pathogen. Applied and Environmental Microbiology, 88(9), e0004422.

Gupta, P. (2023). Role of oxygen absorbers in food as packaging material, their characterization and applications. Journal of Food Science and Technology, 61(2), 242–252.

Hashemi, M., Aminzare, M., Hassanzadazar, H., Roohinejad, S., Tahergorabi, R., & Bekhit, A. E. A. (2023). Impact of sodium alginate-based film loaded with resveratrol and thymol on the shelf life of cooked sausage and the inoculated *Listeria monocytogenes*. Food Science and Nutrition, 11(12), 7855–7869.

Hultman, J., Johansson, P., & Björkroth, J. (2020). Longitudinal metatranscriptomic analysis of a meat spoilage microbiome detects abundant continued fermentation and environmental stress responses during shelf life and beyond. Applied and Environmental Microbiology, 86(24), e01575-20.

Hwang, J., Kim, Y., Seo, Y., Sung, M., Oh, J., & Yoon, Y. (2023). Effect of starter cultures on quality of fermented sausages. Food Science of Animal Resources, 43(1), 1–9.

Jin, H., Park, S.-K., Yun, Y.-G., Song, N.-E., & Baik, S.-H. (2023). Isolation of *Latilactobacillus curvatus* with enhanced nitric oxide synthesis from Korean traditional fermented food and investigation of its probiotic properties. Microorganisms, 11(9), 2285.

Kamilari, E., Efthymiou, M., Anagnostopoulos, D. A., & Tsaltas, D. (2021). Cyprus sausages' bacterial community identification through metataxonomic sequencing: Evaluation of the impact of different DNA extraction protocols on the sausages' microbial diversity representation. Frontiers in Microbiology, 12, 662957.

Kawecki, K., Stangierski, J., & Cegielska-Radziejewska, R. (2021). The influence of packing methods and storage time of poultry sausages with liquid and microencapsulated fish oil additives on their physicochemical, microbial and sensory properties. Sensors, 21(8), 2653.

Kim, H. S., Lee, S. Y., Kang, H. J., Joo, S. T., & Hur, S. J. (2019). Effects of six different starter cultures on mutagenicity and biogenic amine concentrations in fermented sausages treated with vitamins C and E. Food Science of Animal Resources, 39(6), 877–887.

Klaper, K., Hammerl, J. A., Rau, J., Pfeifer, Y., & Werner, G. (2021). Genome-based analysis of *Klebsiella* spp. isolates from animals and food products in Germany, 2013–2017. Pathogens, 10(5), 573.

Laranjo, M., Potes, M. E., & Elias, M. (2019). Role of starter cultures on the safety of fermented meat products. Frontiers in Microbiology, 10, 853.

Łaszkiewicz, B., Szymański, P., Zielińska, D., & Kołożyn-Krajewska, D. (2021). Application of *Lactiplantibacillus plantarum* SCH1 for the bioconservation of cooked sausage made from mechanically separated poultry meat. Applied Sciences, 11, 1576.

Matthews, K. R., Kniel, K. E., & Montville, T. J. (2017). Food microbiology: An introduction. 4th ed. ASM Press, Washington.

Melnychuk, I. (2024). Gut microbiota metabolites connections with echocardiography atrial fibrillation characteristics in patients with coronary artery disease. Eastern Ukrainian Medical Journal, 12(1), 137–147.

Molognoni, L., Daguer, H., Motta, G. E., Merlo, T. C., & Lindner, J. D. (2019). Interactions of preservatives in meat processing: Formation of carcinogenic compounds, analytical methods, and inhibitory agents. Food Research International, 125, 108608.

Niazi, A., Manzoor, S., Asari, S., Bejai, S., Meijer, J., & Bongcam-Rudloff, E. (2014). Genome analysis of *Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5113: A *Rhizobacterium* that improves plant growth and stress management. PloS One, 9(8), e104651.

Popov, S. V., Smiian, O. I., & Profatylo, A. O. (2021). The present conception of neonatal microbiome formation. Eastern Ukrainian Medical Journal, 9(1), 18–28.

Rahman, M., Islam, R., Hasan, S., Zzaman, W., Rana, M. R., Ahmed, S., Roy, M., Sayem, A., Matin, A., Raposo, A., Zandonadi, R. P., Botelho, R. B. A., & Sunny, A. R. (2022). A comprehensive review on bio-preservation of bread: An approach to adopt wholesome strategies. Foods, 11(3), 319.

Ramaroson, M., Guillou, S., Rossero, A., Rezé, S., Anthoine, V., Moriceau, N., Martin, J. L., Duranton, F., & Zagorec, M. (2018). Selection procedure of bioprotective cultures for their combined use with high pressure processing to control spore-forming bacteria in cooked ham. International Journal of Food Microbiology, 276, 28–38.

Rocha, J. M., Kovacevik, B., Veličkovska, S. K., Tamame, M., & Teixeira, J. A. (2024). Diversity of microorganisms and their metabolites in food. Microorganisms, 12(1), 205.

Rodríguez-Medina, N., Barrios-Camacho, H., Duran-Bedolla, J., & Garza-Ramos, U. (2019). *Klebsiella variicola*: An emerging pathogen in humans. Emerging Microbes and Infections, 8(1), 973–988.

Safronova, L. S., Skorochod, I. A., & Ilyash, V. M. (2021). Antioxidant and antiradical properties of probiotic strains *Bacillus amyloliquefaciens* ssp. *plantarum*. Probiotics and Antimicrobial Proteins, 13(6), 1585–1597.

Shevchenko, L. V., Dobrozhan, Y. V., Mykhalska, V. M., Osipova, T. Y., & Solomon, V. V. (2019). Contamination of hen manure with nine antibiotics in poultry farms in Ukraine. Regulatory Mechanisms in Biosystems, 10(4), 532–537.

Simons, A., Alhanout, K., & Duval, R. E. (2020). Bacteriocins, antimicrobial peptides from bacterial origin: Overview of their biology and their impact against multidrug-resistant bacteria. Microorganisms, 8(5), 639.

Smaoui, S., Echegaray, N., Kumar, M., Chaari, M., D'Amore, T., Shariati, M. A., Rebezov, M., & Lorenzo, J. M. (2023). Beyond conventional meat preservation: Saddling the control of bacteriocin and lactic acid bacteria for clean label and functional meat products. Applied Biochemistry and Biotechnology, in press.

Spampinato, G., Candeliere, F., Amaretti, A., Licciardello, F., Rossi, M., & Raimondi, S. (2022). Microbiota survey of sliced cooked ham during the secondary shelf life. Frontiers in Microbiology, 13, 842390.

Vovkotrub, V., Iakubchak, O., Horalskyi, L., Vovkotrub, N., Shevchenko, L., Shynkaruk, N., Rozbytska, T., Slyva, Y., Tupitska, O., & Shtonda, O. (2023). The microscopic structure of pork neck after cooling with showering stiving and processing by culture *Lactobacillus sakei*. Potravinarstvo, 17, 759–776.

Wang, Y., Wu, J., Lv, M., Shao, Z., Hungwe, M., Wang, J., Bai, X., Xie, J., Wang, Y., & Geng, W. (2021). Metabolism characteristics of lactic acid bacteria and the expanding applications in food industry. Frontiers in Bioengineering and Biotechnology, 9, 612285.

Webb, M. D., Barker, G. C., Goodburn, K. E., & Peck, M. W. (2019). Risk presented to minimally processed chilled foods by psychrotrophic *Bacillus cereus*. Trends in Food Science and Technology, 93, 94–105.

Yang, W., Xie, Y., Jin, J., Liu, H., & Zhang, H. (2019). Development and application of an active plastic multilayer film by coating a plantaricin BM-1 for chilled meat preservation. Journal of Food Science, 84(7), 1864–1870.

Yu, H. H., Chin, Y. W., & Paik, H. D. (2021). Application of natural preservatives for meat and meat products against food-borne pathogens and spoilage bacteria: A review. Foods, 10(10), 2418.

Zhang, Y., Zhu, L., Dong, P., Liang, R., Mao, Y., Qiu, S., & Luo, X. (2018). Bio-protective potential of lactic acid bacteria: Effect of *Lactobacillus sakei* and *Lactobacillus curvatus* on changes of the microbial community in vacuum-packaged chilled beef. Asian-Australasian Journal of Animal Sciences, 31(4), 585–594.