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MICROBIOLOGICAL COMMUNITY DURING SHELF LIFE AND SPOILAGE OF BEEF MEET AND PLANT-BASED BURGER IN BULGARIA

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Abstract

The aim of the present study was to compare microbial community during spoilage processes in beef meat and plant-based burger by monitoring the total bacterial count over several days at three different storage temperatures. All key microorganisms isolated during spoilage were identified by MALDI-TOF MS. After six days of storage at 25°C, the bacterial counts for the plant-based and meat burgers were increased from basic 4.6 to 8.9 log10 CFU/g and from 4.9 to 9.0 log10 CFU/g, respectively. On the tenth day of storage at 12°C, the bacterial counts were enumerated as 7.9 log10 CFU/g for the vegetable burger and 9.0 log10 CFU/g for the meat burger. At the lowest temperature of 6°C on the 10-th day, the total count of microorganisms reached 9.9 log10 CFU/g for vegetable burger and 9.8 log10 CFU/g for meat burger. The identification of 304 isolates showed that the plant-based burger was dominated by lactic acid bacteria of the genera Lactococcus, Leuconostoc, and Lactobacillus, while the beef meat burger contained most often bacteria belonging to Streptococcus, Staphylococcus, Pseudomonas, Carnobacterium, and Lactococcus.

Key words*: food safety, MALDI-TOF MS, plant-based meat alternatives, spoilage bacteria.*

INTRODUCTION

Meat is an integral part of the human diet today, with its consumption influenced by various factors, the most important being its biological and nutritional value. Various anthropological evidence is presented, followed by an analysis of the health implications of meat consumption in the modern world. The discussion addresses issues related to saturated fats and omega-3 fatty acid intake, as well as the significance of key nutrients provided by animal-derived foods, with a particular focus on their role in brain development and function (Mann, NJ, 2018).

In recent years, however, there has been a surge in market demand for alternatives to meat products, particularly the so-called plant-based meat alternatives (PBMA). These products offer an excellent way to incorporate more plant proteins into the diet, including seeds, beans, nuts, whole grains, and vegetables. However, it remains unclear whether these substitutes are merely a short-term trend or will establish themselves as a long-term, sustainable dietary shift. Plant-based meat alternatives are products designed to mimic the taste, texture, and nutritional profile of meat using plant-based ingredients (Bakhsh et al., 2021; Boukid et al., 2021; Pingali et al., 2023).

While much is known about the growth of microorganisms responsible for the spoilage of meat and meat products, information on their development in plant-based meat alternatives remains limited (Liu et al., 2023). The composition of meat analogs includes textured plant protein, plant-based lipids, polysaccharides, flavor enhancers, and colorants (Boukid et al., 2021; Moll et al., 2023). The technological process involves procedures such as texturization and extrusion to create a meat-like texture. Additionally, meat analogs provide a relatively different nutritional environment, pH, and internal structure for microorganisms, which may influence their growth and survival ability (Luchansky et al., 2020; Hadi and Brightwell, 2021). Neutral pH and relatively high water activity, combined with a high protein content, provide an optimal environment for bacterial growth (He et al., 2020; Chen et al., 2022; Wang et al., 2022). Although temperatures exceeding 130°C are reached during the processing of textured plant protein,

the possibility of bacterial introduction through other ingredients or secondary contamination cannot be eliminated (He et al., 2020; Liu et al., 2023).

The aim of this study is to analyze and compare the microbial communities present in plant-based and raw meat burgers, with a focus on species composition and their potential impact on product safety, quality, and shelf life. By identifying specific microbial profiles for each burger type, the study seeks to provide insights into the differences in the microbial ecosystem between alternative food products and traditional meat products.

MATERIALS AND METHODS

The study includes two types of food products: a plant-based meat alternative (PBMA) and a beef burger (BB). The main ingredients of the vegan burger are structured soy protein, starch, wheat gluten, and wheat fiber, while the beef burger consists of beef, plant fibers, and potato starch.

Both products are commercially available in Bulgaria as frozen semi-finished products intended for human consumption. The vegan burger is sold in packs of two, while the meat burger comes in packs of five, with both products requiring thermal processing before consumption.

For the purposes of the study, selected samples from 4 different batches were transported to the microbiology laboratory at Trakia University in thermo-insulated bags. Upon delivery, they were left to thaw at a controlled refrigeration temperature of 4°C for 18 hours.

The experimental design included three storage temperatures: 6°C – conditions of refrigerated storage, typical for household and commercial refrigerators; 12°C – moderately elevated temperature, imitating insufficient cooling or temporary storage during transport; 25°C – temperatures corresponding to room temperature, favorable for rapid microbial growth. For each temperature condition, individual packages of plant-based and meat products were used, with samples tested at different time intervals every 2 days (0, 2, 4, 6, and 10 days). Each individual package contained two burgers, vacuum-sealed.

The laboratory microbiological analyses included the following parameters: total bacterial count (CFU/g), expressed as a decimal logarithm (log CFU/g) – an indicator of the microbial load in the product; pH value, measured using Portable pH meters pH 7 Vio Set 1, Italy – an indicator of biochemical changes in the medium caused by microbial metabolic activity; laboratory identification of colonial growth – performed using MALDI Biotyper RUO (Research Use Only) (Server Version: 4.1.100 (PYTH)), (Bruker, Germany).

Microbiological analysis

The determination of the total bacterial count was performed according to the ISO 4833-1:2013 method. Briefly, 10 g of each sample were minced using sterile instruments and placed in a sterile Stomacher bag. Ninety milliliters of MRD (HiMedia, India) were added, and the sample was homogenized using an automatic peristaltic homogenizer.

One milliliter of the resulting homogenate was transferred into 9 mL of MRD, followed by serial dilutions. From each dilution, two Petri dishes were inoculated with Plate Count Agar (HiMedia, India) and incubated at 30°C for 48 hours. The grown colonies were counted, and the result was calculated in CFU/g.

Additionally, morphologically distinct bacterial colonies from PCA plates were selected and subcultured on CASO agar to obtain pure cultures. These isolates were then frozen in BHI broth with 15% glycerin for subsequent identification using MALDI-TOF MS.

MALDI-TOF MS identification

The identification of the presumptive isolates was performed using MALDI Biotyper RUO (Research Use Only) (Server Version: 4.1.100 (PYTH)) (Bruker, Germany). The methodology included defrosting each isolate and culturing it on Plate Count Agar (HiMedia, India), followed by incubation at 37°C for 24 hours.

Using direct bacterial transfer, in accordance with the manufacturer's recommendations (Bruker, Germany), a small portion of a 24-hour fresh single bacterial colony was applied with a toothpick onto a 96-position polished steel target plate (MSP 96; Bruker, Germany). Each sample was carefully spread within the well and left to dry at room temperature for 5 minutes. Then, 1

μL of HCCA Matrix (saturated solution of α-cyano-4-hydroxycinnamic acid) was added to each sample and left to dry at room temperature for 5-10 minutes.

Based on the database embedded in the MALDI Biotyper RUO (Research Use Only) (Server Version: 4.1.100 (PYTH)) (Bruker, Germany) software, the spectral peaks of the analyzed samples were compared to the reference peaks (MBT 4.1 Bruker) (Figure 2). A statistical algorithm generated identification scores ranging from 0.000 to 3.000. Identification scores ≥2.000 were considered valid at the species level, while scores ranging from 1.70 to 1.99 were accepted at the genus level.

Statistical analysis

All data were recorded in electronic spreadsheets (Microsoft Office Excel 2016). The obtained experimental data (CFU/g) were presented as a decimal logarithm ($\log_{10}$), with mean values and standard deviations calculated. Statistical significance was determined at $P < 0.05$.

RESULTS AND DISCUSSIONS

The present study analyzes the microbiological profile and pH dynamics in plant-based and meat burgers at different temperatures (6°C, 12°C, and 25°C), with a focus on the bacterial species identified using MALDI-TOF MS. The changes in bacterial counts (expressed as log CFU/g) and the corresponding variations in pH over different time intervals are graphically represented.

At 6°C, the pH initially increased in PBMA. The recorded values started at 6.4 on day 0, reached 6.86 on day 6, but later decreased to 6.7. In the meat burger, a sharper pH decline was observed, with values of 6.5, 6.7, and 5.9 on days 0, 6, and 10, respectively.

At 12°C, pH fluctuations were minimal. On day 2, the measured pH for PBMA was 5.2, showing almost no change by day 6 (5.7), followed by a slight decrease to 5.5 on day 10. In the meat burger (BB), the pH values remained relatively stable at 5.6, 5.8, and 5.8 for the same time points. At 25°C, pH changes in both products were minor, mainly affecting decimal places. In PBMA, the pH on day 2 reached 5.4, while on days 4 and 6, it stabilized at 5.1. For the meat burger, the pH was 5.9 on day 2, followed by values of 5.7 and 5.6 on days 4 and 6, respectively (Figure 1).

The relatively stable pH values in both samples align with widely known data and the product ingredient labels, where the addition of antioxidants and acidity regulators helps stabilize pH in the mildly acidic range (pH 5.5-6.5), preserving freshness and preventing food oxidation. At 6°C, the initial TBC values were 4.6 log CFU/g for PBMA and 4.9 log CFU/g for BB. A slow but steady growth was observed over the 10-day period. A study by Dušková et al. (2024) reported TBC values ranging from 1.0 to 7.2 log CFU/g in various meat analog samples. In burger samples (n=16), the TBC values ranged from 1.5 to 5.1 log CFU/g, which closely aligns with our results.

The addition of protective cultures (lactic acid bacteria) would likely result in higher TBC values, as seen in the study by Kabisch et al. (2024), where TBC levels between 1 and 8.31 log CFU/g were recorded in raw plant-based ground meat products. The researchers found that lactic acid bacteria constituted the majority of mesophilic bacteria in the samples, with counts ranging from 0.70 to 7.98 log CFU/g.

In our samples, no protective cultures were present, but since the burgers were purchased frozen and stored at –18°C, the lower bacterial load can be explained. Although the initial bacterial concentration was lower in PBMA, the difference between the two products decreased over time. By day 10 at 6°C, the bacterial load in the meat burger remained higher compared to the plant-based analog, with values of 9.07 log CFU/g and 7.9 log CFU/g, respectively.

At 12°C, bacterial growth accelerated compared to 6°C. By day 6, the total bacterial load in PBMA reached 8.55 log CFU/g, while in the meat burger, it was 8.7 log CFU/g, with almost no difference between the two samples. This result supports the claim by Wild et al. (2014) that, due to their nearly neutral pH, as well as high protein and moisture content, meat analogs are highly susceptible to spoilage, similar to traditional ground beef or pork.

By day 10, bacterial counts increased significantly, reaching 9.9 log CFU/g for PBMA and 9.8 log CFU/g for the meat burger. More intense bacterial proliferation was observed in the meat

sample, particularly between days 0 and 2. At 25°C, bacterial growth was the most dynamic, reaching its maximum levels as early as day 6 (8.9 log CFU/g for PBMA and 9.04 log CFU/g for BB). The differences between the meat and plant-based products in this case were minimal, suggesting that high temperatures favor rapid microbial proliferation regardless of the ingredients used in burger production (Figure 1).

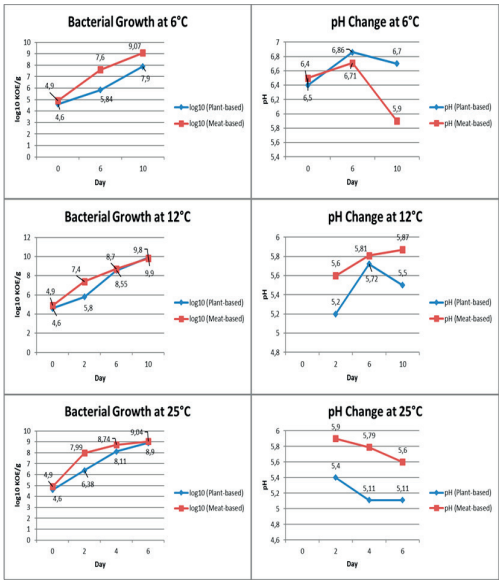

Figure 1. Bacterial growth and pH dynamics in PBMA and BB at 6°C, 12°C, and 25°C

Despite the common perception that plant-based meat alternatives are safer and more resistant to microbial contamination due to undergoing various processing steps, it is important to emphasize that they are not sterile. Microorganisms can be introduced into meat analogs both through the addition of raw ingredients and as a result of contamination after processing (Sampson et al., 2023).

Lupo (2019) notes that PBMA formulations often include various additives such as vitamins, minerals, flavor enhancers, and colorants to achieve the desired taste and visual characteristics. Since these components do not undergo thermal processing, they can introduce microorganisms into the final product.

The growth potential values were calculated for both plant-based and meat burgers. In the plant-based burger, an increase in bacterial counts of 3.3 log CFU/g, 5.3 log CFU/g, and 4.3 log CFU/g was recorded at 6°C, 12°C, and 25°C, respectively.

For the meat burger, the recorded values at the same temperatures were 4.17 log CFU/g, 4.9 log CFU/g, and 4.14 log CFU/g, respectively.

The statistical analysis was performed using a t-test to determine whether there was a significant difference in bacterial growth between the plant-based burger (PBMA) and the meat burger (BB) at different temperatures. In all cases, the p-value was greater than 0.05 ($p > 0.05$), indicating that there was no statistically significant difference between the two products at the respective temperature.

The growing trend toward healthy and sustainable eating has led to increased interest in plant-based meat alternatives in many European countries and worldwide. A new group of consumers, known as "flexitarians", who reduce their meat consumption in daily diets, is rapidly expanding (Wild et al., 2014). To our knowledge, however, there is still insufficient data on the microbial community in meat alternatives available on the Bulgarian market.

In the microbiological analysis of the meat and plant-based burger samples, a total of 304 bacterial colonies with different morphological characteristics were isolated and identified using MALDI Biotyper RUO (Research Use Only) (Server Version: 4.1.100 (PYTH)) (Bruker, Germany). Of the 304 analyzed isolates, 223 (73.35%) were identified at the species level, 64 (21.05%) at the genus level, and for 17 isolates, no reference spectral peaks were found in the MALDI Biotyper RUO 4.1.100 database, classifying them as unidentified.

A total of 39 bacterial genera were detected in both samples, with species distribution presented in Figure 2. The distribution of bacterial isolates shows a clear dominance of a few genera, which may be explained by their ecological role or industrial significance. The most frequently occurring genera include *Leuconostoc* spp. (n=38), *Pseudomonas* spp. (n=30), *Lactococcus* spp. (n=26), and *Lactobacillus* spp. (n=22).

Additionally, several other genera were present in relatively high numbers, such as *Kocuria* spp., *Psychrobacter* spp., *Bacillus* spp., and Enterococcus spp. On the other hand, isolates with very low frequency (1-2 isolates) may not be typical for the studied environment or may be

difficult to detect, including *Actinomyces oris*, *Exiguobacterium maxicanum*, *Luteococcus japonicus*, and *Kurthia zopfii*. Barmettler et al. (2025) also reported dominant isolates identified using MALDI-TOF MS, including *Lactobacillus* spp., *Leuconostoc* spp., *Bacillus* spp., *Bronchothrix thermosphacta*, and *Kocuria rhizophila*. The predominant bacteria belong to the group of lactic acid bacteria, which have been described in previous studies (Duthoo et al., 2022; Geeraerts et al., 2020; Roch et al., 2024).

They may play a role in the stability of PBMA products (Roch et al., 2024), but they can also contribute to acidification, gas accumulation in packaging, or slime formation, even when stored at low temperatures (Barmettler et al., 2025) it reached 57,312 thousand hl, being by 13.19% less than in the year 1990.

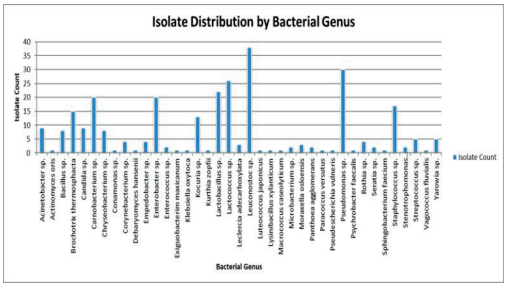

Figure 2. Frequency of bacterial isolates across different genera

Figure 3 presents the percentage distribution of isolates from different bacterial genera in two types of burgers – plant-based (PBMA) and meat-based (BB). *Carnobacterium* spp., *Brochothrix thermosphacta*, and *Acinetobacter* spp. appear to be typical for the meat burger, while others dominate in the plant-based burger, such as *Enterobacter* spp. and *Staphylococcus* spp.

Although bacteria from the genus *Enterobacter* are most commonly associated with urinary and respiratory tract infections in humans, as well as multidrug-resistant nosocomial infections, some studies have investigated the role of environmental strains isolated from meat in the development of antimicrobial resistance (Messaoudi et al., 2009). In a study by Messaoudi et al. (2009), the authors identified a total of 25 *Enterobacter* isolates from 15 meat samples, including chicken, turkey, beef, lamb, pork, as well as meat from dromedary camel, ostrich, and fish. Our results indicate that *Enterobacter* spp. isolates were more commonly found in the plant-based product. Another study detected *Enterobacter* spp. species in air samples of aerosolized compost. According to the authors, such findings suggest possible contamination of the compost with fecal material. (Nasir et al., 2018). The expected bacterial species *Brochothrix thermosphacta, Carnobacterium* spp., and *Acinetobacter* spp. in the meat burger correspond to the typical microbiota of meat products. The predominant bacteria associated with the spoilage of beef and pork include representatives of the genera *Brochothrix thermosphacta, Carnobacterium, Enterobacteriaceae, Lactobacillus, Leuconostoc*, *Pseudomonas*, and *Shewanella putrefaciens*. The main undesirable changes in meat caused by these microorganisms include the development of unpleasant odors and off-flavors, as well as discoloration and gas formation (Borch et al., 2006). In our study, *Acinetobacter* spp. were more frequently detected in the meat burger, which can be explained by their natural presence in the microbiota of meat products. These bacteria can enter meat during processing, storage, or through contact with the environment. Both burger types contained significant amounts of lactic acid bacteria (*Lactococcus* spp., *Lactobacillus* spp.), which can influence fermentation processes and product quality. *Pseudomonas* spp. were also detected in considerable quantities, particularly in the meat burger, which is expected since these bacteria are commonly associated with food spoilage.

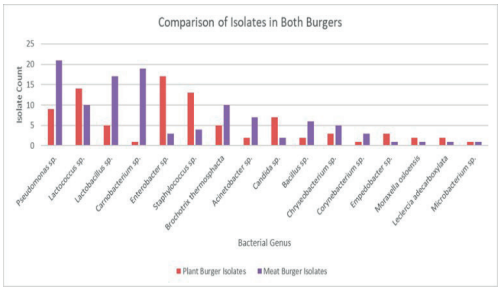

Figure 3. Frequency of bacterial isolates across different genera

Figure 4 presents the percentage distribution of the isolated bacterial genera in the plant-based

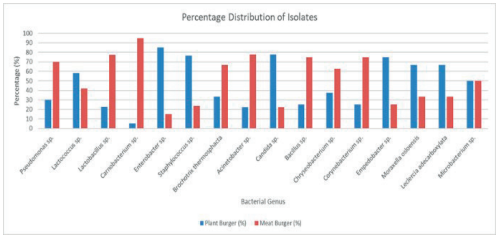

Figure 4. Percentage Distribution of Bacterial Isolates in Plant-Based and Beef Burger

Figures 5 and 6 present the unique bacterial species isolated from PBMA and BB. The study of these specific isolates is essential for understanding the microbiological profile of both products, as well as for assessing potential risks related to food safety and quality. In PBMA, there is a strong dominance of *Leuconostoc* spp., with 38 isolates, followed by *Kocuria* spp., with approximately 13 isolates. In contrast, BB exhibits a more even distribution of species, with Streptococcus spp. (n=5), *Yarowia* spp. (n=5), and *Rothia* spp. (n=4) being the most common, while the remaining species are represented by a smaller number of isolates (1-4).

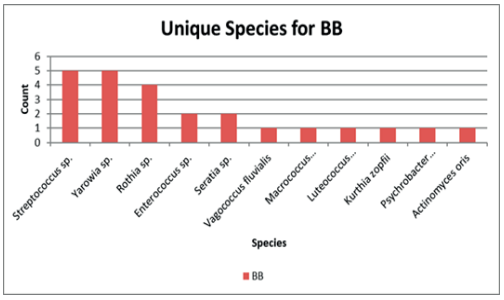

Figure 5 Unique Bacterial Species Identified in beef burger

A key focus of our study is the isolation of opportunistic pathogens, including *Lactococcus garvieae*, *Pseudescherichia vulneris*, *Bacillus pumilus*, and *Empedobacter falsenii*, which can cause infections in immunocompromised patients.

Since the early 1990s, *Lactococcus garvieae* has been associated with various human infections, most commonly endocarditis. Over the past five years, an increase in infections caused by this bacterium has been observed, likely due to advancements in microbiological identification methods and increase awareness among physicians. The primary sources of infection include the consumption or handling of contaminated raw fish and seafood. A recent genetic study also found that meat, raw milk, and dairy products can be potential sources of *Lactococcus garvieae* infections in humans (Gibello et al., 2016). In our samples, *L. garvieae* was isolated from the meat burger.

Although *Bacillus pumilus* is rarely reported as a cause of human infections, Shah et al. (2019) described a clinical case of food poisoning in a 51-year-old man after consuming a stew made with rice and minced meat in a restaurant in Kenya. We isolated this bacterium from the plant-based sample.

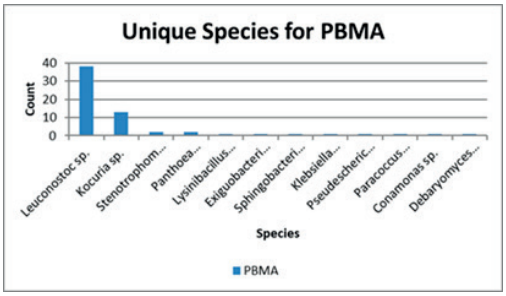

Figure 6. Unique Bacterial Species Identified in plant based meat analog burger and meat burgers

Empedobacter falsenii was first described in 2006. To date, there are only a limited number of reports of its isolation from respiratory, urinary, and abscess samples. In our study, *E. falsenii* was isolated from the meat burger. In addition to clinical specimens, this bacterium has also been found in industrial metalworking fluids and aerosols, carpet surfaces, and polluted soils (Martinez et al., 2023).

As an opportunistic pathogen, *Pseudescherichia vulneris* has a broad host range, including humans, animals, and the environment. Infections caused by this microorganism can affect both immunocompromised and immunocompetent individuals, regardless of age. Clinical manifestations range from localized infections, such as wound infections and localized peritonitis, to systemic diseases, including sepsis, meningitis, and bacteremia (Mustapha et al., 2024). In our study, this bacterium was isolated from PBMA.

In addition to the previously described isolates, our samples also contained and identified well-known pathogens such as *Bacillus cereus*, *Stenotrophomonas maltophilia*, and *Klebsiella oxytoca*, which are recognized for their potential to cause serious infections. Both species possess a variety of antibiotic resistance mechanisms, making them challenging to treat. *Stenotrophomonas maltophilia* has intrinsic resistance to carbapenems and aminoglycosides, while *Klebsiella oxytoca* can develop extended-spectrum β-lactamase (ESBL) and carbapenemase resistance in hospital settings (Brooke, J. S., 2012; ECDC, 2023).

Bacillus cereus is known not only as a causative agent of gastrointestinal diseases but also as a highly virulent ocular pathogen associated with conjunctivitis, panophthalmitis, keratitis, iridocyclitis, and orbital abscesses. Additionally, it can cause various opportunistic infections, including respiratory and wound infections (Griffiths and Schraft, 2017). Our isolate originated from BB; however, due to its widespread environmental presence, B. cereus has also been isolated from milk and dairy products, meat and meat products, grains, legumes, fresh fruits and vegetables, as well as ready-to-eat foods.

The obtained results confirm the necessity of strict microbiological control in the food industry, not only during production but also throughout storage. The presence of these bacteria in food samples highlights potential public health risks and underscores the importance of good hygiene practices in minimizing microbiological contamination.

CONCLUSIONS

The present study provides an in-depth analysis of the microbiological profile and bacterial growth dynamics in plant-based and meat burgers at different temperatures. The obtained results indicate that despite their different compositions, both types of products exhibit similar levels of bacterial contamination, particularly at higher storage temperatures.

Both opportunistic pathogens (*Lactococcus garvieae, Pseudescherichia vulneris, Bacillus pumilus, Empedobacter falsenii*) and clearly pathogenic microorganisms (*Bacillus cereus, Stenotrophomonas maltophilia, Klebsiella oxytoca*) were isolated, which may pose a potential public health risk. The presence of lactic acid bacteria (*Lactococcus* spp., *Leuconostoc* spp., *Lactobacillus* spp.) suggests a possible impact on product quality and stability, including changes in pH, gas accumulation, and slime formation.

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