



Profiling of microbial populations present in ground beef and plant-based meat analogues

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ABSTRACT

Plant-based meat analogues have gained significant popularity in recent years. Different from ground beef, plant-based meat analogues are derived from a broader range of raw ingredients and undergo more complex manufacturing processes. In this study, 16S rRNA and internal transcribed spacer sequencing techniques were employed to profile and compare the microbial populations present in ground beef (GB), soy-, and pea-based meat analogues (SBM and PBM), and their changes during refrigeration (4 °C) and ambient temperature storage (22 °C). The sequencing results illustrated remarkable microbial differences among GB, PBM, and SBM. *Pseudomonas*, *Brochotrich*, and *Lactobacillus* were the top dominate bacterial genera, *Cladosporium*, *Candida*, and *Komagataella* were the top dominant fungal genera present in GB, PBM, and SBM respectively. At the end of storage, dominant microorganisms present in meat samples, in general, were determined by meat types and storage temperatures, except for SBM. For example, in PBM, refrigeration favored the proliferation of *Leuconostoc*, while *Lactococcus* emerged as the primary genus at ambient temperature. For SBM, *Lactobacillus* and *Komagataella* were dominate bacterial and fungal species regardless of the storage temperatures. These findings provide crucial information for the development of preventive measures to ensure the microbial safety and quality of plant-based meat analogues.

1. Introduction

With the rapid expansion of the global population and the economic development of developing countries, there is an increasing demand for reliable protein sources (Hadi & Brightwell, 2021). However, conventional practices of animal protein production and consumption, particularly in the case of cattle, have imposed a substantial environmental burden (Bruinsma, 2009; Grossi, Goglio, Vitali, & Williams, 2019; Koneswaran & Nierenberg, 2008). The transformation of grains and other crops into animal proteins is energy intensive and demands large quantities of arable lands, water and other valuable resources (Cassidy, West, Gerber, & Foley, 2013; de Boer, Schöslar, & Aiking, 2017). On top of that, public health concerns such as type 2 diabetes, cardiovascular ailments, and meat-related cancers have been associated with the consumption of red meat (Huang et al., 2021; Micha, Wallace, & Mozaffarian, 2010; Zhang, Guan, Yu, Zhou, & Chen, 2022). In the light of these concerns, there has been a remarkable shift towards plant-derived proteins as preferred alternatives to animal-derived counterparts over the past few years (Boukid, Rosell, & Castellari, 2021; Wang et al., 2022).

This shift has led to a rapid expanding market for plant-based proteins, such as pulses, wheat gluten, and soy protein, processed into meat-like products, also known as meat analogues. These plant-based ingredients are meticulously processed to mimic the taste, appearance, sensory, and nutritional elements of animal meat (Kyriakopoulou, Dekkers, & van der Goot, 2019).

However, as the popularity of this meat analogues increases, so do the concerns about their safety and microbiological risks, both among consumers and manufacturers (Liu et al., 2023; Tóth et al., 2021). Plant-based meat analogues often contain many processed ingredients and involve complex manufacturing processes, introducing new safety and quality concerns that aren't present in traditional meat products (Boukid et al., 2021). Many of these concerns arise due to the novelty of these ingredients and our limited knowledge towards the food safety and quality aspects of the plant-based meat analogues. Plant-based meat analogues are characterized by high protein and moisture levels as well as a neutral pH value, all of which create favorable conditions for the proliferation of spoilage microorganisms and foodborne pathogens (Hadi & Brightwell, 2021; Liu et al., 2023; Luchansky et al., 2020;

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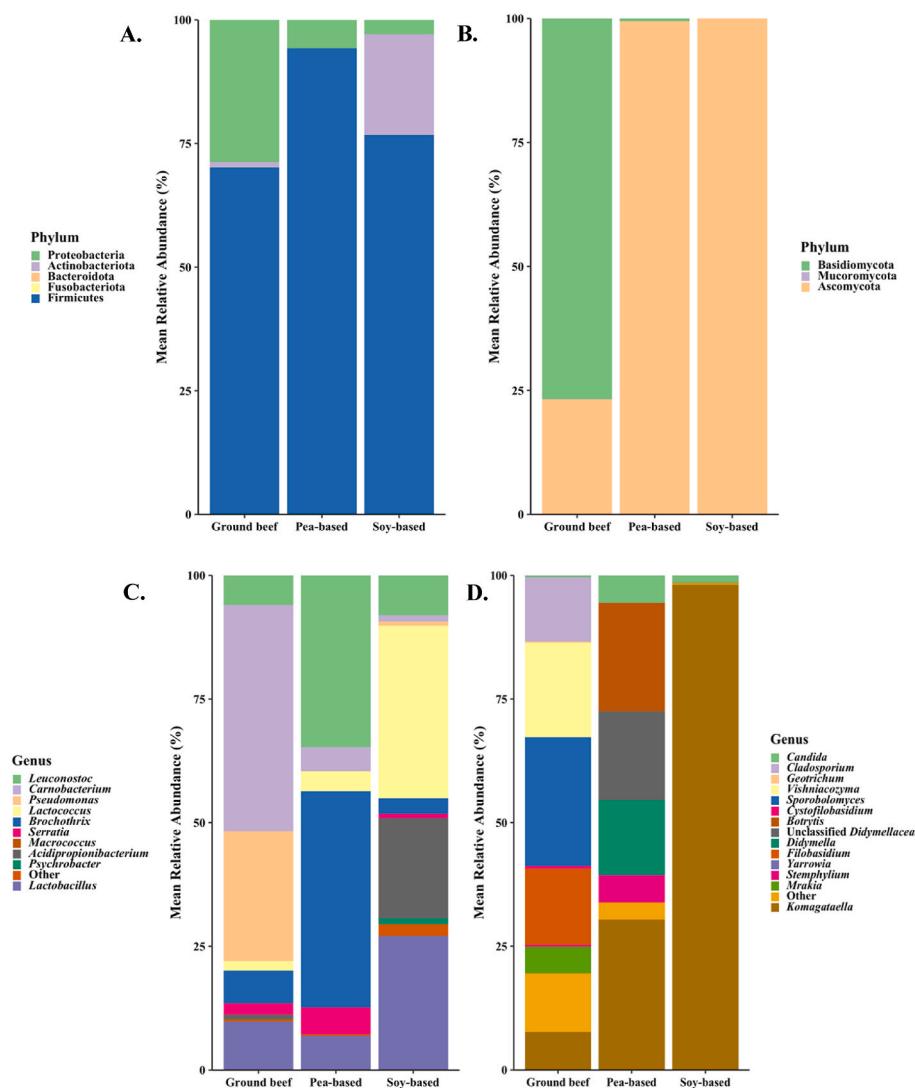


Fig. 1. Native microbial populations of ground beef (GB), pea-based meat (PBM), and soy-based meat (SBM). A, Bacterial communities at phylum level; B, Fungal communities at phylum level; C, Bacterial communities at genus level; D, Fungal communities at genus level. Genera with a relative abundance less than 5% were grouped into “Other”.

Nychas & Panagou, 2011). A microbial survey conducted by Tóth et al. (2021) revealed significant quantities of Enterobacteriaceae and yeast species during storage in both refrigerated and unrefrigerated meat analogues. Similarly, a recent study by Liu et al. (2023) suggested that both pea-based (PBM) and soy-based meat analogues (SBM) could support the survival and even growth of spoilage including *Pseudomonas fluorescens* and *Brochotrich thermosphacta*, as well as pathogenic microorganisms including *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes*.

Microbial spoilage is a complex ecological process influenced by several extrinsic and intrinsic factors. Microorganisms are highly sensitive to changes in the environment (de Boer et al., 2015). The intricacy of this process is often underestimated when relying on culture-dependent analysis, which is typically designed to promote the growth of specific bacteria in relatively homogenous environments (de Boer et al., 2015). In contrast, high-throughput sequencing offers a promising opportunity for characterizing microbial niches in their native state, reducing bias introduced by culturing (Beck et al., 2021). This allows for sample-to-sample comparisons of microbial compositions through normalization. Specifically, 16S and internal transcribed spacer (ITS) ribosomal RNA sequencing are widely used techniques in amplicon sequencing for the identification and comparison of bacteria

and fungi in food samples. The 16S rRNA gene in prokaryotes is about 1500 base pairs long and contains nine variable regions interspersed with conserved areas. These variable regions are often employed to determine the phylogenetic classification of genus or species within varied bacterial communities (Weisburg, Barns, Pelletier, & Lane, 1991). The ITS1 region within the rRNA cistron serves as a frequently utilized DNA marker for fungal species identification (Schoch et al., 2012). Therefore, the primary objective of this study is to employ both 16S rRNA and ITS sequencing to investigate and compare the microbial composition changes in ground beef (GB) with commercially available meat analogues, specifically soy-based and pea-based meat products, thus revealing the major spoilage microorganisms during the refrigerated and ambient storage and providing the information needed for control strategy development.

2. Material and method

2.1. Study design

Two separate batches of PBM, SBM and 80/20% GB were purchased from local grocery store for this study. All three types of meat were aseptically transferred to sterilized Ziploc bags, stored at 4 °C right after

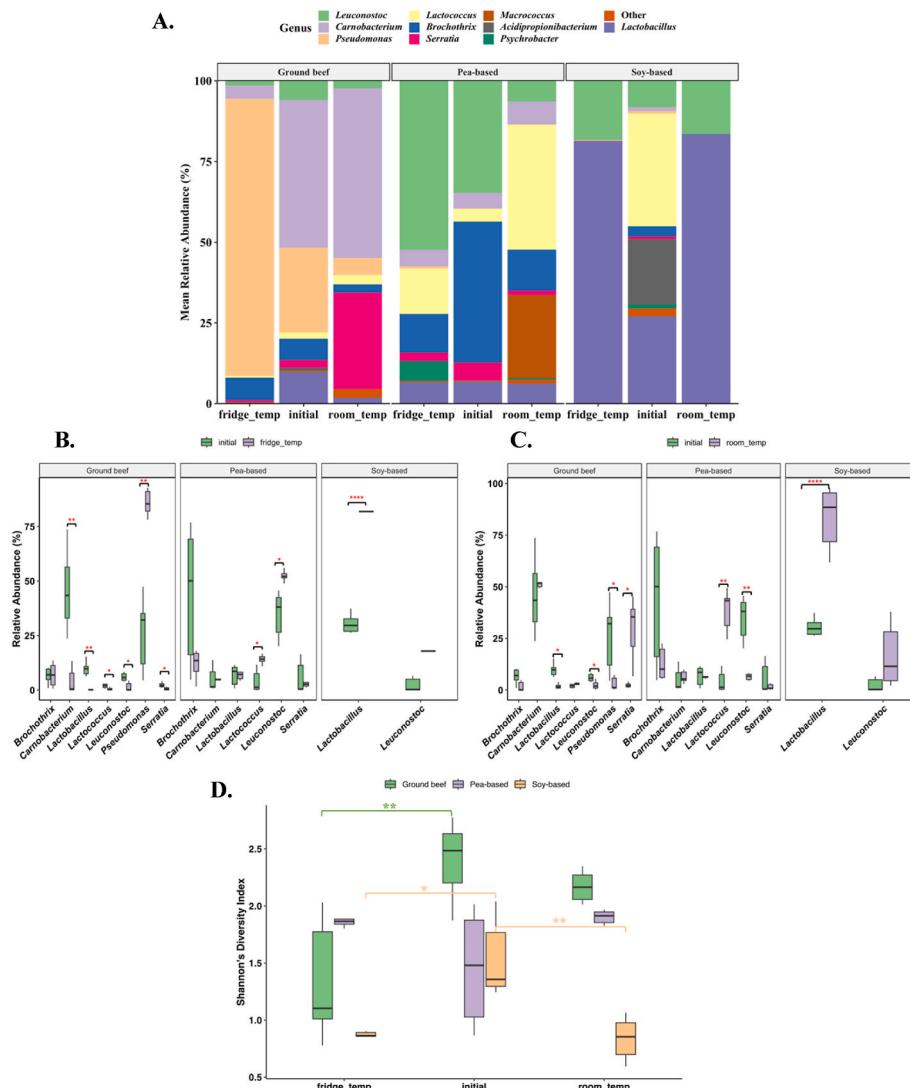


Fig. 2. Changes of bacterial community in ground beef (GB), pea-based meat (PBM), and soy-based meat (SBM) during refrigeration and ambient storage. A, Bar plot represents the relative abundances of bacteria at the genus level. Genera with a relative abundance less than 5% were grouped into “Other”. Samples are grouped by sample types (GB, PBM, and SBM). Storage conditions (initial: control, right after purchase; fridge_temp: stored at 4 °C for 7 days; room_temp: stored at 22 °C for 1 day) are labeled under each sample type. B, Dynamics in relative abundances of major bacterial taxa at the genus level in meat and meat analogues during 7-day storage at 4 °C. C, Dynamics in relative abundances relative abundance of major bacterial taxa at the genus level in meat and meat analogues during 24-h storage at 22 °C. D, Shannon’s diversity index for GB (n = 6), PBM (n = 6) and SBM (n = 6). ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

purchased and processed within the same day. After the initial preparation, the samples were divided into two sets. The first set was stored under refrigeration temperature (4 °C) for seven days to mimic home storage time and condition. The second set of samples were stored under room temperature (22 °C) for one day to replicate temperature abuse condition. Samples were taken for each type of meat at day 0, day 1 for the temperature-abuse condition and day 7 for the refrigeration condition. The overall appearance and color changes of the meat samples at each sampling points were recorded and evaluated. Color changes of the juice samples during storage were analyzed using the Hunter colorimeter (Hunter ColorFlex EZ, USA). The lightness, green-red, and blue-yellow values were represented by L* a* and b* respectively.

2.2. DNA extraction and library preparation

At each sampling point, 25 g of each sub sample were added in 75 mL of phosphate buffered saline (PBS, pH ~ 7.2), hand massaged for 30s, and homogenized in a stomacher for 1 min (Liu et al., 2023). Then 1.8 mL of every meat suspension were added to a 2 mL collection tube

(Thermo Fisher, MA) and subjected to DNA extraction using the Qiagen PowerFood Microbial Kit following the manufacturer’s instruction (QIAGEN, Valencia, CA). The purity and the concentration of DNA samples were evaluated with a plate reader spectrophotometer (Nano-Drop Technologies, DE, USA) at 260 and 280 nm. Further quantification of DNA was performed using the Qubit fluorometer (Thermo Fisher). A total 54 samples with high DNA qualities were subjected to subsequent library preparation and sequencing. The 16S rRNA and internal transcribed spacer (ITS) library preparation was performed using the QIAseq 16S/ITS Screening Panel kit targeting the V3-V4 and ITS regions (QIAGEN) following the manufacturer’s instructions. Subsequently, the sizes and concentrations of the library were checked by a bioanalyzer and sequenced with the Illumina MiSeq platform at the UC Davis Genome Center to obtain 300-bp paired-end reads.

The obtained raw FASTQ files were denoised by using DADA2 in the QIIME2 pipeline (Callahan et al., 2016) with default parameters. Taxonomy was assigned by using the QIIME2 q2-feature-classifier plugin. A Naïve Bayes classifier trained on the SILVA database and a the UNITE database were used for molecular identification of bacteria and fungi,

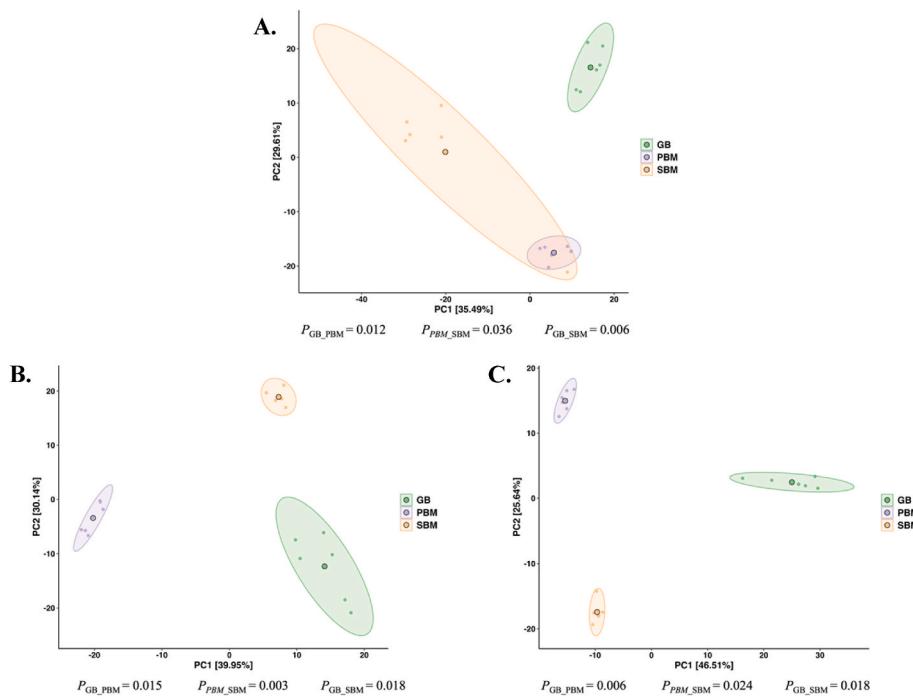


Fig. 3. Principal component analysis of the bacterial communities presents in ground beef (GB), pea-based (PBM) and soy-based meat analogues (SBM) at ASV level at the initial stage (A), after 7 days of refrigeration storage (B), and after 24 h of room temperature storage (C). Pairwise-comparisons were performed using pairwise adonis test and adjusted with the Bonferroni method. P -values are indicated in figure.

respectively (Bolyen et al., 2019; Nilsson et al., 2018). Sequences classified as Archaea, Eukaryota, chloroplasts, or mitochondria were culled. Additional filters were applied in R to remove amplicon sequence variants (ASVs) with fewer than ten copies across all samples, as well as ASVs present in less than five samples.

2.3. Statistical analysis

Statistical analysis was performed in R (version 4.2.2) (R Core Team, 2022). The alpha-diversity was calculated by using observed species and the Shannon index as implemented in the phyloseq package (McMurdie & Holmes, 2013). Beta-diversity was estimated using Aitchison distances, which were calculated based on CLR-transformed data and visualized through Principal Coordinates Analysis (PCoA). Beta dispersion was calculated using the betadisper function from the vegan package (version 2.6.4) (Dixon, 2003). Variations in microbial composition and microbial structure across different sample types and storage conditions/durations was conducted utilizing the Kruskal-Wallis test (Kruskal & Wallis, 1952) and permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2017, pp. 1–15) as implemented in the vegan package (version 2.6.4) (Dixon, 2003). Pairwise comparisons were performed using Dunn's test (Simple Fisheries Stock Assessment Methods package; version 0.9.5) (Ogle, Doll, Wheeler, & Dinno, 2023) and pairwise adonis test (pairwiseAdonis; version 0.4 (Arbizu, 2020) with Bonferroni correction, respectively.

3. Results

3.1. Bacterial and fungal communities present in meat and meat analogues

After being stored at 4 °C for 7 days or at 24 °C for 24 h, all meat samples exhibited clear signs of spoilage. Supplemental Table S1 and Fig. S1 show the changes. Briefly, for all meat samples, both a^* and b^* values decreased as the storage time increased, except for soy-based meat and pea-based meat when being stored at 4 °C for 7 days. The

color change of great beef was more significant compared to plant-based meat at both storage temperatures. The bacterial compositions of GB, PBM, and SBM were first analyzed using 16S rRNA gene sequencing, as illustrated in Fig. 1A and C. The native bacteria of fresh GB and PBM were dominated by Firmicutes (GB: 78.3%; PBM: 94.2 %) and Proteobacteria (GB: 28.7%; PBM: 5.7 %). While Firmicutes were also found in SBM with the highest relative abundance, Actinobacteria (20.3%) was shown to be the second most prevalent bacterial phylum in SBM (Fig. 1A). At the genus level, GB's bacterial profile was primarily characterized by *Carnobacterium*, *Pseudomonas*, *Lactobacillus*, *Brochothrix*, and *Leuconostoc* (Fig. 1C). For PBM, the predominant bacterial genera included *Leuconostoc*, *Carnobacterium*, *Brochothrix*, *Serratia*, and *Lactobacillus* (Fig. 1C). SBM's bacterial community was simpler compared to GB and PBM, consisting mainly of LAB such as *Lactobacillus*, *Lactococcus*, and *Leuconostoc*, along with *Acidipropionibacterium* (Fig. 1C). It's worth noting that while LAB was observed in all three meat types, the dominant genera varied. Furthermore, *Brochothrix*, a well-known spoilage bacterium, was substantially more abundant in PBM than in GB and SBM.

While molds and yeasts may not be the primary microbial safety/quality concerns in conventional meat and meat products, they serve as essential food safety indicators for plant-based foods (Tóth et al., 2021). Their presence in food is typically attributed to raw material or post-processing contamination. Given that plant-based meat products often undergo more complex manufacturing processes compared to conventional meat, the detection of molds and yeasts can serve as an important indicator of hygiene during production. Previous study by Tóth et al. (2021) have identified yeasts in both cooled vegan spaghetti Bolognese and unrefrigerated vegan cabbage casserole. Hence, in this study, ITS sequencing was conducted to analyze the fungal community compositions of both meat and meat analogues, in addition to the bacterial communities.

Results showed that over 75% of GB's fungal community comprised Basidiomycota, whereas the two plant-based meat analogues were predominantly characterized by Ascomycota (Fig. 1B). At genus level, the major fungal genera identified in GB included *Cladosporium*,

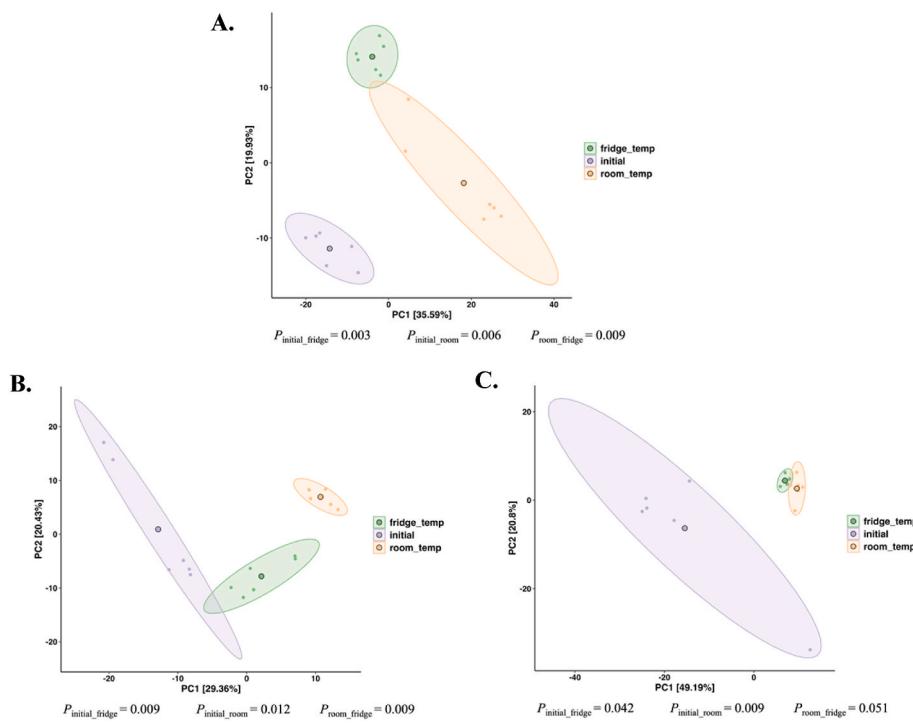


Fig. 4. Principal component analysis of the bacterial communities presents in ground beef (A), pea-based (B), and soy-based meat analogues (C) at the ASV level at different storage time (Day 0 vs. 4 °C for 7 days vs. 22 °C for 1 day). Pairwise-comparisons were performed using the pairwise adonis test and adjusted with the Bonferroni method. P -values are indicated in the figures.

Sporolomyces, *Vishniacozyma*, *Filobasidium*, *Mrakia*, and *Komagataella*. For PBM, the dominant genera were *Candida*, *Botrytis*, Unclassified *Didymellaceae*, *Didymella*, *Cystofilobasidium*, and *Komagataella*. The fungal community of SBM was again notably straightforward, consisting of approximately 98% *Komagataella* and less than 2% *Candida*.

3.2. Changes of the bacterial community of meat and meat analogues during storage

The 7-day refrigeration storage significantly altered the microbial populations present in meat and meat analogues. By the end of the refrigeration storage period, there was a notable shift in GB. *Pseudomonas*, a typical meat spoilage bacterium, became the dominant genus, with its relative abundance increased from 26.3% to 85.9% ($p < 0.05$; Fig. 2A and B). The Shannon's diversity index of GB decreased significantly after refrigeration storage, indicating decreased bacterial diversity (Fig. 2D). On the contrary, the bacterial community of PBM remained relatively stable throughout the refrigeration storage as Shannon's diversity index for PBM did not change significantly, except for the emerging of *Psychrobacter*, which can be attributed to the low-temperature storage conditions (Fig. 2A and D). Significant increases in the relative abundance of *Lactococcus* and *Leuconostoc* were observed on Day 7 ($p < 0.05$), suggesting that these microorganisms might play a major role in the spoilage of PBM during refrigeration storage (Fig. 2B). In SBM, *Leuconostoc* and *Lactobacillus* became the only two dominant genera after refrigeration storage, with the mean relative abundance of *Lactobacillus* significantly increased from 27.06% to 81.40% ($p < 0.05$; Fig. 2A and B). Similar to GB, the Shannon's diversity index of SBM significantly decreased after 7 days of refrigeration storage, primarily due to the absolute dominance of a single bacterial genus ($p < 0.05$; Fig. 2D).

Improper storage of food has been a common food safety concerns in consumers' home (Worsfold, 1997). Therefore, changes of the indigenous microflora in GB, PBM and SBM during storage at an abused temperature were studied (Fig. 2A and C). In GB, unlike the samples

stored under refrigeration, there was a significant decrease in the relative abundance of *Pseudomonas* and a significant increase of the relative abundance of *Serratia* ($p < 0.05$; Fig. 2C). In PBM, a significant increase in the relative abundance of *Lactococcus* was observed ($p < 0.05$), along with the emerging of *Macroccoccus* as one of the dominant genera (Fig. 2A). The presence of *Macroccoccus* raises potential food safety concerns, as *Macroccoccus* spp. can be an opportunistic pathogen in both veterinary and human clinical contexts (Carroll, Pierneef, Mafuna, Magwedere, & Matle, 2023). No significant changes were observed in Shannon's diversity index for both GB and PBM ambient storage ($p > 0.05$; Fig. 2D). With regard to SBM, the microbial populations were dominated by *Leuconostoc* and *Lactobacillus* on Day 1, similar to the pattern observed after refrigeration storage (Fig. 2A and C). This was accompanied by a significant decrease in Shannon's diversity index ($p < 0.05$; Fig. 2D).

Principal component analysis (PCoA) revealed distinct clustering between GB, PBM and SBM at the initial stage. The two plant-based meat variants, PBM and SBM, exhibited closer proximity to each other compared to GB (Fig. 3A). This difference persisted under both storage conditions (Fig. 3B and C). When examining each meat type separately, storage conditions played more notable impact on GB and PBM compared to SBM by showing more significant P values among three conditions (Fig. 4A and B). In contrast, the microbiota of refrigerated SBM samples slightly overlapped with that of SBM samples stored under room temperature (Fig. 4C).

3.3. Changes of fungal community present in meat and meat analogues during storage

For GB, the refrigeration storage increased the relative abundance of *Komagataella* from 7.7% to 32.6%, although the change was not statistically significant (Fig. 5A and B). New dominant genera, namely *Yarrowia* and *Cystofilobasidium*, emerged by the end of the refrigerated storage (Fig. 5A). The diversity of the fungal community in GB decreased after refrigerated storage, as indicated by the significantly decrease in

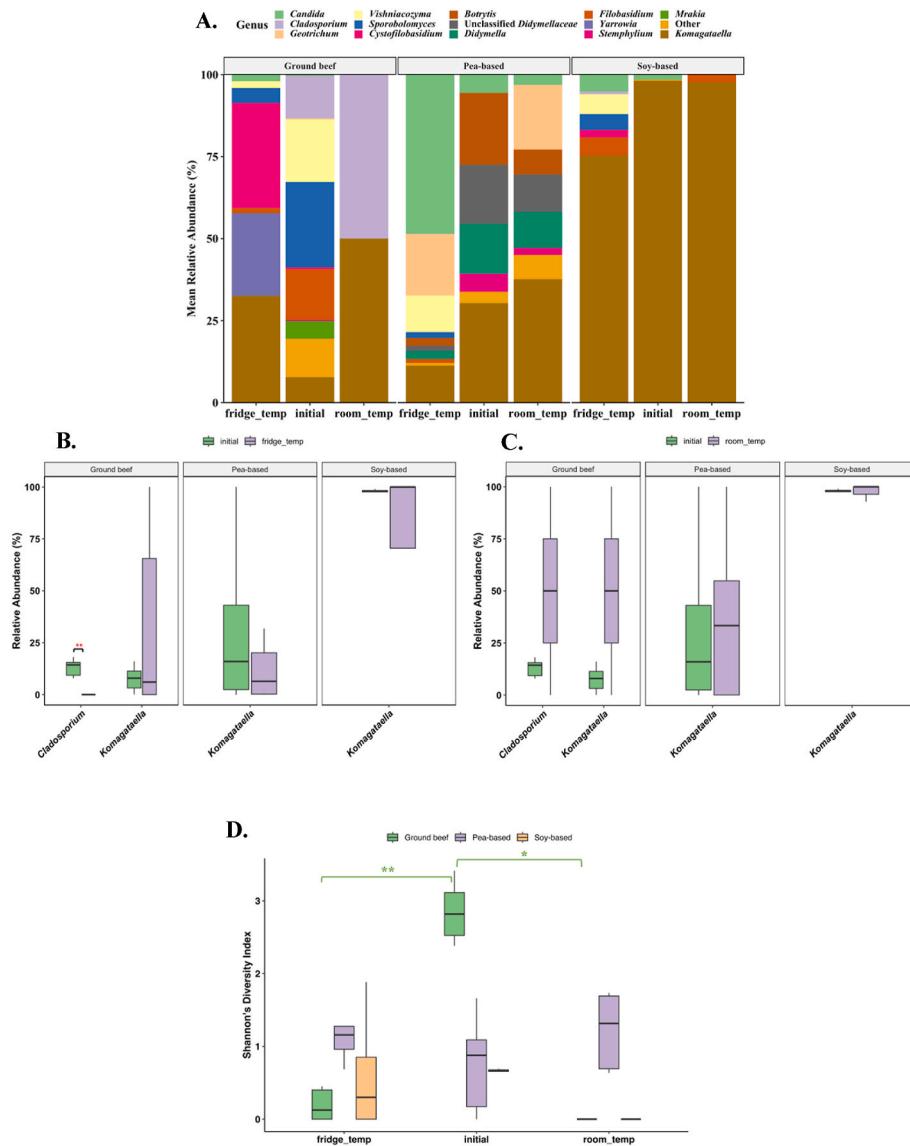


Fig. 5. Changes of fungal community present in ground beef (GB), pea-based meat (PBM), and soy-based meat (SBM) under different storage conditions. A, Bar plot represents the relative abundances of fungi at the genus level. Genera with a relative abundance less than 5% were grouped into “Other”. Samples are grouped by meat types, and the storage conditions (initial: control, right after purchase; room temp: stored at 22 °C for 1 day; fridge temp: stored at 4 °C for 7 days), are labeled under each meat type. B, Dynamics in relative abundances of major fungal taxa at genus level in meat and meat analogue samples after 7 days of storage at 4 °C. C, Dynamics in relative abundances of major fungal taxa at genus level in meat and meat analogue samples after 24 h of storage at 22 °C. D, Shannon’s diversity index for GB (n = 6), PBM (n = 6) and SBM (n = 6) ***p < 0.0001, **p < 0.001, **p < 0.01, *p < 0.05.

the Shannon’s diversity index (Fig. 5D). In contrast, the diversity of the fungal communities presents in plant-based meats (PBM and SBM) increased after the refrigeration storage. A notable increase in fungal genera such as *Candida*, *Geotrichum* and *Vishniacozyma* was observed PBM after refrigerated storage (Fig. 5A). Regarding SBM, although the product remained primarily dominated by *Komagataella*, a few other fungal genera emerged after storage, including *Filobasidium*, *Candida*, and *Vishniacozyma* (Fig. 5A).

The 24-h temperature-abuse storage condition (22 °C) decreased the diversity fungal community in GB. After one day in storage, the only two fungal genera that remained were *Cladosporium* and *Komagataella*, and each accounted for exactly half of the total fungal population (Fig. 5A and D). In contrast, the composition of the majority of the fungal community remained relatively stable in PBM, with only minor fluctuations in their relative abundances (Fig. 5A). *Geotrichum* emerged as a new dominant taxon, with its relative abundance increased from 0% to 19.78% (Fig. 5A). Similar to the refrigeration storage condition,

Komagataella remained as the dominant fungal genus in the SBM after 24 h of storage at room temperature (Fig. 5A and C).

As indicated by the β-diversity analyses, the fungal profiles for each type of meat were distinct from each other at the initial stage (Fig. 6A). By the end of the refrigeration storage (Fig. 6B), GB and SBM samples overlapped with each other, while PBM samples were grouped separately from GB and SBM. At the end of the room temperature storage, similar observations were made as GB and SBM overlapped with each other. Based on the P values, only GB and PBM were significant different, while PBM vs. SBM and GB vs. SBM were not significant different (Fig. 6C). When examining the effect of storage on the fungal community of each type of meat, the samples after either room- or fridge-temperature storage resulted in different fungal communities in GB and SBM (Fig. 7A and C). For PBM, on the other hand, only the 7-day refrigeration temperature storage led to a distinct cluster (Fig. 7B).

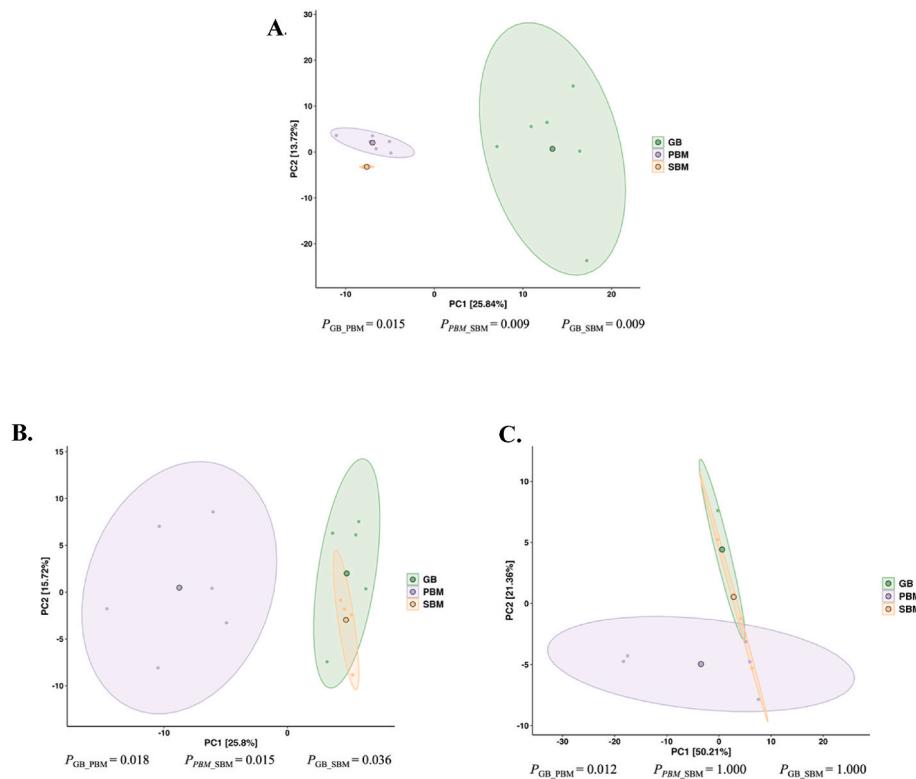


Fig. 6. Principal component analysis of fungal communities presents in ground beef (GB), pea-based (PBM) and soy-based meat analogues (SBM) at ASV level at the initial stage (A), after 7 days of storage at 4 °C (B), and after 24 h of room temperature storage (22 °C, C). Pairwise-comparisons were performed by using the pairwise adonis and adjusted with the Bonferroni method. P -values are indicated in the figures.

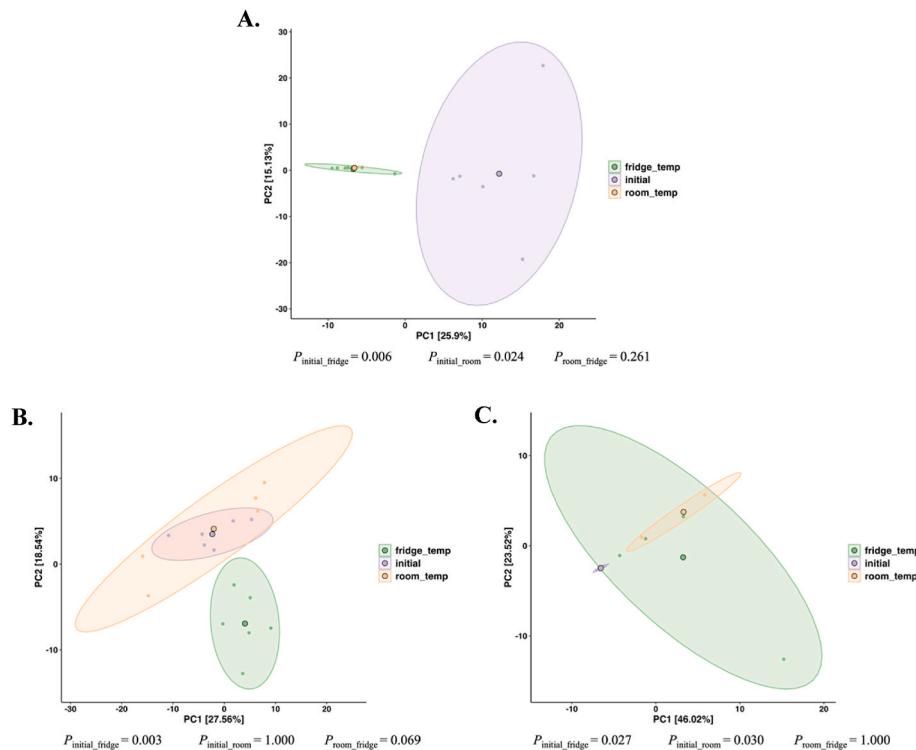


Fig. 7. Principal component analysis of the fungal communities presents in ground beef (A), pea-based (B), and soy-based meat analogues (C) at the ASV level at different storage time (Day 0 vs. 4 °C for 7 days vs. 22 °C for 1 day). Pairwise-comparisons were performed using the pairwise adonis test and adjusted with the Bonferroni method. P -values are indicated in the figures.

4. Discussion

Plant-based meat analogues are gaining popularity in the market and have been promoted as an environmentally friendly, and ethical alternative of traditional meat (Elhalis, See, Osen, Chin, & Chow, 2023). For a long time, the primary challenge of the plant-based meat industry is to ensure these products maintain adequate protein content while delivering a meat-like taste, texture, color, and overall consumer experience (Tóth et al., 2021). However, the food safety and quality aspects of these emerging foods have received relatively less attention and investigation. It has been well-established that the composition of the native food microbiome plays a crucial role in determining food safety and quality. Therefore, in our study, we addressed this issue by employing 16S and ITS sequencing techniques to characterize the microbial communities present GB and two types of plant-based meats (pea- and soy-based) and their changes during refrigeration and ambient storage.

Our results indicate that the initial indigenous microbial compositions differ significantly among GB, PBM, and SBM, with GB displaying higher microbial diversity. This disparity is anticipated, as plant-based meat analogues and GB are derived from distinct raw materials and undergo different manufacturing processes (Egbert & Borders, 2006; Kyriakopoulou et al., 2019). Current meat analogue production processes involve heat treatments, such as extrusion or frying, which may potentially reduce microbial loads and diversity. This is consistent with a previous study conducted by Liu et al. (2023), which reported that the initial APC in GB was 1–2 Log CFU/g higher than in PBM and SBM.

Under refrigeration temperature, *Pseudomonas* dominated the microbiota of GB, followed by *Brochothrix*. There two bacterial genera were previously identified as the primary spoilage-causing bacteria in refrigerated meat (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015; Doulgeraki, Ercolini, Villani, & Nychas, 2012). Under temperature abuse storage condition, the bacterial composition of GB exhibited greater diversity compared to that under refrigeration storage. *Serratia* as well as a few LAB, including *Carnobacterium*, *Leuconostoc*, and *Lactococcus*, dominated the GB microbiota, replacing *Pseudomonas*. This difference is likely due to the mesophilic nature of *Serratia* spp., while *Pseudomonas* spp. are typically categorized as psychrotrophic. As demonstrated in a study by Ercolini, Russo, Nasi, Ferranti, and Villani (2009), *Serratia* spp. were most frequently identified among mesophilic isolates, while *Pseudomonas* spp. were commonly found among psychrotrophs in packaged beef. Furthermore, their research revealed that mesophilic bacteria exhibited significantly faster growth at 20 °C compared to 7 °C. In contrast, most psychrotrophic *Pseudomonas* strains required over 3 days at 7 °C for growth and showed no growth at 30 °C even after 10 days.

In addition to bacteria, yeasts and molds are also able to grow in GB (Liu et al., 2023; Tóth et al., 2021). Refrigeration temperature appeared to promote the growth of *Yarrowia* and *Stemphylium*. *Yarrowia* is known for its ability to tolerate various environmental conditions, including cold temperatures, which makes it well-suited for growth in refrigerated environments (Jach & Malm, 2022). *Yarrowia lipolytica* are commonly associated with meat and milk products spoilage due to its pronounced lipolytic and proteolytic activities (Edina Szandra, 2014). Specifically, it has been implicated in causing spoilage and undesirable changes in poultry stored at 5 °C (Ismail, Dea, Abd El-Rahman, Yassien, & Beuchat, 2001). On the other hand, *Stemphylium* spp. are primarily known as plant pathogens, causing leaf spot diseases (Das et al., 2019; Prencipe & Spadaro, 2021). Therefore, its increased relative abundance in GB after refrigeration storage likely resulted from significant decreases in the relative abundances of other fungal community members. Under the temperature abuse condition, the predominant spoilage fungus found in GB is *Cladosporium*. This fungus is characterized by dark mycelia, which can vary in color from brown to blackish-brown or gray-green and often leads to the formation of dark spots on food items. The primary impact of *Cladosporium* is food spoilage and discoloration (Bullerman, 2003).

Plant-based meat analogues typically consist of a blend of plant

proteins, vegetable oils, yeast extract, salt, and flavoring agents (Boukid et al., 2021). This composition leads to a distinct nutritional profile compared to animal-derived products. Additionally, the native microorganisms present in the raw ingredients may vary, contributing to differences in microbial profiles between meat analogues like PBM and conventional meat products such as GB.

In PBM, the predominant bacterial genus after refrigeration storage was *Leuconostoc*, with the presence of a few other LAB such as *Lactococcus* and *Lactobacillus*. This competitive success and predominance of *Leuconostoc* under chilled condition in spoiled food can possibly be explained by its distinct metabolic activity. Andreevskaya et al. (2018) compared the transcriptome profiles of three prevalent psychrotrophic spoilage LAB (*Leuconostoc gelidum*, *Lactococcus piscium*, and *Lactobacillus oligofermentans*). They found that in the presence of other LAB, the fast-growing *Le. gelidum* upregulated its carbohydrate catabolic pathways, pyruvate fermentation enzymes, and ribosomal proteins to improve its nutrient-scavenging and growth capabilities. In contrast, the slower-growing *Lc. piscium* and *Lb. oligofermentans* downregulated these functions (Andreevskaya et al., 2018). Interestingly, under room temperature/temperature abuse storage, *Lactococcus* emerged as the most abundant genus in PBM. This could be related to the emerging of another LAB, *Macrococcus*. Previous research has demonstrated that adding attenuated *Lactococcus* cells to Caciotta cheese resulted in significantly greater relative abundances of *M. caseolyticus* and indigenous *Lactococcus* spp. in the cheese microbiota, implying a positive correlation between them (Calasso et al., 2020). In addition, the appearance of *Macrococcus* in PBM following temperature abuse storage is a matter of concern. *Macrococcus* species are Gram-positive, catalase-positive, oxidase-positive, and coagulase-negative cocci (Ramos, Vigoder, & Nascimento, 2021). They have been increasingly recognized for their potential as opportunistic pathogens. Notably, since 2018, various strains of *Macrococcus*, including *M. goetzii*, *M. epidermidis*, *M. boemicus*, *M. canis* and *M. caseolyticus* subsp. *hominis*, were identified in human clinical samples, isolated from different infection sites (Mašlaňová et al., 2018; Jost, Schwendener, Liassine, & Perreten, 2021), underscoring the importance of monitoring these bacteria in food.

Compared to PBM, the microbial community of SBM was considerably less complex, primarily consisting of LAB (i.e., *Leuconostoc*, *Lactococcus*, and *Lactobacillus*) and *Acidipropionibacterium* at the initial stage. After storage, the composition became even simpler, consisting solely of *Leuconostoc* and *Lactobacillus*, with *Lactobacillus* constituting over 80 percent of the community regardless of the storage temperature. Similarly, the study conducted by Liu et al. (2023) revealed that common foodborne pathogens including *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* grew better in PBM and SBM. This phenomenon could be attributed to the fact that the SBM product is derived from fermented soybean (Elhalis et al., 2023). Lactic acid fermentation is a traditional method used to enhance the shelf-life of legumes, protecting them against spoilage and pathogenic microorganisms (Emkani, Oliete, & Saurel, 2022). The process of fermentation results in a reduction in pH levels and has the potential to develop antimicrobial chemicals that disrupt the integrity of cell membranes. Consequently, this interference with proton gradients and enzyme function (Leroy & De Vuyst, 2004). Additionally, LAB cultures that are already adapted to a specific substrate create an environment where undesirable microorganisms cannot effectively compete (Steinkraus, 2002).

When looking at the fungal community, PBM stored under refrigeration temperature mainly harbored yeast cells of the genus *Candida*. While *Candida* is a heterogenous genus comprised of over 200 species with different physiological and metabolic activities, the majority of them are mesophilic. Only certain strains of *C. lipolytica* and *C. scottii* isolated from seafood samples have been reported to have active proteolytic activity at low temperatures (Kobatake, Rij, Plácido, & Uden, 1992). Hence, the increase in the relative abundance of *Candida* in this context could again purely be a consequence of the reduction in other fungal genera due to the low temperature. This is particularly evident

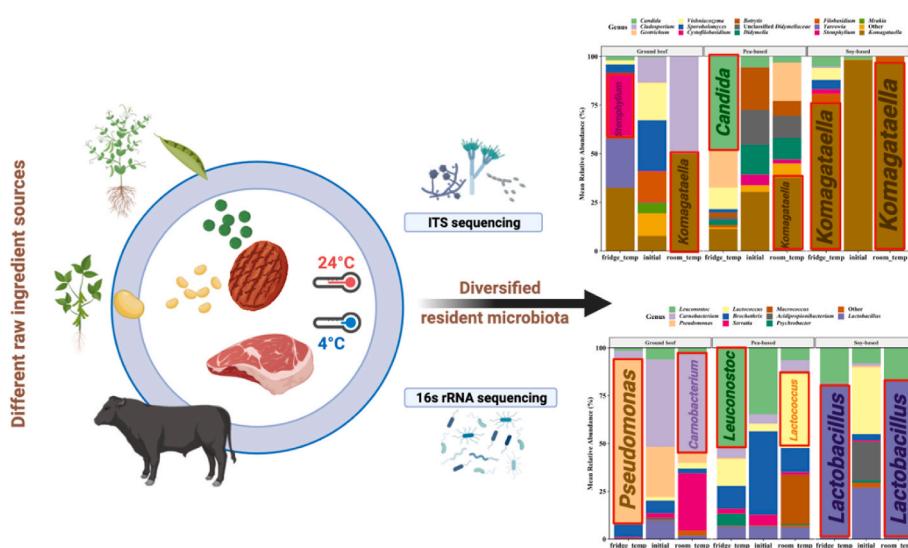


Fig. 8. Graphic abstract of the study. Plant-based meat analogues and ground beef were stored at two temperatures and the major spoilage microorganism were profiled with 16S rRNA and ITS sequencing.

when assessing the fungal community of PBM stored at room temperature, where most of the initially present fungal genera were preserved. In addition to *Candida*, another noteworthy genus present in the fungal community of PBM was *Geotrichum*. Within this genus, the only species of significance in foods is *G. candidum*, often referred to as 'machinery mold' due to its association with food-processing equipment. Unclean equipment provides a favorable environment for rapid growth of this organism (Bullerman, 2003). Fortunately, the relative abundance of *Geotrichum* was minimal in the fresh PBM samples, indicating it was present but in very low numbers in the fresh samples. In SBM, the overwhelming prevalence of *Komagataella* is noteworthy but not unexpected, as *K. phaffii* is intentionally used in SBM to produce legume hemoglobins (LegH), crucial for imparting the meaty flavor and aroma in plant-based meat to replicate the characteristics of animal meat (Reyes, Chen, Fraser, Chan, & Li, 2021). In addition, each LegH includes a cofactor heme, which constitute 95% of functional iron in the human body and is considered as an essential nutrient for humans (Hooda, Shah, & Zhang, 2014).

5. Conclusion

In this study, the microbial populations (both bacterial and fungal) of two plant-based meat analogues, one derived from pea protein and the other from soy protein, were investigated and compared with GB (Fig. 8). Results highlight the significant differences in the microbiota of plant-based meats compared to GB. The dominant spoilage genera in GB were found to be different from those in plant-based meats. While *Pseudomonas*, *Carnobacterium*, and *Serratia* were prevalent in spoiled GB, spoiled plant-based meats were dominated by *Leuconostoc*, *Lactococcus*, and *Lactobacillus*. The specific dominant taxa in each type of meat/meat analogue were also influenced by storage temperature and time. The identification of potential spoilage and pathogenic fungi in this study underscores the importance of developing efficient environmental monitoring systems and other control measures for the production of plant-based meat analogues. While this study provides valuable insights into the microbial quality and safety of plant-based meat, additional research is still needed to find out the sources of these microorganisms. Omics tools, such as metagenomics, metatranscriptomic, and metabolomics, provide opportunities to bridge these knowledge gaps.

CRediT authorship contribution statement

Xiran Li: Writing – original draft, Visualization, Software, Formal analysis, Data curation. **Hongye Wang:** Writing – review & editing, Validation. **Chenxi Guo:** Data curation. **Luxin Wang:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2024.115845>.

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