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

INTRODUCTION

Meat is an integral part of the human diet, with its consumption influenced by various factors, the most important being its biological and nutritional value as well eating quality and consumer's attitudes. Components as saturated fats and omega-3 fatty acid intake, as well as essential amino acids and key nutrients in food of animal origin, with a focus on their role in brain development and function are often within meat research topics (Mann, 2018).

In the 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 sustainable consumer and market demand. 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).

Bacterial contamination and growth of microorganisms responsible for the spoilage of meat and meat products are well studied during the years, whereas such facts for microbiota 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 analogues provide a relatively different nutritional environment, pH and internal structure, which may influence growth and survival ability of most microorganisms (Luchansky et al., 2020; Hadi and Brightwell, 2021). Acidity with pH close to neutral 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). During processing of textured plant protein, the temperature exceeds 130°C, which temperature is abiotic, but in the later processing steps

appears new bacterial contamination with variety of genera as secondary and cross-contamination and by additional seasoning ingredients (He et al., 2020; Liu et al., 2023).
The aim of this study is to analyse and compare the microbial communities present in raw plant-based and meat burgers, with a focus on species variety and their 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 diversity of microbial ecosystem available within alternative food products and traditional meat products during storage and spoilage.

MATERIALS AND METHODS

Plant-based meat alternative (PBMA) and a beef burger (BB) are analysed in the present study. The main ingredients of the vegan burger are structured soy protein, starch, wheat gluten, and wheat fibre, while the beef burger consists of beef, plant fibres, and potato starch.
Both products are commercially available in Bulgaria as raw frozen products, for human consumption after heat treatment. The vegan burger is sold in packs by two, while the meat burger comes in packs by five.
For the purpose of the study, samples from 4 different batches were collected and transported in thermo-insulated bags to the veterinary food safety laboratory at Trakia University. Later the samples were left to thaw at a 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 as in consumer's kitchen, which is favourable for fast microbial growth. At each temperature, individual packages of plant-based and meat products were placed, with individual samples tested in every 2 days' interval (0, 2, 4, 6, and 10 days). Each individual package contained two burgers, vacuum-sealed.
The laboratory analyses were on: total bacterial count (CFU/g), expressed as a decimal logarithm (log CFU/g) - an indicator of the microbial contamination in the product; pH value, measured using Portable pH meters pH 7 Vio Set 1, Italy - an indicator of biochemical changes in the products caused by microbial activity; individual microorganisms identification of obtained colonial growth in total bacterial count - performed by analytical instrument 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 millilitres of MRD (HiMedia, India) were added, and the sample was homogenized in an peristaltic homogenizer at 256rpm^{-1}.
One millilitre of the initial homogenate was transferred into a glass test tube with 9 mL of MRD, followed by decimal serial dilutions. From each dilution, two Petri dishes with Plate Count Agar (HiMedia, India) were inoculated 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% glycerine for subsequent identification by MALDI-TOF MS instrument.

MALDI-TOF MS identification
The identification of the presumptive isolates was performed by instrument MALDI Biotyper RUO (Research Use Only) (Server Version: 4.1.100 (PYTH)) (Bruker, Germany). All collected isolates were cultured on Plate Count Agar (HiMedia, India) at 37°C for 24 hours.
Using direct bacterial transfer, in accordance with the manufacturer's recommendations (Bruker, Germany), a small single bacterial colony from fresh 24-hours culture was placed with a toothpick onto a single position in 96-positions polished steel target plate (MSP 96; Bruker, Germany). Each sample was carefully spread within the well position 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 library database in the MALDI Biotyper RUO (Research Use Only) (Server Version: 4.1.100 (PYTH)) (Bruker, Germany) software, the spectral peaks of each of the analysed bacterial isolate 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 $P < 0.05$ by Graphpad software.

RESULTS AND DISCUSSIONS

The present study analyses 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 variations during the storage 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 also relatively stable with 5.6, 5.8, and 5.8 in the same time-slot points. At 25°C, pH changes in both products were minor. In PBMA, the pH on day 2 reached 5.4, while on days 4 and 6, it was constant 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 constant pH values in both samples align with the product ingredients, such as an antioxidants and acidity regulators which 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 in the range from 1.0 to 7.2 log CFU/g in various meat analogue samples. In burger samples (n=16), the TBC values were from 1.5 to 5.1 log CFU/g, which results is similar to our results.

Research data on the recipes with protective cultures (lactic acid bacteria) reported that it would 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 detected 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 from 0.70 to 7.98 log CFU/g.

In burgers analysed in this study, protective cultures were not used in the recipe by producer. Although the initial bacterial concentration was lower in PBMA compared to the beef meat burger, but later during the storage the difference in the bacterial count number between the two products decreased. By day 10 at 6°C, the bacterial count in the meat burger remained higher compared to the plant-based analogue, 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. 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 analogues are highly susceptible to bacterial spoilage, similar to traditional ground beef or pork meat products.

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 favour fast microbial proliferation regardless of the ingredients used in burger production (Figure 1).

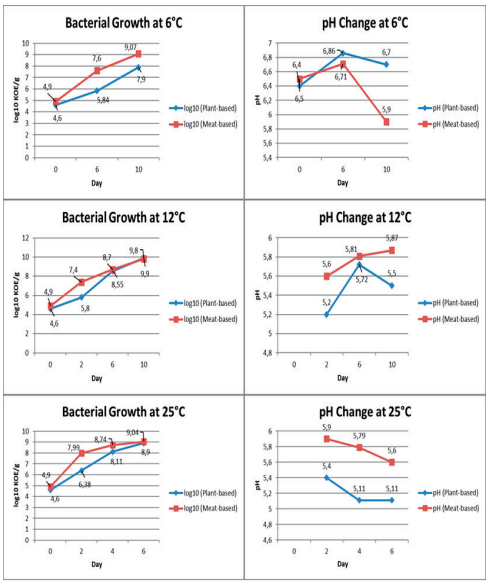

Figure 1. Bacterial growth and pH dynamics in PBMA and BB at 6°C, 12°C, and 25°C

Despite the common perception that plant-based meat alternatives are safer and more resistant to microbial contamination due to undergoing specific extrusion processing steps, it is important to note that they are not sterile. Microorganisms can be introduced into meat analogues both through the addition of raw ingredients as well as a result of cross-contamination during processing or later in the kitchens (Sampson et al., 2023).

Lupo (2019) notes that PBMA formulations often include various additives such as vitamins, minerals, flavour 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 detected at 6°C, 12°C, and 25°C, respectively.

For the meat burger, the 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 habits and sustainable ecology 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 proceed for identification by MALDI Biotyper RUO (Research Use Only) (Server Version: 4.1.100 (PYTH)) (Bruker, Germany). Of the 304 analysed isolates, 223 (73.35%) were correctly identified at the species level, 64 (21.05%) at the genus level, and only 17 isolates were without reference spectral peaks found in the MALDI Biotyper RUO 4.1.100 library database (classified as unidentified isolates).

A total of 39 bacterial genera were detected in both samples, with species distribution and prevalence 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 were *Leuconostoc* spp. (n=38), *Pseudomonas* spp. (n=30), *Lactococcus* spp. (n=26) and *Lactobacillus* spp. (n=22).

Additionally, several other genera were 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 burgers or environment or may be difficult to be detected, including *Actinomyces oris*, *Exiguobacterium maxicanum*, *Luteococcus japonicus*, and *Kurthia zopfii*. Barmettler et al. (2025) also reported as dominating *Lactobacillus* spp., *Leuconostoc* spp., *Bacillus* spp., *Bronchothrix thermosphacta*, and *Kocuria rhizophila*. identified also by MALDI-TOF MS. 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 shelf-life robustness model evaluation of PBMA products (Roch et al., 2024), but they can also contribute to acidification, gas accumulation in retail packages, or slime formation, even when stored at low temperatures (Barmettler et al. 2025).

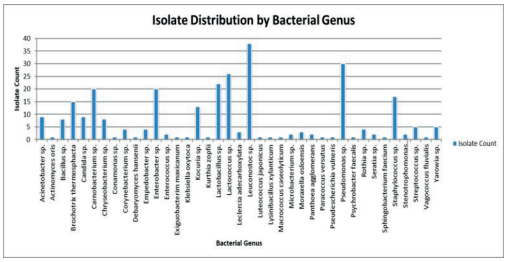

Figure 2. Frequency of bacterial isolates across different genera

Bacterial genera distribution in the tested types of burgers identify *Carnobacterium* spp., *Brochothrix thermosphacta* and *Acinetobacter* spp. as 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. Research studies have analysed the role of environmental strains, isolated from meat, in the growing 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 microbiota typical for 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*.

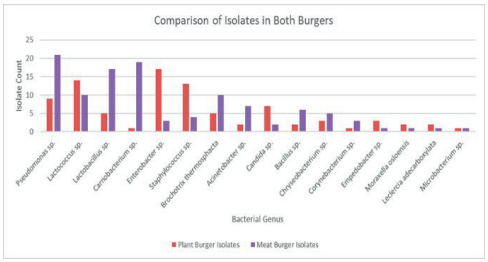

Figure 3. Frequency of bacterial isolates across different genera

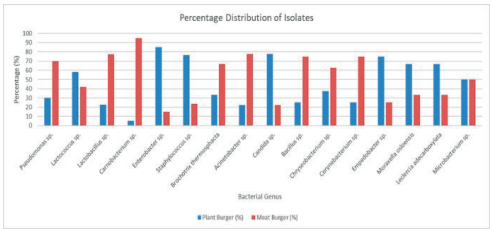

Figure 4. Percentage Distribution of Bacterial Isolates in Plant-Based and Beef Burger

The main quality changes in meat caused by these microorganisms include the appearance of unpleasant odours and off-flavours, as well as discoloration and gas formation (Borch et al., 2006). In our study, *Acinetobacter* spp. was more frequently detected in the meat burger compared to meat analogue burger, which can be explained by their presence as natural microbiota in raw meat products. These bacteria can be wider distributed during processing, storage or through contact surfaces. Both burger types contained significant amounts of lactic acid bacteria (*Lactococcus* spp., *Lactobacillus* spp.), which can lead to deterioration by

fermentative type and product quality changes. *Pseudomonas* spp. was also detected in considerable quantities, particularly in the meat burger, which is expected since these bacteria are commonly associated with food contamination and spoilage processes.

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

Figures 5 and 6 present the unique bacterial species isolated from PBMA and BB. Research analyses 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 (n=1 to 4).

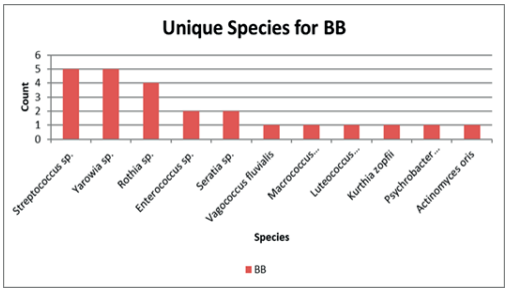

Figure 5 Unique Bacterial Species Identified in beef burger

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). We identified six isolates of *Lactococcus garvieae* from the beef burger, which indicates contamination rather than primary source infection and presence. Although the identification score exceeded 2.3, the application of molecular methods would be beneficial in further isolate analyses of closely related species *Lactococcus formosensis* and *Lactococcus petauri*.

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 burger samples.

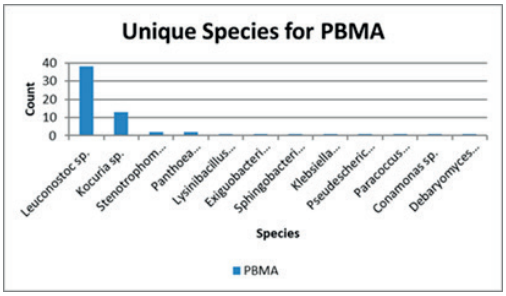

Figure 6. Unique Bacterial Species Identified in plant based meat analog burger and meat burgers

Empedobacter falsenii was first described in 2006. 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 waste 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 persons, regardless of their age. Clinical manifestations range from localized infections, such as wound infections and localized peritonitis, to systemic diseases, including sepsis, meningitis, and bacteraemia

(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 *Bacillus cereus* 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 three different temperatures. The obtained results indicate that despite their different compositions, both types of products exhibit similar levels of total bacterial count and 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 changes and shelf-life robustness, including changes in pH, gas accumulation and slime formation.

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