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Microbial communities in fermented products: Current knowledge and future prospects

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Microbial communities in fermented products: Current knowledge and future prospects

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Editorial: Microbial communities in fermented products: current knowledge and future prospects

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Editorial on the Research Topic
Microbial communities in fermented products: current knowledge and future prospects

Fermentation has been applied since antiquity for the production of palatable food with excellent preservation capacity. It is driven by microbial consortia that are adapted to the nutrient and energy sources of the raw materials and specific ambient conditions. Through the interplay between abiotic and biotic factors, the production of fermented products with enhanced organoleptic characteristics, preservation potential and nutritional value, is achieved.

Assessment of the microbial communities that drive fermentation has attracted significant scientific attention. Characterization of the physicochemical properties of the raw materials employed, identification of the microorganisms that dominate or participate as secondary microbiota in these microecosystems, their metabolic potential, as well as the effect that the fermentation conditions may have on the biotransformations that take place during fermentation, have been in the epicenter of scientific scrutiny for decades. The aim of this Research Topic was to provide with a collection of articles that would facilitate our understanding on the aforementioned topics, identify research gaps and pave the road for future research.

The paramount importance of the raw materials and their effect on the physicochemical, sensorial, and functional quality of the final product was highlighted by the studies of Asif et al. and Wei et al.. In the first study, buttermilk of increasing fat content was used for the production of Cheddar-type cheese. Increase of the fat content resulted in the increase of the lactic, propionic, acetic, and citric acids, vitamins A and E, as well as the free fatty acid content, after 90 days of ripening. In addition, the highest fat content was awarded with the highest texture and sensory scores. Wei et al. studied the effect of broken egg addition to the composting mixture of *Pleurotus floridanus* cultivation, on the physicochemical properties and the bacterial microcommunities

of the substrate as well as the agronomic and nutritional properties of the fruiting bodies. The physicochemical properties of the substrate after composing ranged within values that would not affect negatively the mycelial growth ratio and mushroom yield. After composting, the bacterial communities of the control and the compost made with the addition of broken eggs mixture consisted of members of the phyla Actinobacteriota, Firmicutes, and Proteobacteria. In all cases, the genera *Streptococcus* and *Streptomyces* seemed to prevail before composting and the genus *Acinetobacter* after composting. Regarding the agronomic properties of the fruiting bodies, addition of broken eggs mixture above 16.8 Kg, resulted in the increase of incubation period and contamination rate and the decrease of yield of first flush, total yield and biological efficiency; therefore, it was regarded as not suitable for *Pleurotus floridanus* cultivation. On the other hand, the addition of broken eggs resulted in the improvement of the nutritional quality and flavor of the fruiting bodies.

The characterization of the microbiota that is involved in fermentation, necessarily includes their identification at species or subspecies level and the assessment of their metabolic properties that are associated with the safety, the technological and the functional characteristics of the product. The genetic and technological diversity of dairy lactic acid bacteria isolates has been extensively assessed. Their probiotic potential has been in the epicenter of intensive study, since fermented dairy products are excellent vehicles for probiotic delivery. The latest research regarding the health benefits attributed to probiotic cultures, their mode of action, as well as the emerging applications in the food industry, were collected and comprehensively presented by Latif et al.. In addition, the probiotic potential of 23 lactic acid bacteria strains isolated from different fermented food products was presented by Megur et al.. The extensive variety of properties that were assessed were associated with the safety of their use, their capacity to withstand the harsh conditions of the human GIT and colonize it, to inhibit the growth of pathogenic bacteria and to possess functional potential. The results obtained revealed that promising probiotic candidates can be retrieved from spontaneously fermented products.

Spontaneous fermentations are driven by microbial consortia that are subjected to qualitative and quantitative changes, due to the dynamic nature of their microenvironment. Indeed, a succession at species and subspecies level is very frequently reported. However, studies assigning specific attributes of the final product to specific members of these microbial consortia, are generally lacking. In order to address this literature gap, Martini et al. investigated the bacterial dynamics at species and strain level during ripening of Parmigiano Reggiano cheese, which was fermented by natural whey starter, and the possible correlation with the evolution of the peptide profiles. The most frequently isolated non-starter lactic acid bacteria species were *Lacticaseibacillus rhamnosus*, *La. paracasei,* and *La. zeae*. More than 520 peptides were detected in the cheese samples, most of them originating from β-caseins. Occurrence of *La. zeae* was positively correlated with the incidence of 8 anti-hypertensive peptides. Similarly, Dong et al. studied the dynamics of the members of the microbial community during maturation of sauce-flavor Daqu of three colors, namely black, yellow and white and their effect on the compounds that affect the quality of the final product. It was reported that Daqu microecosystem consisted of fungi and bacteria, with the phyla Ascomycota and Firmicutes, respectively, being the most prevalent. Regarding fungi, genus *Thermoascus* was prevalent in all Daqu types. On the contrary, the bacterial diversity was more pronounced, with genera *Kroppenstedia*, *Virgibacillus,* and *Bacillus* being prevalent in black, yellow and white Daqu, respectively. Acidity was reported as the most important factor affecting the composition of the microcommunities. In addition, the key role of *Kroppenstedia* in color formation and of molds in pyrazine compounds formation were highlighted.

The development of autochthonous starter cultures is an emerging trend, which aims to improve the organoleptic quality of the final products and enhance their typicity and locality. Therefore, there is a need for studies assessing the potential of autochthonous strains and their effect on the quality of the final product. The studies by Grizon et al., Tzamourani et al., and Kamarinou et al. may serve as examples of studies that are aligned to this need. In the first study, the genetic and functional diversity of *Streptococcus thermophilus* strains isolated from different farms in the Saint-Nectaire cheese-producing PDO area in France, was investigated. A total of 22 non-commercial *S. thermophilus* strains were included in the study along with 4 commercial ones, and their genetic and technological properties were comparatively assessed. Pan-genome analysis revealed that the 41% of the genes could be characterized as hard-core genes, as they were present in 25 out of the 26 genomes, while the 56% of the genes could be characterized as accessory ones, as they were present in <24 of the 26 genomes. This remarkable genetic diversity was partially exposed through the different acidification and proteolytic capacity of the strains. In the study by Tzamourani et al. a novel approach for rapid and efficient screening and classification of autochthonous yeast isolates with enological potential, was proposed. This approach allowed an effective technological classification by employing a phenotype-based, technologically oriented preselection procedure followed by biostatistical data treatment. The capacity of the proposed approach was validated and verified by micro-fermentation trials. Finally, in the study by Kamarinou et al., Feta cheese was produced by employing a commercial lactococcal starter culture that was supplemented with a mixture of autochthonous lactic acid bacteria strains belonging to the species *Lactococcus lactis*, *Levilactobacillus brevis*, *La. paracasei*, *Lactiplantibacillus plantarum,* and *Leuconostoc mesenteroides*. The final product was characterized by enhanced quality and safety, and distinctive organoleptic characteristics.

In conclusion, although the microbial communities of fermented products have been extensively assessed, especially in the case of products with commercial significance, novel approaches and insights are frequently presented. These, apart from improving our understanding of microecosystem development, reveal new possibilities and alternatives for future research.

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Probiotics: mechanism of action, health benefits and their application in food industries

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Probiotics, like lactic acid bacteria, are non-pathogenic microbes that exert health benefits to the host when administered in adequate quantity. Currently, research is being conducted on the molecular events and applications of probiotics. The suggested mechanisms by which probiotics exert their action include; competitive exclusion of pathogens for adhesion sites, improvement of the intestinal mucosal barrier, gut immunomodulation, and neurotransmitter synthesis. This review emphasizes the recent advances in the health benefits of probiotics and the emerging applications of probiotics in the food industry. Due to their capability to modulate gut microbiota and attenuate the immune system, probiotics could be used as an adjuvant in hypertension, hypercholesterolemia, cancer, and gastrointestinal diseases. Considering the functional properties, probiotics are being used in the dairy, beverage, and baking industries. After developing the latest techniques by researchers, probiotics can now survive within harsh processing conditions and withstand GI stresses quite effectively. Thus, the potential of probiotics can efficiently be utilized on a commercial scale in food processing industries.

KEYWORDS

probiotics, lactic acid bacteria, immunomodulation, anti-allergic and gastrointestinal diseases, functional foods

1. Introduction

Probiotics, in the form of supplements or food products, have emerged as the most prominent ingredient in the era of functional foods. Probiotics have always been a vital component and commercial target for providing potential health benefits (Sanz et al., 2016; Hamad et al., 2022). The term "probiotic" was first presented by Werner Kollath in 1953, which is known to be a derivative of the Latin word *pro* and the Greek word *βιο* meaning "for life." Kollath defined probiotics as active bodies with essential functions for promoting various health aspects (Gasbarrini et al., 2016). Food and Agriculture Organization (FAO) and World Health Organization (WHO) described them as "live microbes when administered in adequate quantities, confer health benefits on host organisms" (Munir et al., 2022). Several bacteria belonging to the genera *Pediococcus, Lactococcus, Enterococcus, Streptococcus, Propionibacterium*, and *Bacillus* are considered potential microbes for probiotic status (de Brito Alves et al., 2016; Hamad et al., 2022).

The frequently used strains belong to the divergent group of *Bifidobacterium* and *Lactobacillus* that significantly affect health with various actions. They detoxify xenobiotics and environmental pollutants (Reid, 2015), bio-transform mycotoxins in foods (Hamad et al., 2022), synthesize vitamin K, riboflavin, and folate (Reid, 2015; Hamad et al., 2022), and ferment undigested fiber in the colon (Warman et al., 2022). Probiotics prevent pathogenic bacteria by restricting binding sites on mucosal epithelial cells and modulating the host immune response, thus improving intestinal barrier integrity (Fusco et al., 2023). The advantages of probiotics are related to the modulation of gut microbiota, mitigation of nutritional intolerances (lactose intolerance), increase in bioavailability of macro and micronutrients, and alleviation of allergic incidences in susceptible individuals (Roobab et al., 2020).

Probiotics can be consumed either by incorporating them into foods or drinks in the form of dairy or non-dairy foodstuffs or as supplements (Fenster et al., 2019). Various fermented foods have active microbes genetically similar to the strains utilized as probiotics. It has been observed that fermented foods enhance the functional and nutritional aspects by transforming substrates and producing bioactive and bioavailable end-products (Marco et al., 2017). The approximate consumption of 10^9 colony-forming unit (CFU)/day have been revealed as an effective dose (Hill et al., 2014). By keeping in view, the effective dosage, probiotics are being incorporated into many foods like beverages, ice cream, yogurt, bread, and many others by the food industry. The most significant barrier associated with probiotics in the food industry is their susceptibility to processing conditions and sensitivity to gastrointestinal (GI) stresses. However, regarding their health benefits, the consumer always showed an inclined interest in probiotic products (Konuray and Erginkaya, 2018). Now scientists have developed new and innovative methods like nanoencapsulation and genetic modification, which enable probiotics to withstand harsh conditions of both processing and GI stresses in the body (Putta et al., 2018). This review paper provides a profound insight into the mechanistic approach and current perspective on the beneficial aspects of probiotics in preventing and treating various diseases. The application and safe utilization of probiotics in major food industries have also been described.

2. Mechanisms of action

Outstanding advances have been made in the field of probiotics, but there has yet to be a key breakthrough in the documentation of their mechanism of action. Probiotics possibly exert a positive potential on the human body through these main mechanisms; competitive exclusion of pathogens, improvement in intestinal barrier functions, immunomodulation in the host's body, and production of neurotransmitters (Figure 1; Plaza-Diaz et al., 2019). Probiotics compete with pathogens for nutrients and receptor-binding sites, making their survival difficult in the gut (Plaza-Diaz et al., 2019). Probiotics also act as anti-microbial agents by producing substances; short chain fatty acids (SCFA), organic acids, hydrogen peroxide (Ahire et al., 2021), and bacteriocins (Fantinato et al., 2019) thus decreasing pathogenic bacteria in the gut. Moreover, probiotics improve the intestinal barrier function by stimulating the production of mucin proteins (Chang et al., 2021), regulating the expression of tight junction proteins, including occluding and claudin 1, and regulating the immune response in the gut (Bu et al., 2022; Ma et al., 2022).

Probiotics also regulate the innate and adaptive immune response modulating dendritic cells (DC), macrophages B and T lymphocytes. Probiotics also increase the production of anti-inflammatory cytokines while interacting with intestinal epithelial cells and attracting macrophages and mononuclear cells (Petruzziello et al., 2023). Furthermore, probiotics can produce neurotransmitters in the gut through the gut-brain axis. Specific probiotic stains can modulate the serotonin, gamma-aminobutyric acid (GABA), and dopamine levels, affecting mood, behavior, gut motility, and stress-related pathways (Srivastav et al., 2019; Sajedi et al., 2021; Gangaraju et al., 2022).

3. Health attributes of probiotics

The health benefits of probiotics are associated with preventing and reducing many diseases, i.e., allergic diseases, cancer, hypercholesterolemia, lactose intolerance, inflammatory bowel disease, diarrhea, and irritable bowel syndrome (Grom et al., 2020), as shown in Figure 2. Table 1 shows different studies regarding the application of probiotics in different diseases.

3.1. Antiallergic effect of probiotics

Allergy is a hypersensitive disorder of the immune system, termed as type I hypersensitivity and defined as a "disease following a response by the immune system to an antigen." With escalating incidence rate, allergies affect nearly half of the population of Europe and North America. These allergic reactions occur due to one or more common environmental substances or antigens (Prakash et al., 2014). The most common allergic reactions include asthma, rhinitis, atopic eczema, dermatitis, urticaria, angioedema, hay fever, and food, drug, and insect hypersensitivity (Lopez-Santamarina et al., 2021). The gut microbiome is a viable therapeutic target for managing allergic diseases (Harata et al., 2016), as they modulate the immunological and inflammatory response that consequently affects the development of sensitization and allergy (Fiocchi et al., 2015).

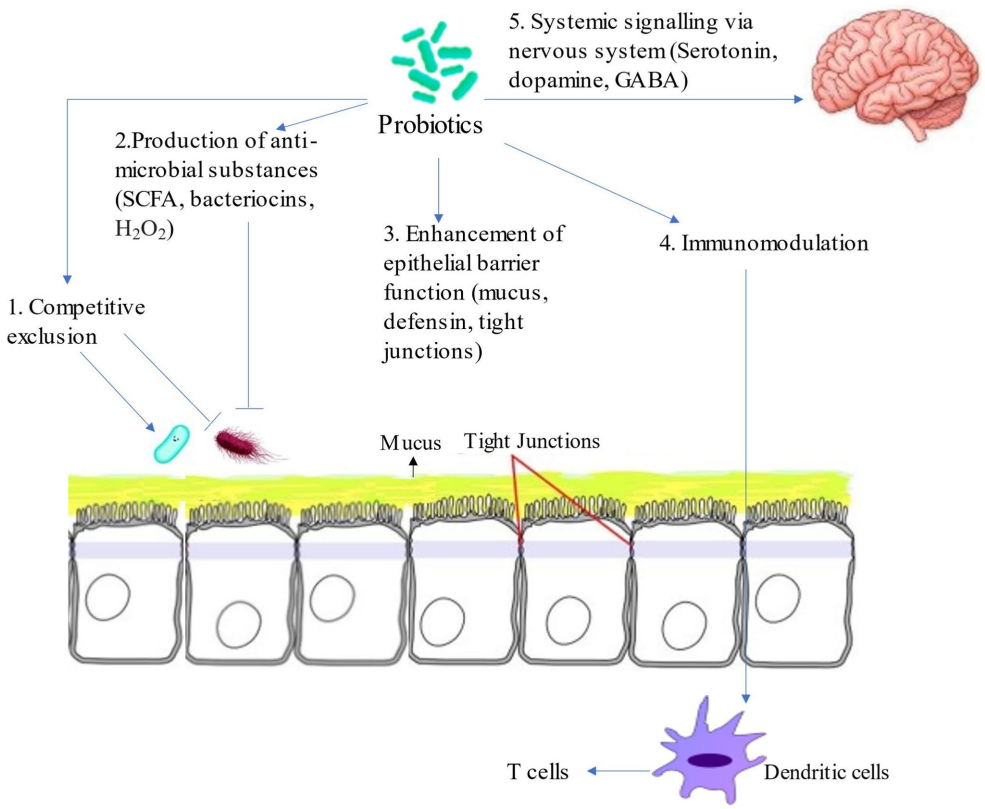

FIGURE 1
Mechanism of action of probiotics. 1. Probiotics perform their function by competing with pathogens for nutrients and receptors for binding thereby making their survival and adherence to gut mucosa difficult. 2. Probiotics produce anti-microbial substances which inhibit pathogens growth. 3. Probiotics promote epithelial barrier function by enhancing mucus production and increasing the expression of tight junction proteins which prevents the translocation of pathogens from intestine into the blood. 4. Probiotics regulate immunity of the host by modulating maturation and function of dendritic cells subsequently increasing the activity of T cells which play important role in immune homeostasis. 5. Probiotics also regulate the production of neurotransmitters including serotonin, dopamine and gamma aminobutyric acid (GABA).

Allergic diseases are characterized by an imbalance in lymphocyte-governed immunity in which the immune response becomes overly biased toward T helper 2 lymphocytes dominated response (Th2 cells) (Di Costanzo et al., 2016). Allergen-sensitized Th2 cells produce various interleukins such as IL-1, IL-4, and IL-5, thus recruiting granular effector cells, i.e., mast cells, eosinophils, and basophils toward the site of allergic inflammation. In addition, the interleukins switch B lymphocyte immunoglobulin isotype, which upsurges the circulating level of total and allergen-specific IgE (Galli et al., 2020). Although the precise mechanism is not entirely known, it is expected that the probiotics improve mucosal barrier functions, stimulate the immune system, reduce leakage of antigen through the mucosa, produce anti-inflammatory cytokines, increase the production of secretory IgA (exclude antigens from intestinal mucosa), degrade dietary antigen and up-regulate anti-inflammatory cytokines as IL-10 (Liang et al., 2022).

The proposed mechanism for the antiallergic effect of probiotics is the augmentation of T helper cells (Th)1/Th2 immune balance by suppressing Th2 skewed immune response and favoring Th1 cell response (Di Costanzo et al., 2016). Ma et al. (2019) explain that probiotics modulate the function of dendritic cells, which in turn have the ability peripheral Tregs. Tregs control the excess immune response and maintain a balance between Th1 and Th2 cells (Figure 3). Besides, lactobacilli stimulate regulatory T cells which play a paramount role in balancing immune response through the production of immunosuppressive cytokines and modulation of IgE, IgA, and IgG production (Owaga et al., 2014).

The antiallergic effect of *Lactiplantibacillus plantarum* SY12 and *L. plantarum* SY11 was studied using RAW 264.7 (murine macrophage) cell line. Both species showed a reduction in the production of nitric oxide, T helper 2 linked cytokines, tumor necrosis factor-α, and cyclooxygenase-2 as well as inducible nitric oxide synthase compared to the control group (Lee et al., 2014). In this regard, the *Limosilactobacillus reuteri* effect was also investigated against the food allergy in ovalbumin (OVA)-sensitized BALB/c mice. Oral intake of *L. reuteri* helped restore the deteriorated profile of colonic microflora and attenuated allergic diarrhea. It also increased the activation of mast cells, enhanced the production of serum immunoglobulin E (IgE), suppressed the T helper 1 and 2 cytokines production, down-regulated the GATA3 expression, and increased the expression of TGF-b, IL-10, and Foxp3. The findings confirmed the anti-allergic activities of *L. reuteri* promoted by the modulation of enteric flora and enhancement of tolerogenic immune responses (Huang et al., 2017).

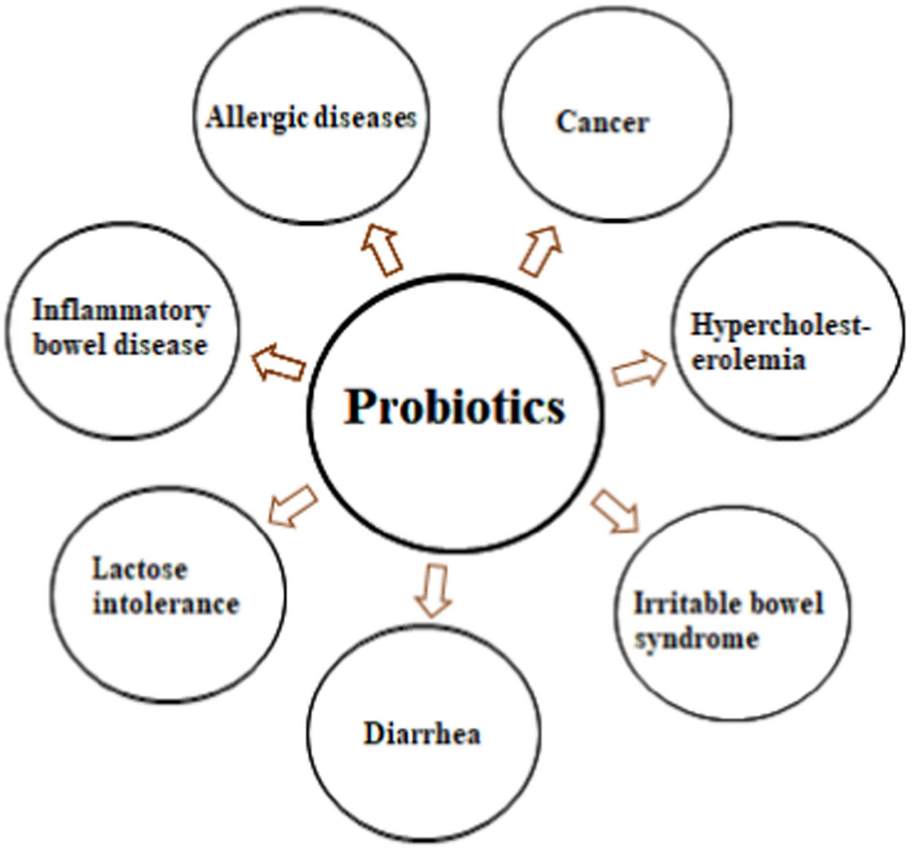

FIGURE 2
Health attributes of probiotics. Probiotics help in the prevention and management of allergic diseases, cancer, hypercholesterolemia, irritable bowel syndrome, diarrhea, lactose intolerance, inflammatory bowel disease.

3.2. Cancer suppressor activity of probiotics

Probiotics could be used as an adjuvant for various types of cancers based on their potential to modulate enteric flora and enhance local and systematic immunity. They prevent the initiation, progression, and metastasis of transplantable or chemically induced tumors (Samanta, 2022). The effect of probiotics can be observed in suppressing both intestinal and extraintestinal cancers (So et al., 2017). The interaction of probiotics and their metabolites (bacteriocin, peptides, and organic acids) with critical metabolic pathways such as cellular proliferation, inflammation, apoptosis, angiogenesis, and metastasis has been revealed by many researchers (Harikumar et al., 2013). Moreover, the probiotics inhibit carcinogenesis by inhibiting pathogens through competitive exclusion, increasing short-chain fatty acid production (Chong, 2014), reducing carcinogenic bile salts production, binding carcinogens and mutagens, down-regulating NF-kappa B dependent genes products for cell proliferation (Cox-2, cyclin D1) and cell survivability (Bcl-3, Bcl-xL) and enhancing apoptosis (Konishi et al., 2016). Probiotics also upregulate TNF-related apoptosis-inducing ligand (TRAIL) (Kłłonowska-Olejnik, 2004), modulate cell cycle by rapamycin (mTOR)/4EBP1 (Islam et al., 2014) and inhibit the formation of aberrant crypt foci (Yu and Li, 2016). Figure 4 describes the anti-cancer effect of probiotics.

Previous studies have scrutinized that the ERK1/2 pathway modulates cell survival, proliferation, differentiation, and cell motility by regulating the BCL-2 protein family in mitochondria (Passaniti et al., 2022). *Saccharomyces boulardii,* both *in vitro* and *in vivo*, inhibited the activation of ERK1/2 mitogen-associated protein kinase. In the same way, probiotic *L. reuteri* induced apoptosis in human myeloid leukemia-derived cells by modulating NF-kappa B and MAPK signaling pathways (Saber et al., 2017). The colonic microflora has also been related to the development of liver disorders such as liver fibrosis (De Minicis et al., 2014), nonalcoholic fatty liver diseases (Zhuge et al., 2022), and more recently, liver cancer (So et al., 2017). Probiotics have been demonstrated to inhibit hepatocellular carcinoma (HCC) progression by reducing liver tumor size and down-regulating angiogenic factors. The mechanistic approach to this is the level of T helper (Th) 17 cells in the gut and its recruitment to tumor sites was lower in probiotic-treated mice (Li et al., 2016). In breast cancer apart from immunomodulation, the hypoxia-inducible factor (HIF) pathway was also reported to be significantly suppressed by *Lactobacillus* cultures supernatant (Esfandiary et al., 2016).

In addition to this, experimental studies were carried out to reduce the mutagenic potential of a powerful carcinogen; *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) by *Lacticaseibacillus rhamnosus* Vc. Oral feeding of *L. rhamnosus* Vc (10^9 CFU) to *Gallus gallus* (chicks) for 30 days significantly detoxified the parent compound reducing its mutagenicity (61%) and genotoxicity (69%)

TABLE 1 Therapeutic effect of probiotics in gastrointestinal disorders.

Disease	Strain	Dosage	Subjects	Results	References
Allergic reactions	*L. plantarum*	5×10^{10} cells once a week for 4 weeks	Mice sensitized with peanut allergen	↓ Interleukin-10 ↑ Interferon-γ	Yang et al. (2021)
Allergic reactions	*Lactobacillus* multiple strains	10^9 CFU lactobacilli every day for 28 days	30 BALB/c mice model of soybean sensitization	↑ Interferon-γ and IL-2 ↓ IL-4, IL-6 Promoted Tregs	Yang et al. (2021)
Cancer	*Lactobacillus fermentum*	–	CCD18-Co, HCT-116, and HT-29 cell lines	Activation of intrinsic apoptosis	Lee et al. (2019)
Cancer	*Pediococcus acidilactici* TMAB26	–	HT-29 and Caco-2 cell lines	Significant toxicity on cancer cells	Barigela and Bhukya (2021)
Hypercholesterolemia	*L. casei* pWQH01 *L. plantarum* AR113	1×10^9 CFU for 5 weeks	30 male C57BL/6J mice	Have Bile Salt Hydrolase activity ↓ hepatic levels of TC and LDL-C ↑cholesterol 7α-hydroxylase (CYP7A1) gene	
Hypercholesterolemia	*L. fermentum* MJM60397	5×10^{10} CFU	Male mice	↓ cholesterol and low-density lipoprotein (LDL) cholesterol levels ↑ LDLR gene	Palaniyandi et al. (2020)
Ulcerative colitis	*Bifidobacterium longum* 536 (BB536)	$2–3 \times 10^{11}$ three times daily for 8 weeks	56 patients with mild to moderate UC	↓ Mayo subscore ↓Rachmilewitz endoscopic index (EI)	Tamaki et al. (2016)
Ulcerative colitis	*L. lactis* NCDO 2118	2.5×10^6 CFU/g	36 mice	↓ Severity of colitis ↓ disease activity index ↑ gene expression of tight junction proteins (*zo-1*, *zo-2*)	Cordeiro et al. (2021)
Lactose intolerance	*L. acidophilus*	1×10^{10} once daily for 4 weeks	60 human participants	↓Abdominal pain ↓Abdominal cramping ↓Vomiting	Pakdaman et al. (2015)
IBS	*L. delbruekii* and *L. fermentum*	10 billion bacteria twice daily for 4 weeks	90 human subjects	↓Abdominal pain ↓IL-8 Restore normal intestinal flora	Husein et al. (2017)
Radiation-induced diarrhea	*L. acidophilus* and *B. animalis*	1.75 billion lyophilized live bacteria three times daily	53 patients receiving external beam pelvic radiotherapy	↓Moderate and severe diarrhea ↓Grade II abdominal pain	Linn et al. (2019)
Chronic diarrhea	*L. plantarum* CCFM1143	3.52×10^9 CFU per day	55 human patients with chronic diarrhea	Improved clinical symptoms of diarrhea Improved immune response Modulated gut microbiota	Yang et al. (2021)
Antibiotic associated diarrhea	*Lactobacillus* and *Bifidobacterium* strains	1×10^9 CFU once a day	36 human subjects	Delayed recurrence of diarrhea (5.39 days) ↓ Average no. of daily stools 45% positive evaluation	Trallero et al. (2019)
Chron's disease	*B. longum* and inulin/ oligofructose	2×10^{11} freeze-dried viable *B. longum* twice daily for 6 months	35 human subjects	↓Crohn disease activity indices ↓Histological scores ↓TNF-α expression	Steed et al. (2010)

↓ shows the reduction in different parameters while ↑ shows increasing trend.

(Pithva et al., 2015). In another study, the role of *Saccharomyces cerevisiae* on the activation of apoptotic pathway Akt/NF-kB was explored in cancer. Heat-killed *S. cerevisiae* induced apoptosis in cancer cells, the SW480 cell line, by up-regulating Bax, cleaved caspase 3 and cleaved caspase 9, and down-regulating p-Akt1, Bcl-XL, Rel A, procaspase 3 and procaspase 9 expressions. Hence, it was concluded that probiotics modulate Akt/NF-kB pathway following the apoptotic cascade and play an essential role in cancer prevention (Shamekhi et al., 2020).

3.3. Hypocholesterolemic effect of probiotics

Probiotics can be used as an effective tool for lowering blood cholesterol levels. They can act directly or indirectly to decrease cholesterol levels in the body. The direct mechanism includes the inhibition of *de novo* synthesis of cholesterol by hypocholesterolemia factors like uric acid, lactose, orotic acid, and whey protein as well as the reduction in intestinal absorption of dietary cholesterol in three

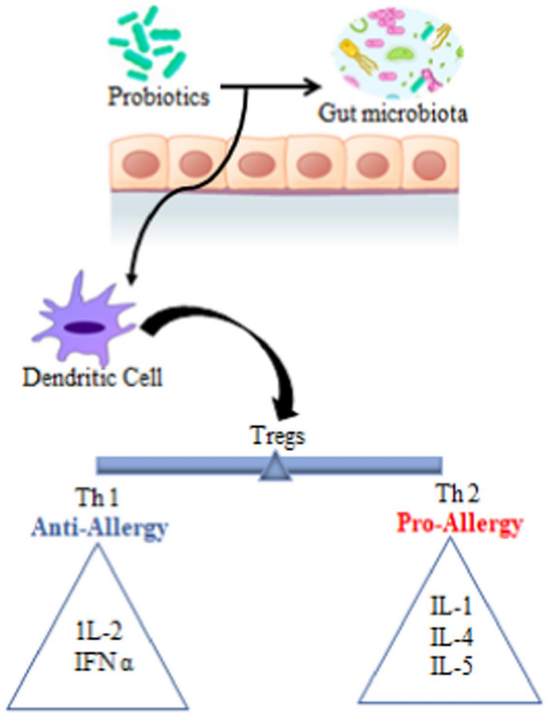

FIGURE 3
Anti-allergic effect of probiotics. Tregs, T regulatory cells; Th 1, T helper cells type 1; Th 2, T helper cell type 2; IL, interleukin; IFN α, interferon α. Probiotics help in the migration and maturation of dendritic cells via modulating the composition of gut microbiota. Dendritic cells in the gut-associated lymphoid tissues have the ability to induce the development of peripheral Tregs and to play a central role in the development of immune homeostasis. Tregs maintain the proper level of Th 1, Th 2 cells as well as anti-allergy and pro-allergy cytokines.

ways- assimilation, binding, and degradation (Thakkar et al., 2016). The indirect mechanism for curtailing cholesterol by probiotics is deconjugating bile salts (conjugated glycodeoxycholic acid and taurodeoxycholic acid) via bile salt hydrolase (BSH) production. Deconjugated bile salts are less reabsorbed through the intestine, thus inhibiting enterohepatic circulation of the bile and higher excretion in the feces (Figure 5; Rezaei et al., 2017).

Human and animal studies have provided evidence for the hypocholesterolemic properties of probiotics. In a study, the hypocholesterolemic properties of *Levilactobacillus brevis* MT950194 and *L. brevis* MW365351 were observed both *in vitro* and *in vivo*. The strains reduced cholesterol content, increased fecal cholesterol excretion, and converted bile into free cholic acid (Munir et al., 2022). The potential of a probiotic complex comprising *Pediococcus*, *Lactobacillus*, and *Bifidobacteria* was also investigated in lipid metabolism. After 10 weeks of the experimental period, the results showed significantly reduced cholesterol levels in medium and high-dose groups (Galli et al., 2020). The cholesterol reduction potential of a new strain, *L. plantarum* DMDL 9010, was investigated by using *in vivo* model. The intake of strain resulted in the reduction of serum cholesterol, hepatic cholesterol, triglycerides, and an increase in fecal excretion of bile acids. A significant decrease in total cholesterol, low-density lipoprotein, and atherosclerosis index by 23.03, 28.00, and 34.03%, respectively was observed with the use of *L. plantarum* DMDL 9010 (10^9 cells per day) (Liu et al., 2017).

Recently, research regarding gene expression by probiotics in hypercholesterolemia was conducted by Dehkohneh and his colleagues. The role of *Lacticaseibacillus paracasei* TD3 was examined in modulating two significant genes involved in cholesterol metabolism; 3-hydroxy-3-methyl glutaryl coenzyme (HMGCR) and cytochrome P450 7A1 (CYP7A1). A dose of 1×10^{10} CFU was given to male Wistar rats for 21 days. The cholesterol level was significantly decreased along with the reduction of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes. The dramatic decline of HMGCR and CYP7A1 genes in adipose tissues was also observed using real-time polymerase chain reaction (Dehkohneh et al., 2019).

3.4. Impact of probiotics on intestinal diseases

The gut plays a pivotal role in the digestion and absorption of nutrients and maintains mucosal barrier integrity. Numerous commensal bacteria reside in the human GI tract constituting an active community, which strongly affects human physiology (Shehata et al., 2022). The modification in intestinal microflora can be achieved by administering antibiotics, probiotics, prebiotics, and fecal transplant (Shahverdi, 2016).

The metabolic activity of the intestinal microbiome affects the host's health, both favorably and unfavorably (Saber et al., 2017). The exact balance in the microflora (eubiosis), when disturbed, results in acute and chronic clinical disorders like antibiotic-associated diarrhea (AAD), ulcers, inflammatory bowel disease (IBD), and irritable bowel syndrome (IBS) (Saber et al., 2017). In addition, several researchers have supported the theory that microbial dysbiosis participates in the etiology of some human cancers (Su et al., 2021), especially GI cancers (Pereira-Marques et al., 2019). Restoring healthy gut microbiota can be used as a practical approach to managing intestinal diseases. Probiotics can increase microbial richness and diversity, increase enzyme (Lactase) production, improve immune micro-environment (Jang et al., 2019), and improves intestinal permeability (Stratiki et al., 2007). In this way, probiotics can alleviate intestinal diseases. Studies regarding the use of probiotics in intestinal diseases are given in Table 1.

4. Application of probiotics in the food industry

The public awareness of diet-related issues and ever-increasing evidence about probiotic health benefits have increased consumer interest in probiotic foods. A large number of food items, including yogurt, powdered milk, frozen fermented dairy desserts, cheese and cheese products, ice creams, baby foods, cereals, and fruit juices, are among numerous probiotic foods (Papademas and Kotsaki, 2019). The most prominent barrier to using probiotics in the food industry is their sensitivity toward heat treatments during processing and GI stresses in the human body. However, researchers and food industries are trying to find new and innovative methods and techniques to overcome the issues (Zhang et al., 2022). The global increase in sales

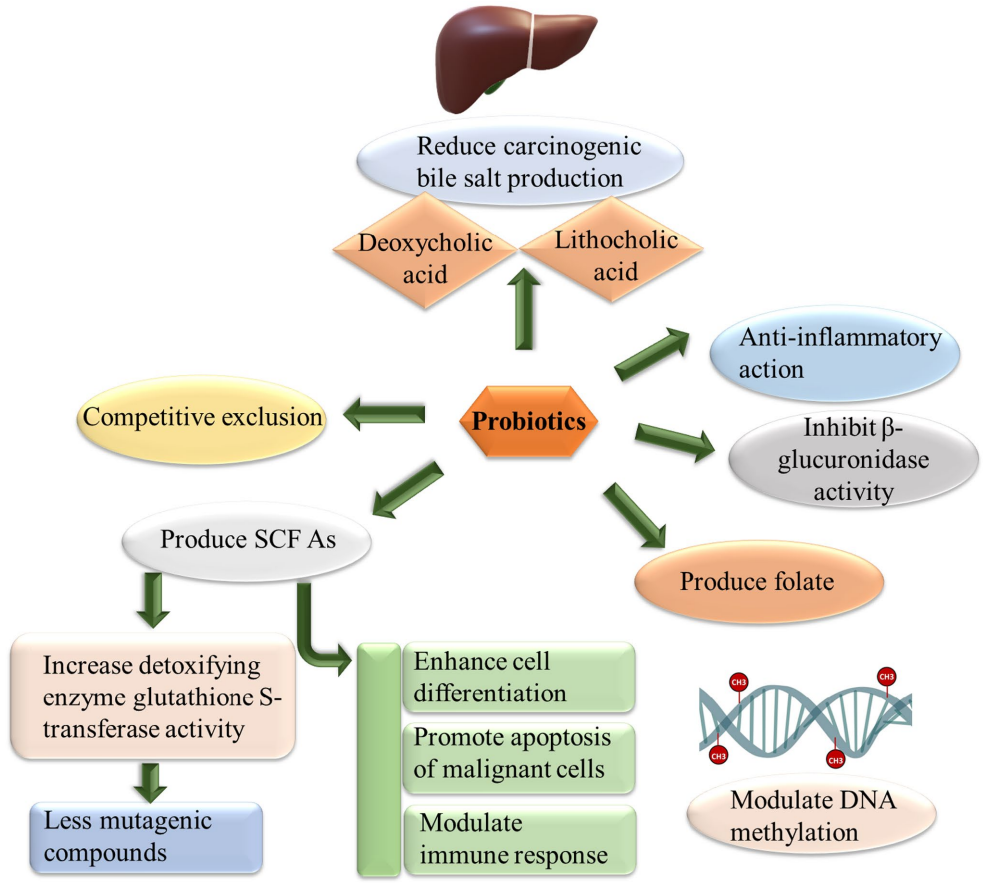

FIGURE 4
Cancer suppressor activity of probiotics. Probiotics use different pathways to fight against cancer. Probiotics inhibit β glucuronidase activity, produce folate which ultimately modulate DNA methylation patterns protecting the integrity of genome, produce short chain fatty acids (SCFA) enhancing cell differentiation and apoptosis of cancerous cells, exclude pathogens involved in chronic inflammation which may lead to cancer development.

of probiotics-based products is estimated to reach 75 billion dollars by 2025. This exponential growth in sales of probiotic products has already gained much interest from food producers to develop new products with probiotics. Probiotics are commonly used in dairy, beverage, baking, and edible film industries (Reque and Brandelli, 2021).

4.1. Probiotics in the dairy industry

Food producers have been showing great interest in developing new probiotics products due to their large acceptability among consumers. Dairy-based products are prepared as natural products to promote health and prevent diseases (Nami et al., 2019). Lactic acid bacteria (LAB) in dairy products help increase the shelf life of fermented products. LAB act as antimicrobial agents against many pathogens living inside the human body, thus improving human health (de Souza da Motta et al., 2022). Table 2 refers to the application of probiotics in the dairy industry. Considering the demand for functional dairy products in markets, it has been estimated and forecasted that the industry will jump up to a market value of 64.3 billion USD globally by the end of 2023, apart from traditional dairy products (Iqbal et al., 2017; FAO, 2022).

Many products, such as pasteurized milk, infant formula, fermented milk, and ice creams are being produced and consumed worldwide as probiotic-based dairy products. Some products like cheese and fermented milk are preferred as probiotics carriers because their pH buffering capacity and fat contents give additional protection to probiotics while passing through the GI tract (Meybodi and Mortazavian, 2017). Yogurt, including reduced lactose or lactose-free, functional ingredient-supplemented yogurts such as vitamins, minerals, sterols, stanols, conjugated linoleic acids, prebiotics, and probiotics have also gained good market success for quite a long period (Fernandez and Marette, 2017).

Nowadays, probiotics-based dairy products have been recommended as safe and healthy due to their beneficial effects on health, such as aiding mineral absorptions in the body, being efficient against *Helicobacter pylori* infection, and preventing diarrhea and constipation (Gao et al., 2021). Nami and his team (Nami et al., 2019) found the hypocholesterolemic effects of *L. plantarum* from homemade yogurt. They found the most substantial cholesterol-removing potential in growing cells (84%), moderate removal of cholesterol in the resting cell (41.1%), and the lowest in dead cells (32.7%). *L. plantarum* showed a positive potential for controlling serum cholesterol. At the same time, it was found that *L. plantarum* was resistant to BSH activity,

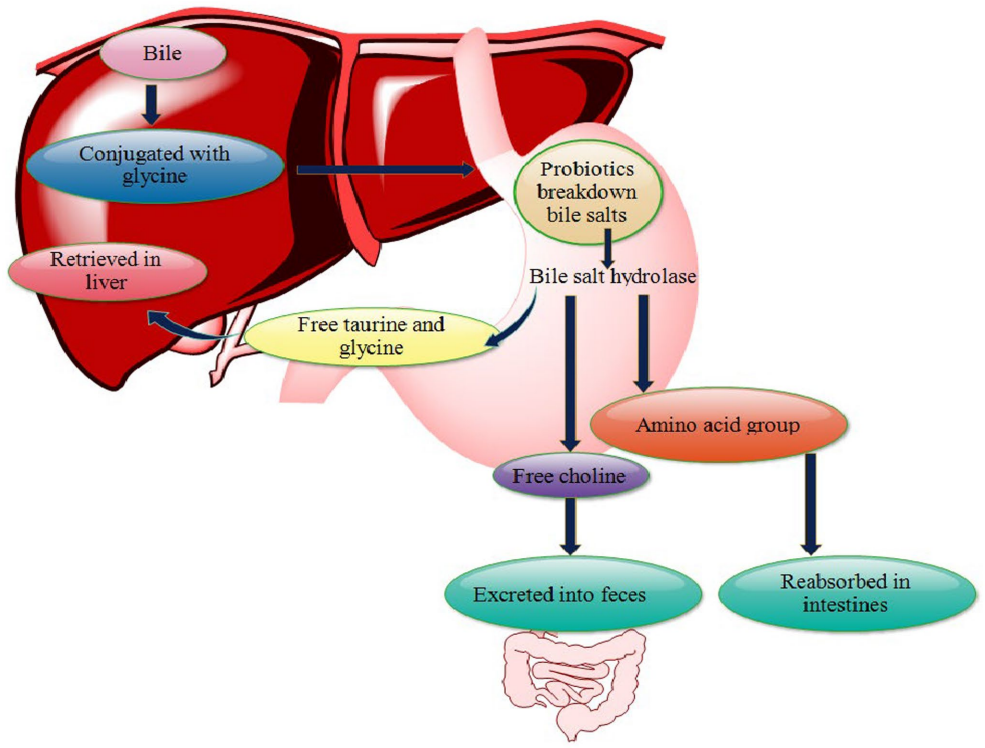

FIGURE 5
Mechanism of lowering cholesterol level by probiotics. Probiotics breakdown or deconjugate bile salts into free choline, glycine and amino group by synthesizing bile salt hydrolase. Free choline excreted via choline, amino acid group is absorbed in the intestine, and free taurine and glycine return back to the liver. This increases the elimination of bile from body and more cholesterol is used to synthesize bile thereby, reducing the cholesterol level in the blood.

antibiotics, and hemolytic activity (Nami et al., 2019). Lee et al. (2020) prepared *L. plantarum* B710 containing fermented milk, which showed bone-protective effects. Moreover, Prezzi et al. (2020) examined that the addition of *L. rhamnosus* inhibited the growth of *Listeria monocytogenes* in Minas Frescal cheese. *L. rhamnosus* showed no negative effect on the textural and physiochemical properties of cheese and survived during storage and after simulated gastrointestinal conditions.

Arbex et al. (2018) investigated six *Leuconostoc mesenteroides* strains from three different sources of dairy and non-dairy products provided each sample showing probiotic properties. One strain of *L. mesenteroids* from camel milk coded as CM9 showed high dextran production and the best resistance to intestinal stresses. CM9 had a strong antimicrobial potential against *Staphylococcus aureus* and *Escherichia coli* (Arbex et al., 2018; Azam et al., 2021). In another research, the effect of *Lactobacillus acidophilus* and *L. rhamnosus* were investigated on soft cheese. It was found that *L. acidophilus* had good overall quality with a better immune-modulation response in mice. At the same time, they also controlled pro-inflammatory cytokines and interleukin regulation and enhanced the secretion of secretory immunoglobulin A (Cuffia et al., 2019). In a study, Nguyen et al. (2019) and Riaz et al. (2019) investigated the survival of *Bifidobacterium bifidum* encapsulated in zein. The results suggested that probiotic bacteria survived well after 32 days of storage (Nguyen et al., 2019).

4.2. Probiotics in the beverage industry

The demand for non-dairy probiotic foods has been increasing steadily, especially when the consumer has become aware of the side effects associated with medicine. Consuming probiotic food is more readily acceptable to consumers as it is a more natural way of receiving their daily dose of probiotics (Reque and Brandelli, 2021). Fruit juices supplemented with probiotics have been reported as a more unique and appropriate method in the probiotic beverage industry. Fruit juices have been accepted widely among all consumers regardless of age, gender, and geographic region around the globe due to the presence of essential nutrients (Mantzourani et al., 2018a,b). The viability of probiotics is shorter in non-dairy foods when compared to dietary supplements due to the harsh environments faced by probiotics in beverages. Processors must consider many factors in the production of probiotic juices, such as pH, temperature, anthocyanins, and most importantly a vegetative form of probiotics (Min et al., 2019; Azam et al., 2022).

To overcome these complexities, microencapsulation techniques have been introduced. Using these techniques, probiotics can be employed as an essential ingredient in the functional food industry. The micro or nanoencapsulation of probiotics allows them to withstand harsh processing and storage environments due to the protective coating around them (Afzaal et al., 2022). It was reported that the acid sensitivity of *Bifidobacterium* and *Lactobacillus* was improved after their microencapsulation with gelatin or plant gums

TABLE 2 Application of probiotics in food industries.

Food industry	Product	Probiotic strain	Storage time	Viability at the end of storage	References
Dairy	Ricotta cheese	*B. animalis* subsp. *lactis* (Bb-12) *L. acidophilus* (La-05)	7 days at 7°C	~10^6 CFU/g	Meira et al. (2015)
	Yogurt	*B. Lactis*	29 days at 4°C	10^6–10^7 CFU/g	Danielle (2015)
		L. acidophilus B. animalis subsp. *lactis*	45 days at 5 ± 1°C	8.84 log CFU/g 8.01 log CFU/g	Lucatto et al. (2020)
	Cheddar cheese	*L. lactis* subsp. *lactis L. helvetics S. thermophilus L. rhamnosus*	4 weeks at 16°C	10^8 CFU/g	Ulpathakumbura et al. (2016)
	Mango juice enriched dairy drink	*L. acidophilus*	5 weeks at 4 °C	7.72 log CFU/mL	Leaf et al. (2016)
Beverages-fruit based	Pineapple juice	*L. acidophilus, L. plantarum*, and *L. lactis*	60 days at 4°C	9–10 log CFU/mL	Nguyen et al. (2019)
	Orange juice	*P. acidilactici*	35 days at 4°C and 30°C	7.2–8.5 log CFU/mL	Cristiny de Oliveira Vieira et al. (2020)
	Pomegranate	*L. plantarum* ATCC 14917	28 days at 4°C	8.8 log CFU/mL	Mantzourani et al. (2018a)
	Cornelian cherry juice	*L. plantarum*	4 weeks at 4°C	9.95 log CFU/mL	Mantzourani et al. (2018b)
Beverages-vegetable based	Carrot blended with orange juice	*L. plantarum* CECT 220	30 days at 4°C	10^8–10^9 CFU/mL	Al-Sheraji et al. (2013)
	Beet	*L. plantarum*	21 days at 4°C	7–8 log CFU/mL	Barbu et al. (2020)
	Melon, carrot	*L. plantarum* CICC22696 and *L. acidophilus* CICC20710	28 days at 4°C	10^8–10^9 CFU/mL	Do and Fan (2019)
Bakery	Pan bread	Sodium alginate and 2% whey protein concentrate *L. rhamnosus* GG	7 days at room temperature	7.57–8.98 and 6.55–6.91 log CFU/portion	Lu et al. (2018)
	Bread	Encapsulating *L. acidophilus* and *L. casei* in calcium alginate	4 days at ambient temperature	7.2×10^8 CFU/g	Seyedain-Ardabili et al. (2016)

(Ozturk et al., 2021). Besides this, low-temperature processing is also an effective strategy to control metabolic activity and protect probiotic cell viability throughout the shelf life of juices so that an adequate and safe dose of microbes is delivered to the consumer (Tyutkov et al., 2022). Some studies regarding probiotics in the beverage industry are shown in Table 2.

Miranda et al. (2019) have investigated the direct addition of an activated and microencapsulated form of probiotics in orange juice to check their effect on physical, chemical, rheological, microbial, and sensory parameters. They found that in the inactivated state, the level of organic acids was increased, but the essential volatile compounds were decreased. On the other hand, the encapsulated probiotics showed improved consistency and rheological parameters but their sensory attributes were not up to the mark due to changes in taste. The most optimum treatment was found to be the direct addition of probiotics to juice based on good physicochemical and sensory acceptance that was more similar to the natural pure product having many essential volatile compounds (octanol, o-cymene, α-cubebene, and 1-hexanol, etc.) (Miranda et al., 2019). Secondary packaging is another important technique used to produce shelf-stable beverage products. In this technique, the probiotics are in a separate compartment from food, i.e., bottle cap or straw, and are released only into juices immediately before consumption (Fenster et al., 2019).

In another research, water kefir grains were used to ferment soy whey (a byproduct of tofu) to prepare a functional beverage. After 2 days of fermentation, the polyphenol contents and antioxidant properties increased significantly, supported by good sensory scores and overall acceptability (Fenster et al., 2019). Laali et al. (2018) used *L. plantarum* to make a beverage from coconut water after fermentation. This process not only enhanced the vitamin and mineral (potassium, calcium, and sodium) contents but also improved anti-hypertensive, antioxidant, and antimicrobial properties making it suitable for use (Laali et al., 2018). The beverage prepared from whey, germinated millet flour, and barley extract was treated with *L. acidophilus* in another study, and it was found to be effective in controlling the pathogenicity induced by *Shigella* in mice models. The beverage stimulated the immune response and enhanced the IgA level, thus controlling pathogenicity (Ganguly et al., 2019).

4.3. Probiotics in bakery

Bakery products (bread, biscuits, doughnuts, cookies, etc.) contribute to several major food components such as carbohydrates, proteins, fats, dietary fiber, vitamins, and minerals in varying amounts (Niesche and Haase, 2012; El-Sohaimy et al., 2019). Researchers have been trying to incorporate probiotics in baked products by developing new techniques to deliver thermo-durable bioactive materials so that probiotics can survive high temperatures during baking (Mirzamani et al., 2021).

The microencapsulation technique and the sourdough method have been studied as an alternative to increasing the nutritional value and cell viability of probiotics in bread during baking (Ganguly et al., 2019) and in GI conditions (Champagne et al., 2018; Ashraf et al., 2022). In a study, *L. rhamnosus* was encapsulated in sodium alginate, and higher cell viability was observed during the baking of pan bread and in simulated gastrointestinal conditions (Hauser and Matthes, 2017). Zhang et al. (2018) analyzed the encapsulation of *L. plantarum* into bread-making using different matrices (reconstituted skim milk, gum arabic, maltodextrin, and inulin). The results suggested that bacterial survival was better in gum arabic and reconstituted skim milk than in the other two heating methods (Zhang et al., 2018). Another research studied the incorporation of *L. plantarum* under different baking temperatures (175, 205, and 235°C) and its survival during storage. The bacterial cell viability was counted every 2 min during baking and a decline from 10^9 CFU/g to 10^{4-5} CFU/g was observed after baking. The storage results were remarkable as the probiotic viability was increased by 2–3 logarithmic cycles to 10^8, which was attributed to the decline in the pH of bread during storage (Zhang et al., 2018). Table 2 illustrates the use of probiotics using different strains in the baking industry.

4.4. Probiotics in edible food coatings

Bioactive food packaging is the latest approach promoting the concept of functional foods due to its extraordinary health-promoting benefits. This technique is quite helpful in overcoming the stability and GIT stresses faced by probiotics (Khodaei and Hamidi-Esfahani, 2019). Studies on the use of probiotics with some biopolymers for edible coating are illustrated in Table 3.

The encapsulation of probiotics into edible films protects them from premature degradation and increases their viability in the human body (Singh et al., 2019). The technique of edible films is being used nowadays as a tool for the effective delivery of probiotics to consumers. Still, at the same time, it also enhances the stability and safety of food by inhibiting the growth of spoilage microorganisms (Pavli et al., 2018). The prime difference between active packaging and edible coating or bioactive packaging is that active packaging is usually done to enhance the safety and quality of packaged food, while on the other hand, bioactive packaging affects the health of consumers directly generating healthier packaged foods through edible coated bioactive material which upon consumption promote health (Gagliarini et al., 2019).

Many researchers have shown keen interest in film-forming materials, for instance, biopolymers including cellulose, zein, seaweed extracts, pectins, alginates, and chitosan for entrapping probiotics to enhance the nutritional values of foods (Pop et al., 2019). Therefore, bacterial microorganisms are being incorporated into films and coatings to confer probiotics' ability to the food products or act as antimicrobial agents (Afsah-Hejri et al., 2013). As an example, the fabricated cellulose-based edible films in combination with *L. rhamnosus* using sodium carboxymethyl cellulose (CMC) and hydroxymethyl cellulose (HEC) with citric acid as a crosslinker to control the consistency of film loaded with *L. rhamnosus* (Singh et al., 2019). Moreover, cellulose-based edible films showed the therapeutic effects of probiotics (Singh et al., 2019). The film effect provides a suitable environment to encapsulate bacteria from transport to delivery in the GIT system effectively.

Four probiotic strains (*L. acidophilus*, *L. casei*, *L. rhamnosus*, and *B. bifidum*) were investigated using CMC-based edible coatings in this regard and their effects on storage under refrigerated conditions were also checked. The results suggested that *L. acidophilus* showed the highest viable count during storage with more water vapor permeability and opacity and decreased tensile strength and elongation at break values of film structure. The physical and mechanical properties of edible films remained the same (Ebrahimi et al., 2018). Another research found that after incorporating *L. plantarum* into CMC-based edible coating, the physicochemical properties and microbial characteristics of fresh strawberries were significantly improved. The probiotics population remained constant throughout the storage period, which controlled mold and yeast growth and helped to improve the shelf life of strawberries (Khodaei and Hamidi-Esfahani, 2019).

Bambace et al. (2019) incorporated *L. rhamnosus* into an alginate prebiotic fiber solution to enhance the shelf life of minimally processed and ready-to-eat blueberries by fourteen days. *L. rhamnosus* showed good antimicrobial properties with alginate and sensory acceptability for coated food (Bambace et al., 2019). In another work, kefiran polysaccharides-based films were used to deliver probiotics (*L. paracasei* and *Kluyveromyces marxianus*) to the gut. These films exhibited good antimicrobial properties and protected the probiotics from GIT stresses. *L. paracasei* showed better mechanical properties and good viable count than *K. marxianus* (Gagliarini et al., 2019).

TABLE 3 Use of probiotics in edible film.

Application matrix	Probiotic	Biopolymer material	Viability	References
Baked cereal products	*L. rhamnosus* GG	Sodium alginate	7.57–8.98 log CFU/portion	Lu et al. (2018)
	L. acidophilus L. rhamnosus	Carboxymethylcellulose (CMC)	10^7 CFU/g	Ebrahimi et al. (2018)
Hake fillets	*B. animalis* spp. *lactis*, *L. paracasei* spp. *paracasei*	Agar	–	De Lacey et al. (2014)
	L. rhamnosus GG	Sodium alginate/Pectin/κ-Carrageenan-Locust bean gum/Gelatine/Whey protein concentrate	0.87–3.06 log CFU/g	Soukoulis et al. (2017)
	L. reuteri ATCC 55730 *L. rhamnosus* GG ATCC 53103 *L. acidophilus* DSM 20079	Pullulan and starches (from potato, tapioca, and corn)	12.9 log CFU/mL	Kanmani and Lim (2013)

5. Delivery systems and the strategies to extend viability

The association between probiotics and human health has been well-known for an extended period. When consumed orally, probiotics can regulate the composition of intestinal microbiota (Sharma et al., 2023). However, the severe physicochemical stresses (high temperatures and acidity during processing, storage, and passage to the large intestine) can drastically reduce the viability of probiotics. Researchers have used different encapsulating techniques to overcome these stresses and enhance the viability of probiotics within the human body (Luo et al., 2022). The traditional and most widely used technique is microencapsulation. Microencapsulation is classified into four methods, namely; spray drying, freeze drying, emulsification, and extrusion. One can improve the ability of probiotics to withstand the harsh environment of processing and the human body. Still, these methods have certain limitations, such as extreme temperatures and acidity can ultimately affect the size, stability, and ultimately viability of microstructures of microcapsules (Razavi et al., 2021).

These hindrances paved the way to find new encapsulation strategies to enhance the durability and viability of probiotics. In recent years, the nanoencapsulation technique has been used widely to enhance probiotics-loaded nanoparticles' ability to face severe processing and *in-vivo* stresses. These techniques also facilitate the targeted delivery and control release of probiotics in the intestine (Xu et al., 2022). The unique biological and physicochemical characteristics of nanocapsules, such as smaller particle sizes, higher surface areas, and increased reactivities, improve the efficiency of encapsulated probiotics, thus, providing a logical solution to human health and safety (Singh et al., 2022). The ability of nanoencapsulation to entrap probiotics is analyzed by the potential of electrospun nanofibers, hydrogels, nanocoating, nanoliposomes, and other nanomaterials (Garcia-Brand et al., 2022).

Mojaveri and his colleagues, in their recent work, attempted to improve the viability of *Bifidobacterium animalis* Bb12 by using a nanofiber technique made from chitosan and poly (vinyl alcohol) and inulin as prebiotics. The simulated results of the GI tract showed that the encapsulation of probiotics in electrospun nanofibers significantly enhanced the physicochemical behavior with increased stability of nanoparticles within the human body (Mojaveri et al., 2020). In another study, Li et al. (2019) studied the cellulose-based gels for control release of encapsulated *L. plantarum* with better storage and concluded that cellulose-based gels provide better storage stability and much-enhanced control release pattern in simulated intestinal fluids (Li et al., 2019).

Encapsulation of probiotics with the help of biomaterial-based nanocoating can also protect these beneficial microbes from antibiotics and GI conditions, facilitating the retention of probiotics within the GI tract. It was found that metal-phenolic network-based nano-coating made from iron (III) and tannic acid can help protect probiotic microbes from the detrimental effect of antibiotics (Ashraf et al., 2023; Guo and Wu, 2023). Due to their physicochemical parameters, smaller structures, and thermodynamic properties, nanoliposomes enjoy vast applications for a wide range of products. The stability of *L. rhamnosus* was analyzed by loading them into chitosan-gelatin coated nanoliposomes. The characterization study suggested the successful coating of bifidobacteria with coated nanoliposomes. Further supported by the results of simulated GI fluids with a significant amount of viable cells present in the fluid guiding toward the suitability of nanoliposomes as a potential carrier of probiotics in developing nutraceutical foods (Hosseini et al., 2022).

6. Conclusion

Probiotics have well-documented physiological effects with a definitive mechanism. However, the exact mechanism of how they work to enhance health and prevent different diseases must be explored. Evidence from well-documented clinical trials has revealed that probiotics can potentially alleviate different GI and other disorders. Despite our understanding of some molecular mechanisms underlying beneficial aspects of probiotics, we are still far from clinically proven efficacy in many autoimmune and inflammatory diseases. Moreover, many studies have been done on the animal model, so there is an emergent need to translate these results into humans. Currently, genetically modified commensal lactic acid bacteria are being used to deliver special health-interest compounds. But most of the work regarding recombinant bacteria is related to vaccines. However, genetically modified bacteria can be used for exploring innovative strategies to deliver bioactive molecules to mucosal tissues. More consistent and reproducible clinical trials are required to reveal probiotics efficacy, limitations, and safety, determining their effects on the immune system. Considering all the methodologies discussed in this review, probiotics can be applied easily by food producers to make novel functional foods to promote human health.

Author contributions

All authors wrote the manuscript, read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effect of fat contents of buttermilk on fatty acid composition, lipolysis, vitamins and sensory properties of cheddar-type cheese

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Cheddar-type cheese produced from buttermilk had softer texture than standard cheddar cheese due to lower fat content of buttermilk. Fat is extremely important for the functional characteristics and optimum textural attributes of cheese. The effect of different fat contents of buttermilk on chemical characteristics of cheddar-type cheese is not previously investigated. This investigation was conducted to know the effect of different fat contents of buttermilk on fatty acids composition, organic acids, vitamins, lipolysis and sensory characteristics of cheddar-type cheese. Cheddar-type cheese was produced from buttermilk having 1, 1.75, 2.50 and 3.25% fat contents (control, T_1, T_2 and T_3). Fat content of control, T_1, T_2 and T_3 were 9.81, 16.34, 25.17 and 31.19%. Fatty acids profile was determined on GC–MS, organic acids and vitamin A and E were determined on HPLC. Free fatty acids, peroxide value and cholesterol were determined. Cheddar-style cheese produced from buttermilk (1% fat) showed that it had softer texture and lacking typical cheese flavor. Gas chromatography–mass spectrometry (GC–MS) analysis showed that long-chain unsaturated fatty acids in control, T_1, T_2 and T_3 samples were 45.88, 45.78, 45.90 and 46.19 mg/100 g. High Performance Liquid Chromatography (HPLC) analysis showed that lactic acid, propionic acid, citric acid and acetic acid gradually and steadily increased during the storage interval of 90 days. At the age of 90 days, lactic acid in control, T_1, T_2 and T_3 was 4,789, 5,487, 6,571 and 8,049 ppm, respectively. At the end of ripening duration of 90 days, free fatty acids in control, T_1, T_2 and T_3 were 0.29, 0.31, 0.35 and 0.42% with no difference in peroxide value. Stability of vitamin A after 90 days storage control, T_1, T_2 and T_3 was 87.0, 80.0, 94.0 and 91.0%. Flavor score of cheddar-type cheese produced from butter milk having 1.0, 2.5 and 3.25% fat content was 81, 89 and 91% of total score (9). Hence, it is concluded that cheddar-type cheese can be produced from buttermilk having 2.5 and 3.25% fat contents with acceptable

sensory attributes. Application of buttermilk for the production of other cheese varieties should be studied.

KEYWORDS

cheddar, cheese, butter, organic acid, food development, lactic acid bacteria

1. Introduction

On the basis of acidity, two different types of buttermilk are produced, sweet buttermilk is produced during the churning of unfermented cream, cultured buttermilk is produced by fermentation of cream with mesophilic lactic acid producing bacteria (Walstra et al., 2005). Chemical composition of sweet butter is comprised of non-fat constituents of churned cream, i.e., casein, whey proteins, lactose and minerals, dry matter content of buttermilk varies from 8 to 12%. Fat globules in cream have an outer membrane. Milk fat globule membrane that covers the lipid core it provides stability and structural integrity to milk fat; this membrane is disrupted during churning then migrate to buttermilk. It helps fat globules in milk to stay in suspension because of its components that acts as emulsifier (phospholipids and glycoproteins). Magnitude of phospholipids in buttermilk ranges from 80 to 125 mg/g which is considerably higher than whole milk that contains 2.7–4.8 mg/g polar lipids (Sodini et al., 2006). Emulsifying, water holding and lower foaming capacity in buttermilk are due to phospholipids (Goudédranche et al., 2000). Biological properties of buttermilk such as antibacterial, cholesterol lowering, hypotensive, antioxidant potential, positive impact on nerve tissues, skin and hair are mainly due sphingomyelin, sialic acid and gangliosides (Walstra et al., 2005). High concentration of phospholipids in buttermilk are of great interest because that component possesses certain biological activities which have beneficial impact on human health. Studies indicate that phospholipids have potential to protect human body against bacterial toxins, infections and colon cancer. Along with this, it had emulsifying properties that is why this component had a significant importance as a functional ingredient that improves certain characteristics in food (Kifah et al., 2014).

Buttermilk is rich in lactic acid bacteria which boost immune system helps body to get rid of pathogens. Its probiotic effect lowers the risk of candida in diabetic women (Wang, 2019). A general analysis of buttermilk composition and chemical nature indicates that it has 0.18% acidity, 3.4% protein, 0.8% fat and 4% lactose reported by Shrestha et al. (2015).

One biggest challenge is to find ways for efficient utilization and applications of dairy by-products. One of the most significant by-products produced by the dairy industries is butter milk. During the churning process, considerable content of proteins and phospholipids are released from the membrane of fat globules to buttermilk that is perceived to have therapeutic effects (Sharma et al., 2021). In 2017, approximately 29% of the total milk produced in European Union was used for the production of butter, yield of butter and buttermilk was almost 50% (Eurostat, 2017). Due to its nutritional and technological properties, it is of great interest for the food industries to find/extend the applications of buttermilk for the development of functional foods. The possible application of buttermilk in fermented beverages and fruit drinks (Liutkevičius et al., 2016). However, cheese is supposed to be the biggest application area of buttermilk (Skryplonek et al., 2019).

Fat in cheese is extremely important for the functional characteristics and development of optimum textural attributes. Skryplonek et al. (2019) produced Quark cheese (un-ripened) from sweet and cultured buttermilks, composition, water activity, texture and sensory properties were studied however, fatty acids composition, lipolysis and sensorial prospects of ripened butter milk cheese is not reported in literature. Texture of cheddar-type cheese produced from buttermilk was soft with lack of flowability. Fat content in buttermilk-based cheddar-type cheese was about 10% (Hickey, 2017). Fatty acid composition of fat in buttermilk is considerably different from milk, former has a greater number of polyunsaturated fatty acids than the latter. Therefore, in this investigation, fat extracted from buttermilk was used as source of fat enrichment. Functional and textural properties of buttermilk-based cheddar type cheese can be improved by increasing fat content. Ripening of cheese has a great deal of impact on nutritional profile, texture and sensory properties of cheese. Low fat versions of cheese produced from buttermilk may be a good addition in cheese industry. Therefore, this investigation was conducted to know the effect of different fat contents of buttermilk on fatty acids composition, organic acids, vitamins, lipolysis and sensory characteristics of cheddar-type cheese.

2. Materials and methods

2.1. Starter culture

Cheddar cheese starter culture (CH-4219-CN) *Lactococcus lactis* ssp. lactis, *Lactococcus lactis* ssp. cremoris, Starter culture for butter milk (XT-313) *Lactococcus lactis* ssp. lactis biovar diacetylactis and *Leuconostoc mesenteroides* ssp. cremoris and rennet were obtained from Christian Hansen (Hoersholm, Denmark).

2.2. Buttermilk cheese production

Sweet buttermilk (having 1% fat content) was heated to 50°C in a water bath (Memmert water bath/Memmert GmbH & Co, KG) for the separation of cream from butter milk by cream separator (Packo, Belgium) separated cream was used to adjusted the fat content of butter milk to 1.75, 2.5 and 3.25% (T_1, T_2, T_3 respectively). Buttermilk having 1% fat content was used as control (T_0), pre-acidified for 45 min using 2% bulk starter culture of *Lactococcus lactis* spp. *lactis* and *Lactococcus lactis* spp. *cremoris*. Calcium chloride 2 mL (35%), rennet 0.02% were added and curd was cut to 1.5–2 cm cubes followed by

cooking at 39°C for 45 min, cheddaring (pH 5.2), milling by cheddar cheese mill salting (1.5%), pressing by hydraulic press at 3.5 bar pressure for 16 h, vacuum packaging (by using a vacuum packaging machine/Tabletop vacuum – Sealing bar 265 mm) and ripening at 6–8°C for 90 days. Analysis was performed in triplicate on days 0, 45 and 90.

2.3. Composition of buttermilk and cheese

Compositional analysis such as fat, protein, lactose, ash, solids-not-fat, total solids content and pH of buttermilk and moisture, fat, protein and pH of cheddar-type cheese were performed as per standard procedures of AOAC (2005).

2.4. Fatty acid profile

First, extracted fat was mixed with methanolic hydrogen chloride (15 mL HCl and 85 mL methanol) in a screw capped test tube and was heated at 100°C for 60 min till fat was fully dissolved. After that, test tube was cooled down to room temperature. Further, 2 mL distilled water and 2 mL n hexane was added in the tubes followed by vortexing (Labnet's Vortex Mixer VX-200) at 1,500 rpm for 2 min. Then the tube was placed down for 5 min or until two layers were separated. Then supernatant was poured in a tube containing 1 g anhydrous sodium sulphate, that were transferred to GC vials and injected to GC–MS, Agilent Technologies (7890-B), USA using a SP-2560 capillary column (75 m x 0.1 mm, DF 0.14 μm). With the split ratio of 50% injectors and 50% detectors were set at 250°C, using FAME-37 (Restek Corporation, USA) standards, flow rate of helium, hydrogen and oxygen were set at 2, 4 and 40 mL/min (Qian, 2003).

2.5. Organic acids by HPLC

Estimation of organic acids was performed on HPLC, 10 g sample with 40 mL dihydrogen phosphate (H_2PO_4) was taken in a tube and vortexed at 200 rpm for 1.5 min. Cheese samples were then incubated at 65°C and centrifugation (Beckman-S241.5) was performed at 2958 g for 5 min. By using Whatman filter paper the upper layer and lactic acid, propionic acid, citric acid and acetic acid were analyzed on a ShodexPSpak KC-118 (8 mm × 300 mm i.d.) ion exchange organic column attached with UV detector 214 nm column.

2.6. Lipolysis

As an indicator of lipolysis, free fatty acids (FFA), cholesterol and peroxide values (POV) were determined of all samples and control at 0, 45 and 90 days (American Oil Chemists' Society, 2011). For measurement of FFA, sample (50 g) was mixed with absolute and neutralized ethanol with 0.1 N NaOH. Samples were then titrated with NaOH and FFA were calculated in terms of oleic acid. For POV, three parts of glacial acetic were blended with two parts of chloroform, 30 mL of this solution was mixed with samples, 1 g potassium iodide was added and flasks were incubated in dark for exactly 5 min and then titrated with standard 0.01 N sodium thiosulfate solution using starch (1% indicator) and POV was reported as ($mEqO_2$/kg) and calculated with the help of following formula.

$$POV = (EQ1 - B) * T * M * F1/W * F2$$

Where as:

B: Blank value; EQ: Consumption of titrant at first Equivalence point; T: Actual concentration of the titrant; M: Molecular weight; W: sample weight in g; F1: 1000 Conversion factor; F2: 1 Conversion factor.

2.7. Vitamin A determination by HPLC

A 20 mL buttermilk sample was taken and mixed with 5 mL of ammonia (25%) and 20 mL ethanol (96%). Supernatant was extracted and added with 0.0025% BHT and sol-vent was evaporated with the help of a rotary evaporator at 35°C. With the help of 30 mL potassium hydroxide (5% in ethanol) saponification was performed and extracted with n-hexane. After evaporation of solvent on rotary evaporator, 20 μL sample was injected into HPLC, Waters Corp., Milford, MA, USA/ (Shimadzu) which was equipped with Spherisorb RP-18 column, Supelcogel C-610H, 300 × 7.8 mm (Supelco Inc., Bellefonte, PA, USA) and water 990 detector. In various concentrations, retinyl palmitate was used as standard. Mobile phase was consisted of acetonitrile-methanol 85:15 (v/v) in isocratic system (Khan et al., 2017).

2.8. Vitamin E determination by HPLC

For vitamin E determination, fat was extracted by standard method. Then 2 mL *n*-hexane (HPLC grade) was added to 200 μg of fat. At 1,500 rpm the content was vortexed for 25 s and then injected (10 μL) into HPLC (HPLC LCM, Waters Corp., Milford, MA, USA/ (Shimadzu) which was equipped with Spherisorb RP-18 column, Supelcogel C-610H, 300 mm × 7.8 mm, Supelco Inc., Bellefonte, PA, USA). Mobile phase was comprised of 0.5% acetic acid and 0.5% ethyl acetate in n-hexane with a flow rate was adjusted at 1.5 mL/min. Vitamin E were expressed as μg/g (Khan et al., 2017).

2.9. Sensory evaluations

All the samples were tested for color, flavor and texture in individual sensory evaluation booths on 0, 45, and 90 days of storage. Ten trained judges performed the sensory evaluation using 9-point scale (Meilgaard et al., 2007).

2.10. Statistical analysis

The experiments were planned in a completely randomized design and data were analyzed using two-way analysis of variance. Duncan multiple range (DMR) tests were used to express significance difference different versions of cheddar-type cheese. DMR Test were used with the aid of SAS 9.4 statistical software (SAS Institute Inc. Cary, North Carolina, U.S.A).

3. Results and discussion

3.1. Cheese composition

Fat, protein, lactose, mineral, lactose and total solids contents in control milk were 1.00, 3.20, 2.95, 0.62 and 7.92%, respectively (Table 1). Difference in fat content of substrate buttermilk had a pronounced effect on fat and moisture content of cheddar-type cheese. It was recorded that conversion ratio of control and all samples were almost 10 times which is normal in cow milk cheddar cheese. From this conversion ratio, it is also perceived that this research work may be highly useful for the industries to develop functional buttermilk-based cheddar-type cheese. Further, cheddar-type cheese was produced from the same equipment which is used for the production of standard cheddar cheese and also standard cheese production procedure without any alteration was used. This will be helpful the industries to save time in the development and adoption of this cheese. Moisture content of control, T_1, T_2 and T_3 were 62.52, 54.29, 45.67 and 40.35% (Table 1). Due to the presence of inverse relationship between moisture and fat, significant decline in moisture content of T_1, T_2 and T_3 can be justified (Collins et al., 2003). Fat content of control, T_1, T_2 and T_3 were 9.81, 16.34, 25.17 and 31.19%. Fat addition did not affect protein content and pH in control and experimental cheese samples. Fat level depends upon milk type, cheese manufacturing process and formulation. Fat level in cheese influences biochemistry, microstructure, yield and rheological properties of cheese (Murtaza et al., 2008). Cheddar-style cheese was produced either by blending buttermilk or buttermilk powder with cow milk, protein and fat content in cheese were 10 and 27%, respectively (Hickey et al., 2018). Ullah et al. (2018) reported that fat and protein content in standard cheddar cheese were 32 and 26%.

3.2. Fatty acids composition of cheddar type cheese

Among all dietary lipids, fatty acid composition of milk fat is highly complicated. Short-chain (C4:0-C10:0; SCFA), medium-chain (C12:0-C16:0; MCFA) and long chain (C18:0 and above LCFA) are present in milk fat. On an average, ratio of saturated and unsaturated fatty acids in milk is 70:30. Typical aroma, functional properties and nutritional characteristics of milk fat is due to mixture of fatty acids (Nadeem et al., 2014). Palmitic acid (C16:0) and stearic acid (C18:0) are the dominant fatty acids of milk. Processing technologies have a pronounced effect on fatty acid composition of fatty acid composition of milk. Fatty acid profile of butter and buttermilk were considerably different from each other, concentrations of oleic acid, linoleic acid and linolenic acid were significantly higher in buttermilk than butter and parent milk (Sakkas et al., 2022). Traditionally, cheddar cheese is ripened for several months to achieve optimum flavor and textural attributes. During this phase, lipolysis in milk fat takes place which leads to the production of free fatty acid (FFA), mono & di-glycerides, organic acids and flavoring compounds. In terms of auto-oxidative stability, cheese is relatively more resistant to lipid oxidation as compared to other dairy products and super oxidative stability of cheese is attributed to lower oxidation–reduction potential (McSweeney and Sousa, 2000). Ripening effect on fatty acid composition of standard cheese has been extensively studied however, transition in fatty acid composition of cheddar-type cheese produced exclusively from buttermilk is not reported in literature. In current investigation, GC–MS analysis showed that raising fat content of cheese buttermilk from 1% to 1.75, 2.5 and 3.25% did not affect fatty acids composition of cheddar type cheeses produced from buttermilk. This non-significant impact on fatty acid composition of control and experimental cheese samples was due to non-variation in source of fat, cream was extracted from buttermilk and fat contents of all the treatments were adjusted using same source of buttermilk cream. Determination of fatty acids composition in 90 days old cheese samples showed that ripening phase of 90 days had a significant influence on fatty acid composition of control, T_1, T_2 and T_3. Cheddar-style cheese was produced either by blending milk with buttermilk or buttermilk powder, cheese samples were ripened for 180 days, it was found that fatty acids composition of 180 days old cheddar cheese was remarkably different from fresh cheese (Hickey, 2017). Most of lipolysis during the ripening phase of cheddar cheese is induced by starter bacteria. Khan et al. (2022) studied the impact of ripening on fatty acid composition of cheddar cheese, it was reported that ripening induced major changes in fatty acid composition of cheddar cheese. Batool et al. (2018a,b) monitored the effect of 42 days ripening period on fatty acid composition of cheddar cheese, fresh and 42 days old cheeses had different fatty acid composition (Ullah et al., 2018). Monitored ripening effect on fatty acid composition of cheddar cheese, freshly prepared curd and 3 months old cheddar cheese had considerably different fatty acid profile. In this investigation, two isomers of conjugated linoleic acids were also found, concentrations of C18:2c9t13 and C18:2c9c12 in control were 0.91 and 2.11 mg/100 g. Intake of 2 g conjugated linoleic acid on daily basis can prevent obesity, cancer, diabetes and cardiovascular diseases (Khan et al., 2021; Table 2).

TABLE 1 Composition of cheddar-type cheese produced from buttermilk.

Treatment	Moisture (%)	Fat (%)	Protein (%)	pH
Control	62.52 ± 0.43^{a}	9.81 ± 0.09^{d}	24.11 ± 0.05^{a}	5.21 ± 0.02^{a}
T_1	54.29 ± 0.37^{b}	16.34 ± 0.15^{c}	24.32 ± 0.08^{a}	5.29 ± 0.05^{a}
T_2	45.67 ± 0.24^{c}	25.17 ± 0.24^{b}	24.61 ± 0.13^{a}	5.24 ± 0.07^{a}
T_3	40.35 ± 0.64^{d}	31.19 ± 0.13^{a}	24.79 ± 0.17^{a}	5.20 ± 0.10^{a}

Control: Butter milk with 1% fat content. T_1: Butter milk with 1.75% fat content. T_2: Butter milk with 2.50% fat content. T_3: Butter milk with 3.25% fat content. In a column, different letters on means indicate statistically significant difference ($p < 0.05$).

3.3. Organic acids

In several ripened cheese varieties, organic acids are an important flavoring compounds, which are produced due to the breakdown milk fat, addition of acids, usual ruminant metabolic courses or the by the growth of bacteria. Organic acids are also produced by the catabolism of carbohydrates by the activities of lactic acid bacteria (Ullah et al., 2018). Organic acids can exert a great deal of control on undesirable bacteria by dropping pH but inhibitory effect largely depends upon the kinds of organic acids produced. Extent of lipolysis varies among different varieties of cheese, in cheddar, Gouda and Emmentaler cheese, medium degree of lipolysis is required however, in certain hard Italian cheese varieties, and extensive lipolysis is required for the

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TABLE 2 Fatty acids composition of cheddar-type cheese produced from buttermilk (mg/100 g).

Fatty Acid	Control		T_1		T_2		T_3	
	0-Day	90-Days	0-Day	90-Days	0-Day	90-Days	0-Day	90-Days
C4:0	1.81 ± 0.01^a	1.62 ± 0.05^b	1.80 ± 0.03^a	1.61 ± 0.08^b	1.79 ± 0.04^a	1.59 ± 0.07^b	1.78 ± 0.02^a	1.48 ± 0.10^c
C6:0	2.15 ± 0.02^a	1.94 ± 0.03^b	2.17 ± 0.06^a	1.91 ± 0.05^b	2.15 ± 0.07^a	1.90 ± 0.02^b	2.14 ± 0.05^a	2.05 ± 0.03^b
C8:0	2.49 ± 0.05^a	2.27 ± 0.01^b	2.51 ± 0.01^a	2.25 ± 0.07^b	2.50 ± 0.01^a	2.23 ± 0.09^b	2.48 ± 0.08^a	2.21 ± 0.05^b
C10:0	3.25 ± 0.10^a	2.88 ± 0.19^b	3.26 ± 0.04^a	2.86 ± 0.09^b	3.25 ± 0.09^a	2.85 ± 0.04^b	3.24 ± 0.16^a	2.84 ± 0.02^b
C12:0	2.77 ± 0.11^a	2.53 ± 0.13^b	2.75 ± 0.07^a	2.49 ± 0.13^b	2.73 ± 0.03^a	2.46 ± 0.17^b	2.71 ± 0.04^a	2.68 ± 0.12^b
C14:0	10.84 ± 0.19^a	9.24 ± 0.26^b	10.88 ± 0.32^a	9.14 ± 0.18^b	10.79 ± 0.27^a	10.99 ± 0.13^b	10.85 ± 0.24^a	10.74 ± 0.34^b
C16:0	17.69 ± 0.24^a	16.21 ± 0.39^b	17.95 ± 0.46^a	16.10 ± 0.34^b	17.82 ± 0.28^a	17.93 ± 0.34^b	17.88 ± 0.41^a	17.16 ± 0.21^b
C18:0	8.77 ± 0.13^a	7.44 ± 0.17^b	8.82 ± 0.21^a	7.35 ± 0.38^b	8.77 ± 0.42^a	8.83 ± 0.19^b	8.91 ± 0.35^a	7.82 ± 0.07^b
C18:1	36.43 ± 0.17^a	34.59 ± 0.48^b	36.55 ± 0.54^a	34.49 ± 0.66^b	36.42 ± 0.77^a	36.79 ± 0.90^b	36.67 ± 0.83^a	35.87 ± 0.25^b
C18:2	7.66 ± 0.09^a	6.89 ± 0.08^b	7.59 ± 0.12^a	6.81 ± 0.02^b	7.78 ± 0.15^a	7.91 ± 0.56^b	7.74 ± 0.27^a	7.13 ± 0.19^b
C18:3	1.79 ± 0.06^a	1.63 ± 0.02^b	1.74 ± 0.05^a	1.61 ± 0.15^b	1.70 ± 0.04^a	1.69 ± 0.47^b	1.78 ± 0.04^a	1.44 ± 0.14^b
C18:2*c9t13*	0.91 ± 0.04^a	0.75 ± 0.01^b	0.89 ± 0.03^a	0.80 ± 0.03^b	0.88 ± 0.02^a	0.82 ± 0.06^b	0.91 ± 0.03^a	0.81 ± 0.02^b
C18:2*c9c12*	2.11 ± 0.03^a	1.94 ± 0.03^b	2.15 ± 0.01^a	1.91 ± 0.02^b	2.10 ± 0.01^a	2.05 ± 0.11^b	2.13 ± 0.02^a	2.06 ± 0.01^b

Control: Butter milk with 1% fat content. T_1: Butter milk with 1.75% fat content. T_2: Butter milk with 2.50% fat content. T_3: Butter milk with 3.25% fat content. In a row, different letters on means indicate statistically significant difference ($p < 0.05$).

TABLE 3 Organic acids of cheddar-type cheese produced from buttermilk (ppm).

Cheese type	Days	Lactic acid	Propionic acid	Citric acid	Acetic acid
Control	45	$4,571 \pm 1.24^h$	217 ± 0.03^g	108 ± 0.05^h	$18,743 \pm 2.56^h$
	90	$4,789 \pm 2.57^g$	342 ± 0.09^f	243 ± 0.03^g	$20,465 \pm 3.17^g$
T_1	45	$4,829 \pm 3.49^f$	379 ± 0.81^e	243 ± 0.15^f	$20,443 \pm 5.77^f$
	90	$5,487 \pm 1.98^e$	614 ± 0.21^d	379 ± 0.13^e	$24,519 \pm 0.98^e$
T_2	45	$5,689 \pm 2.19^d$	527 ± 0.36^c	464 ± 0.24^d	$26,213 \pm 0.73^d$
	90	$6,571 \pm 1.77^c$	884 ± 0.28^c	713 ± 0.17^c	$33,101 \pm 1.35^c$
T_3	45	$7,019 \pm 1.35^b$	$1,034 \pm 0.15^b$	984 ± 0.29^b	$34,641 \pm 1.61^b$
	90	$8,049 \pm 0.65^a$	$1,437 \pm 0.44^a$	$1,481 \pm 0.35^a$	$47,993 \pm 1.43^a$

Control: Butter milk with 1% fat content. T_1: Butter milk with 1.75% fat content. T_2: Butter milk with 2.50% fat content. T_3: Butter milk with 3.25% fat content. In a column, different letters on means indicate statistically significant difference ($p < 0.05$).

development of desired flavor (Murtaza et al., 2014). Fat segment of cheese is extremely important for the production of typical flavor and texture cheese. Standard cheddar cheese contains more than 30% fat, in addition to other factors, degree of lipolysis is also largely determined by the fat content of cheese. Low fat versions of cheddar cheese undergo lower degree of lipolysis than standard cheddar cheese (McSweeney and Sousa, 2000). In current investigation, impact of different fat levels, i.e., 1, 1.75, 2.5 and 3.25% in cheese buttermilk and ripening period of 90 days was investigated on production of organic acids. Results showed that fat contents had a great deal of impact on generation of organic acids, highest organic acids production was recorded in T_3 followed by T_2, T_1 and control ($p < 0.05$). At the age of 45 days, lactic acid in control, T_1, T_2 and T_3 was 4,571, 4,829, 5,689 and 7,019 ppm (Table 3). At the age of 45 days, citric acid in control, T_1, T_2 and T_3 was 18,743, 20,443, 26,213 and 36,641 ppm. Concentrations of lactic acid, propionic acid, citric acid and acetic gradually and steadily increased during the storage interval of 90 days. At the age of 90 days, lactic acid in control, T_1, T_2 and T_3 was 4,789. 5,487. 6,571 and 8,049 ppm. At the age of 90 days, citric acid in control, T_1, T_2 and T_3 was 20,465, 24,519, 33,101 and 47,993 ppm. Conventionally, cheddar cheese is produced from cow milk with a fat content of 3.2 to 3.5%, for the production of cheddar cheese from buffalo milk (4% fat content) was used, impact of higher fat content in cheese milk was studied on production of organic acids. Concentrations of organic acids were higher when high fat cheese milk was used to produce cheddar cheese as compared to standard cheese (Murtaza et al., 2012; Ikram et al., 2021).

3.4. Lipolysis

Due to action of lipases, enzymatic hydrolysis takes place during the ripening of cheese that improve flavor of cheese by producing FFA which are further catabolized to flavoring compounds (McSweeney and Sousa, 2000). Proportions of fatty acids from C6:0 to C18:3 is almost similar to milk fat which shows that hydrolysis of triglycerides and production of FFA is non-specific. Degree of lipolysis varies from cheese to cheese, in several varieties of cheese, lower level of lipolysis of desirable for optimum flavor and too much lipolysis is undesirable. In cheddar, Gouda and Swiss cheeses, moderate levels of free acids are

required. However, extensive lipolysis occurs during the ripening of certain hard Italian, Blue and Feta cheese. Lipolysis is affected by moisture, temperature, ripening duration, fatty acid composition, fat content and oxygen, methyl ketones, thioesters and lactones are produced due to the catabolism of FFA. Oxidation of fatty acid result in the production of ketoacids which are carboxylated to methyl ketones mainly from capric acid to lauric acid (Alewijn et al., 2005). Effect of different contents on lipolysis of cheddar type-cheese is documented in Table 4. Transition in values of FFA, POV and cholesterol were used to estimate the degree of lipolysis. Before ripening, FFA of control, T_1, T_2 and T_3 were similar to each other ($p > 0.05$). FFA of buttermilk cream were 0.08% and same source of cream was used in T_1, T_2 and T_3 levels. Estimation of FFA in 45 days old cheddar-type cheese samples revealed a significant impact on ripening on production of ripening. Treatments having higher fat content yielded more FFA and were in the order of $T_3 > T_2 > T_1 >$ control (Table 4). At the end of ripening duration of 90 days, FFA in control, T_1, T_2 and T_3 were 0.29, 0.31, 0.35 and 0.42%. Butter oil was added to cheese milk and converted to cheese, cheese having buttermilk produced more FFA as compared to cheese prepared without addition of butter oil (Morin et al., 2007). Ahmad et al. (2015) monitored the generation of FFA in cheese during the time frame of 3 months, it was observed that FFA in cheddar cheese slowly steadily increased in the duration of 3 months. Magnitudes of unsaturated fatty acids is normally higher in buttermilk than milk, the resilience of unsaturated fatty acids such as linoleic acid is hundred times lesser than stearic acid. Therefore, control over lipid oxidation in buttermilk-based cheese and other products is challenging for the food scientists. In present study, cheddar-type cheese was produced from buttermilk having four different levels of fat and POV was used to measure lipid oxidation at regular frequencies of 0, 45 and 90 days. Different fat contents in control, T_1, T_2 and T_3 did not affect POV however, POV constantly increased in the ripening phase of cheddar-type cheese. The rise in POV of control, T_1, T_2 and T_3 during the entire ripening phase was not due to the existence of a greater number of unsaturated fatty acids in buttermilk than standard milk and different fat contents in treatments but it was due to the ripening effect. According to the guidelines of European Union, maximum allowable limit of FFA and POV is 0.2% and 10 ($MeqO_2$/kg). At the end of ripening, POV of control was 0.68 (mEqO2/kg) which was almost similar to cow cheddar cheese (Batool et al., 2018a,b). Due to lipid oxidation in food matrix, objectionable flavors may be developed, ripened cheeses usually do not suffer from lipid oxidation because of lower oxidation reduction potential. Addition of buttermilk in several foods inhibited lipid oxidation (Vanderghem et al., 2010). Among all the treatments and control cholesterol significantly varied when analyzed at 0, 45 and 90 days of ripening. Significant variation in cholesterol content was due to the presence of higher fat content in T_1, T_2 and T_3. Ripening period of 90 days remarkably reduce cholesterol in control, T_1, T_2 and T_3. Khan et al. (2018) monitored the changes in concentration of cholesterol during the ripening duration of 90 days in Gouda cheese, results showed that cholesterol content of 90 days old cheese were lower than fresh cheese.

TABLE 4 Lipolysis of cheddar-type cheese produced from buttermilk.

Cheese type	Days	Free fatty acids (%)	Peroxide value ($mEqO_2$/kg)	Cholesterol (mg/dL)
Control	0	0.08 ± 0.01^{g}	0.25 ± 0.02^{c}	112.29 ± 0.68^{e}
	45	0.16 ± 0.02^{f}	0.42 ± 0.05^{b}	103.27 ± 0.07^{f}
	90	0.29 ± 0.01^{c}	0.68 ± 0.03^{a}	89.24 ± 0.09^{g}
T_1	0	0.08 ± 0.01^{g}	0.25 ± 0.02^{c}	132.47 ± 0.51^{c}
	45	0.19 ± 0.03^{e}	0.39 ± 0.07^{b}	$121.49d \pm 0.14^{d}$
	90	0.31 ± 0.02^{c}	0.65 ± 0.04^{a}	108.72 ± 0.26^{e}
T_2	0	0.08 ± 0.01^{g}	0.25 ± 0.02^{c}	157.58 ± 0.13^{b}
	45	0.22 ± 0.02^{d}	0.44 ± 0.01^{b}	142.33 ± 0.25^{b}
	90	0.35 ± 0.01^{b}	0.61 ± 0.03^{a}	125.79 ± 0.18^{d}
T_3	0	0.08 ± 0.02^{g}	0.25 ± 0.02^{c}	177.83 ± 0.91^{a}
	45	0.27 ± 0.04^{c}	0.40 ± 0.07^{b}	161.27 ± 0.19^{b}
	90	0.42 ± 0.03^{a}	0.71 ± 0.06^{a}	139.89 ± 0.12^{c}

Control: Butter milk with 1% fat content. T_1: Butter milk with 1.75% fat content. T_2: Butter milk with 2.50% fat content. T_3: Butter milk with 3.25% fat content. In a column, different letters on means indicate statistically significant difference ($p < 0.05$).

3.5. Vitamin A and E

Milk and dairy products are fundamental part of human diet and their intake may from 5 to 10%. Increased awareness metabolic diseases, nutrition, transition in lifestyles and availability of wide range of functional foods has reduced in the consumption of dairy products all over the word. Food scientists and technologists have not only developed new but also converted the traditional foods to functional foods (Khan et al., 2022). In this study, an effort was made to develop cheddar-type cheese using buttermilk having different fat contents as a substrate as it is a good source of vitamin A and E. Vitamins belong to the group of organic compounds and perform several physiologically important functions in human body. Vitamin A is required for good vision, it helps the eyes to adjust in diffused light, as a catalyst of proteins and part of several enzyme systems. Vitamin E possess antioxidant properties and it can help the body to slower down ageing process. About 10% of the daily requirements of vitamin A is obtained from dairy products (Górska-Warsewicz et al., 2019). Batool et al. (2018a,b) studied the impact of vitamin E addition in cheddar cheese, results showed that vitamin E raised antioxidant capacity and stability with no effect on sensory properties. Neutralization of free radicals is mandatory to prevent oxidative stress that can cause cancer, diabetes, atherogenesis, lipid and protein oxidation. Effect of different contents and ripening on vitamin A and E in cheddar-type cheese was analyzed and documented (Table 5). In fresh cheese, vitamin A and E contents were significantly higher in experimental cheese samples than the control and were in the order of $T_3 > T_2 > T_1 >$ control. Stability of vitamin A in 90 days old control, T_1, T_2 and T_3 was 87, 80, 94 and 91%, respectively. Stability of vitamin E in 90 days old control, T_1, T_2 and T_3 was 82, 87, 86 and 91%, respectively (Ikram et al., 2021). Supplemented cheddar cheese with vitamin A four different concentrations, i.e., 3,500, 4,000, 4,500 and 5,000 IU/kg and stability was analyzed till 3 months in refrigerated stored cheese. In all treatments and control, stability of vitamin A was more than 80%. Stability of vitamin in buttermilk, Gouda cheese, yoghurt and pasteurized milk was more than 80% (Park, 2009). Microcapsules of vitamin A and E were added to cheddar cheese, in mature cheddar cheese, stability of vitamin A and E was 90 and 82%

TABLE 5 Vitamin A and E in cheddar-type cheese produced from buttermilk.

Treatment	Days	Vitamin A (IU/100 g)	Vitamin E (mg/100 g)
Control	0	360.57 ± 0.31^{g}	1.16 ± 0.07^{g}
	90	314.29 ± 0.41^{h}	0.95 ± 0.04^{h}
T_1	0	570.14 ± 0.27^{e}	1.72 ± 0.02^{e}
	90	456.38 ± 0.25^{f}	1.49 ± 0.08^{f}
T_2	0	834.67 ± 0.39^{c}	1.89 ± 0.04^{c}
	90	791.18 ± 0.54^{d}	1.63 ± 0.06^{d}
T_3	0	1136.57 ± 0.44^{a}	2.31 ± 0.09^{a}
	90	1039.47 ± 0.89^{b}	2.10 ± 0.03^{b}

Control: Butter milk with 1% fat content. T_1: Butter milk with 1.75% fat content. T_2: Butter milk with 2.50% fat content. T_3: Butter milk with 3.25% fat content. In a column, different letters on means indicate statistically significant difference ($p < 0.05$).

TABLE 6 Sensory properties of cheddar-type cheese produced from buttermilk.

Cheese type	Days	Color	Flavor	Texture
Control	45	8.1 ± 0.14^{a}	7.2 ± 0.13^{c}	7.0 ± 0.04^{c}
	90	8.3 ± 0.11^{a}	7.3 ± 0.18^{b}	7.4 ± 0.02^{b}
T_1	45	8.2 ± 0.06^{a}	7.3 ± 0.23^{c}	7.1 ± 0.09^{c}
	90	8.4 ± 0.19^{a}	7.6 ± 0.35^{b}	7.3 ± 0.03^{b}
T_2	45	8.2 ± 0.04^{a}	7.9 ± 0.11^{a}	8.0 ± 0.17^{a}
	90	8.3 ± 0.08^{a}	8.0 ± 0.28^{a}	8.2 ± 0.28^{a}
T_3	45	8.1 ± 0.05^{a}	8.0 ± 0.05^{a}	8.1 ± 0.02^{a}
	90	8.3 ± 0.13^{a}	8.2 ± 0.26^{a}	8.3 ± 0.06^{a}

Control: Butter milk with 1% fat content. T_1: Butter milk with 1.75% fat content. T_2: Butter milk with 2.50% fat content. T_3: Butter milk with 3.25% fat content. In a column, different letters on means indicate statistically significant difference ($p < 0.05$).

(Stratulat et al., 2014). Stability of vitamin A and in buttermilk derived cheese was greater than 90% (Król et al., 2020).

3.6. Sensory characteristics

Fat content significantly affected the flavor and texture score of cheddar-type cheese produced from buttermilk having 1, 1.75, 2.5 and 3.25% fat. Flavor score of 45 days old control, T_2 and T_3 were at par with each other ($p < 0.05$). Flavor score of all the treatments was strongly correlated with POV, non-variation in flavor score of control, T_2 and T_3 was due to minimum variation in peroxide value. Flavor score and POV of 90 days old cheddar cheese and control were strongly correlated (Batool et al., 2018a,b). Ullah et al. (2018) reported that fat content had a significant effect on flavor and texture of cheddar cheese. Texture score of 45 days old control, T_2 and T_3 were 7.0, 8.0 and 8.1 ($p < 0.05$). Ripening of 90 days considerably improved flavor and texture score, highest flavor score was obtained by T_3 followed by T_2. After 90 days, highest texture score was obtained by T_3 followed by T_2. Texture scores of T_1 and control were not different from each other. Flavor score of cheddar-type cheese produced from butter milk having 1, 2.5 and 3.25% fat content was 81, 89 and 91% of total score (9). These results suggested that cheddar-type cheese can be produced from buttermilk having 2.5 and 3.25% fat contents with acceptable sensory attributes (Table 6).

4. Conclusion

The aim of this study was to estimate the impact of buttermilk and their cream on fatty acid composition, organic acid, oxidative stability and vitamins contents, as well as sensory properties of experimental cheddar-style cheese samples. The results of samples showed a non-significant effect on fatty acid composition in comparison to the control. In contrast, organic acids in the experimental cheese samples were significantly improved at the end of the 90-day ripening period. Free fatty acid was slightly increased in the cheese sample, while peroxide value of cheddar-style cheese samples resulted in similar values compared to the control. Furthermore, vitamins A and E was stable in the experimental cheese samples during the storage period, and sensory scores for color, taste, texture were within acceptable range. This study may open a new array for emerging innovative product in the healthy cheese market, with relevant potential of nutritional properties. Further research work could be done on the use of buttermilk cream formulation as a method of improving bioactive properties of cheddar-style cheese in the diet.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

MA performed the methods and investigation. MN was involved in conceptualization, funding acquisition, and writing of original draft. MI helped in writing of this manuscript. RU, FA-A, and MT helped in software. JMR, SK, FK, MAR, and TE supported in analysis and supervision of research work. TE supported in funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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In vitro screening and characterization of lactic acid bacteria from Lithuanian fermented food with potential probiotic properties

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The present work aimed to identify probiotic candidates from Lithuanian homemade fermented food samples. A total of 23 lactic acid bacteria were isolated from different fermented food samples. Among these, only 12 showed resistance to low pH, tolerance to pepsin, bile salts, and pancreatin. The 12 strains also exhibited antimicrobial activity against *Staphylococcus aureus* ATCC 29213, *Salmonella Typhimurium* ATCC 14028, *Streptococcus pyogenes* ATCC 12384, *Streptococcus pyogenes* ATCC 19615, and *Klebsiella pneumoniae* ATCC 13883. Cell-free supernatants of isolate 3A and 55w showed the strongest antioxidant activity of 26.37 μg/mL and 26.06 μg/mL, respectively. Isolate 11w exhibited the strongest auto-aggregation ability of 79.96% as well as the strongest adhesion to HCT116 colon cells (25.671 ± 0.43%). The selected strains were tested for their synbiotic relation in the presence of a prebiotic. The selected candidates showed high proliferation in the presence of 4% as compared to 2% galactooligosaccharides. Among the strains tested for tryptophan production ability, isolate 11w produced the highest L-tryptophan levels of 16.63 ± 2.25 μm, exhibiting psychobiotic ability in the presence of a prebiotic. The safety of these strains was studied by ascertaining their antibiotic susceptibility, mucin degradation, gelatin hydrolysis, and hemolytic activity. In all, isolates 40C and 11w demonstrated the most desirable probiotic potentials and were identified by 16S RNA and later confirmed by whole genome sequencing as *Lacticaseibacillus paracasei* 11w, and *Lactiplantibacillus plantarum* 40C: following with the harboring plasmid investigation. Out of all the 23 selected strains, only *Lacticaseibacillus paracasei* 11w showed the potential and desirable probiotic properties.

KEYWORDS

fermented food, lactic acid bacteria, potential probiotics, *in vitro* screening, tryptophan, safety assessment, probiotics LAB-lactic acid bacteria, GIT-gastrointestinal tract

Introduction

The demand for functional foods has increased in recent years due to consumers' interest in their therapeutic applications. The main types of functional foods include probiotics, prebiotics, and synbiotics (which are a mixture of probiotics and prebiotics) (Topolska et al., 2021). The demand for different strains of probiotics has led to consumer awareness of the health benefits and therapeutic effects of modulating the gut microbiota, leading to the amelioration of neurological diseases and metabolic disorders (Amoah et al., 2022). These health benefits are due to the direct effects of the microbes on the host and their fermentation products in the gut. Because these fermented by-products of microbes remain in the gut after consumption, they play a significant role in the functioning of the body and may have direct health benefits for the host.

The Food and Agriculture Organization and the World Health Organization define probiotics as live microorganisms that confer health benefits on their hosts when ingested in an adequate concentration (Salminen et al., 2021). Over the last decades, studies on probiotics have expanded tremendously. Numerous *in vivo* studies have found that, when adequately administered, probiotics modulate the gut microbiota by promoting the growth of beneficial microorganisms in the gastrointestinal tract (GIT) (Vadopalas et al., 2020).

Most strains of lactic acid bacteria (LAB) are commonly used as probiotics in foods (Zapaśnik et al., 2022). LAB are a group of bacteria that include genera such as *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Streptococcus*, are Gram-positive cocci or rods, and are acid-tolerant, non-respiring but aerotolerant bacteria (Shokryazdan et al., 2017). They are naturally present in fermented foods, composts (Tran et al., 2019), GIT (Marchwińska and Gwiazdowska, 2021), vaginal tract (Silva et al., 2022), plant surfaces (Yu et al., 2020), and silages (Bohn et al., 2017).

Probiotic fermentation often produces by-products with diverse health-promoting effects, including protection against infectious agents (Bartkiene et al., 2020; Mileriene et al., 2023), anti-allergenic effects (Liang et al., 2022), immunomodulatory effects (Kober et al., 2022), anti-obesity effects (Liu et al., 2022), antidiabetic effects (Wang et al., 2022; Daliri et al., 2023a), antioxidant effects (Hoffmann et al., 2021), enhancement of the bioavailability of vitamins/minerals (Ballini et al., 2019), anti-anxiety effects (Lalonde and Strazielle, 2022), and attenuation of Alzheimer's disease (Megur et al., 2021).

In recent years, many probiotic candidates have been isolated from traditionally fermented foods and their potential effects on health have been well documented. For instance, *Pediococcus acidilactici* SDL 1402 and *Weissella cibaria* SCCB 2306 isolated from Korean fermented soybean paste were shown to survive simulated gastrointestinal conditions, inhibit pathogenic bacteria, and showed good gut colonization potentials (Oh et al., 2018). The bacteria were found to have no virulent factors and displayed significant cholesterol-reducing potentials *in vivo* (Daliri et al., 2022). Similarly, *Lacticaseibacillus paracasei* L2 isolated from Lben (a Tunisian traditionally fermented dairy product) displayed an excellent gut colonization potential, strong pathogen inhibiting ability, and produced antioxidant metabolites during fermentation (M'hamed et al., 2023). Traditionally fermented foods, therefore, remain a good source of probiotic candidates since most of their commensal LAB are generally regarded as safe or qualified presumption of safety (Koutsoumanis et al., 2021; Grujović et al., 2022).

The criteria for the selection of probiotic strains are considered important before their use in animal and/or human studies. The most important feature of a probiotic is its potential health effect and safety. Desirable properties of probiotics include their ability to survive in the GIT, their antimicrobial activity against pathogenic microorganisms, and their antioxidant properties (Reuben et al., 2020). Furthermore, the binding ability of LAB to HCT116 colon cells has been used as a criterion for assessing the potential gut colonization ability of probiotic candidates in some studies (M'hamed et al., 2023). In addition, their ability to grow in the presence of prebiotics to produce essential metabolites such as tryptophan are considered desirable traits (Kepert et al., 2017). For this reason, the present study aimed to isolate probiotic bacteria from Lithuanian fermented pear, cherry tomato, cucumber, and orange. The isolates were screened for their resistance to simulated gastrointestinal conditions. Strains that survived *in-vitro* gastrointestinal conditions were tested for their functional properties and the selected strains were tested for their safety. Probiotic candidates were identified using whole genome sequencing.

Materials and methods

Sample collection, bacteria isolation, and selection

Strains were isolated from various fermented foods collected at Halės Turgus market (Halle Market, Vilnius, Lithuania). The fermented foods were traditional Lithuanian fermented cherry tomatoes, pears, oranges, and cucumbers. Each sample paste (1 g) was transferred aseptically into separate test tubes containing 9 mL of sterile peptone water (0.1%) (Sigma- Aldrich, Poznań, Poland). Aliquots of 10 µL from appropriate 10^5 CFU/mL dilution were pentagonally streaked on the pre-solidified de Man, Rogosa, and Sharpe (MRS) agar (Oxoid, Wesel, Germany) and incubated at 37°C for 48–72 h under aerobic conditions. Representative colonies of LAB were randomly picked and were purified by repeated streak plating on MRS agar until pure colonies were obtained. The pure colonies were maintained on MRS agar plates and subcultured every 5 weeks until imperative for characterization. Cell morphology and colonial characterization were observed on MRS agar. These isolates were stored and preserved in a −80°C deep freezer (Froilabo, Livingston, United Kingdom) at the Department of Microbiology, Faculty of Life Sciences Centre, Vilnius University, Vilnius, Lithuania.

Cell culture

The human colonic cell lines HCT-116 were obtained from the Department of Biological Models, Vilnius University, Lithuania. The cells were routinely cultured in Dulbecco's modified Eagle's minimal

Abbreviations: LAB, Lactic acid bacteria; GIT, Gastrointestinal tract; CFS, Cell-free culture supernatants; CFU, Colony forming units; TSB, Tryptone soya broth; GOS, Galactooligosaccharides.

essential medium (DMEM; Sigma- Aldrich, Poznań, Poland) supplemented with 10% (v/v) heat-inactivated (30 min, 56°C) fetal bovine serum (Sigma- Aldrich, Poznań, Poland). The cells in a medium were also supplemented with a 1% (v/v) penicillin–streptomycin solution to a final concentration of 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. The incubation was at 37°C in an atmosphere of 5% CO_2 and 95% air. The cells were nourished with complete DMEM every alternate day until the cells reached 70–80% confluency.

Screening of probiotic properties of isolated bacteria

Resistance to low pH

The resistance of LAB to low pH was studied according to a previously described method (Oh et al., 2018), with little modifications. Briefly, LAB cultures incubated at 37°C for 24 h were centrifuged at 10,000 g for 10 min. The pellets were suspended in sterile PBS (Sigma- Aldrich, Poznań, Poland) and adjusted to a pH of 2.0 using 1 M HCl. The mixture was then incubated at 37°C for 4 h. Aliquots of the mixture were taken at time 0 and after 4 h. The samples were serially diluted in peptone water and the viable cells were determined by the spread plate method using MRS agar. The plates were incubated at 37°C for 24 h and the percentage survival of the bacteria was calculated as follows:

$$\%\ \text{Survival} = \left(\frac{\text{CFU} * \text{of viable cells survived}}{\text{CFU} * \text{of initial viable cells inoculated}} \right) \times 100$$

*CFU = Colony forming units.

Resistance to pepsin

To test the viability in the presence of pepsin, simulated gastric juice was prepared by suspending 3 mg/mL pepsin (Sigma- Aldrich, Poznań, Poland) in sterile peptone water (w/v) and adjusted to pH 2.0. The fluid was inoculated with active cultures at an inoculum size of 1% (v/v) and incubated at 37°C for 4 h. The viable cells were determined before (T1) and after incubation (T2) by the spread plate method (Tokatl et al., 2015). The percentage survival of the bacteria was calculated according to resistance to low pH.

$$\%\ \text{Survival} = \left(\frac{\text{CFU} * \text{of viable cells survived}}{\text{CFU} * \text{of initial viable cells inoculated}} \right) \times 100$$

*CFU = Colony forming units.

Resistance to bile salts and pancreatin

Resistance to intestinal juices was tested as reported (Manovina et al., 2022). Briefly, 0.3% (w/v) bile salt (Sigma- Aldrich, Poznań, Poland) and 1 mg/mL pancreatin (Sigma- Aldrich, Poznań, Poland) were dissolved in sterile peptone water (w/v) adjusted to pH 8 cell-free culture supernatants. The fluid was inoculated with 1% (v/v) LAB cultures and incubated at 37°C for 6 h. The viable cells were determined before and after incubation by the spread plate method. The percentage survival of the bacteria was calculated according to the equation below.

$$\%\ \text{Survival} = \left(\frac{\text{CFU} * \text{of viable cells survived}}{\text{CFU} * \text{of initial viable cells inoculated}} \right) \times 100$$

*CFU = Colony forming units.

Assessment of functional properties

Probiotic antimicrobial activity

Antibacterial activity was determined using the agar well diffusion test as previously described (Edith Marius et al., 2018). *Staphylococcus aureus* ATCC 29213, *Salmonella Typhimurium* ATCC 14028, *Streptococcus pyogenes* ATCC 12384, *Streptococcus pyogenes* ATCC 19615, and *Klebsiella pneumoniae* ATCC 13883 were obtained from the Department of Microbiology, Vilnius University and were used as indicator strains for the detection of antimicrobial activity. The LAB were cultured in 3 mL MRS broth medium and incubated for 24 h at 37°C. The MRS broth tubes were subsequently centrifuged (10000 rpm for 10 min) to prepare cell-free culture supernatants (CFS). The pH values of the supernatants were adjusted to approximately 7 by the addition of NaOH. A suspension of 100 µL of 10^7 CFU/mL of each pathogenic strain was then prepared and spread onto the nutrient agar, into which 5-mm-deep wells had been dug. Approximately 100 µL of CFS was poured into each well, and nutrient agar plates were incubated for 24 h at 37°C. Finally, the inhibition zone diameter was measured in millimeters (mm) (Xu et al., 2020).

Trolox equivalent antioxidant capacity

The scavenging effect of the CFS on a 1,1-Diphenyl2-picryl-hydrazyl (DPPH) radical was assessed as described in a previous study (Liao et al., 2012). Briefly, the scavenging ratio of the sample and Trolox (Abcam, Cambridge, United Kingdom) on DPPH (Abcam, Cambridge, UK) at the same time was tested, and then a suitable concentration range of the Trolox and its scavenging percentage was found. Then, a linear regression equation between the Trolox concentration and its scavenging percentage was built, and the Trolox equivalent antioxidant capacity (TEAC) was calculated through the equation. A higher TEAC value meant higher DPPH scavenging activity. Meanwhile, the scavenging percentage on the DPPH radical of the sample solution was tested following the treatment of the Trolox solution. The scavenging effect on the DPPH radical of the samples could be calculated as the Trolox equivalent's antioxidant capacity from the calibration curve: $y = -0.0298x + 0.9995$.

In vitro gut colonization potential

Auto-aggregation ability was determined by the method described previously with slight modification (Botta et al., 2014; Li et al., 2020). The overnight selected LAB culture was centrifuged at 10000 rpm for 10 min to harvest the cell pellets. Pellets were washed thrice with phosphate-buffered saline (PBS; pH 7.4), re-suspended in PBS, and the initial absorbance was noted at 600 nm. The bacterial suspension was incubated at 37°C for 24 h, and the final absorbance of the supernatant was measured at 600 nm at three different times: 4 h, 12 h, and 24 h. The percentage of cellular auto-aggregation was measured by the formula:

$$\%\ \text{Auto-aggregation} = \left(\frac{\text{ODinitial} - \text{ODfinal}}{\text{ODinitial}} \right) \times 100$$

In vitro percent adhesion on HCT116 cells

The concentration of cells in a monolayer was determined by trypsinizing the adhered cells with 3 mL of 0.25% trypsin– EDTA solution for 5–10 min at 37°C. The final cell count in suspension was measured with the help of a hemocytometer (Sigma- Aldrich, Poznań, Poland). For adhesion assay, HCT116 cells were seeded separately in each well of standard 12-well tissue culture plates at a concentration of 1×10^6 cells/mL and incubated for ~48 h or more, until a complete monolayer was obtained. Change of medium was performed every 24–48 h. The spent medium was completely removed 24 h before adhesion assay and cells were fed with DMEM lacking antibiotics. The LAB isolates for adhesion assay were propagated in MRS broth and cultures obtained after 18 h of growth at 37°C were centrifuged at 6000 × g for 10 min. The pellet was washed once with PBS (pH 7.4). The cell density was adjusted approximately to the desired levels by measuring the absorbance at 600 nm. The exact number of viable bacteria used in the assay was determined by plate counting on MRS agar.

The adhesion of LAB isolates was measured as described previously with few modifications (Sharma and Kanwar, 2017). The HCT 116 cells in a monolayer were washed twice with 3 mL of PBS (pH 7.6). The 2 mL of DMEM without serum and antibiotics was added to each well and incubated at 37°C for 40 min before inoculation of bacteria. Different LAB isolates with a concentration of approximately 1×10^7 CFU suspended in 1 mL DMEM without serum and antibiotics were used to inoculate each well of tissue culture plates. The plates were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 3 h. After incubation, the monolayer was washed five times with sterile PBS (pH 7.6) to remove non-adherent bacteria.

The monolayer was washed five times with sterile PBS (pH 7.4) to remove non-adherent bacteria. To enumerate the viable adhered bacteria, the cells from the monolayer were detached by trypsinization. Each well was treated with 1 mL of 0.25% trypsin–EDTA solution and incubated for 15 min at room temperature. The suspension of lysed cells and LAB was serially diluted with saline solution and plated on MRS agar. The enumeration was done after 48 h of incubation at 37°C in an anaerobic atmosphere. The adhesion was expressed as the percentage of the number of adhered bacteria to the total bacteria used for the experiment and calculated as:

$$\text{Percent adhesion} = \frac{B1}{B0} \times 100$$

where B0 and B1 CFU/mL are the initial and final count of bacteria, respectively.

Influence of galactooligosaccharides on strain growth

Prebiotic influence on LAB growth was tested as described previously with slight modifications (Wang et al., 2019). Briefly, LAB cultures incubated at 37°C for 24 h were administered in the MRS broth containing prebiotic 2% Galactooligosaccharides (GOS) and 4% GOS, i.e., the lowest concentration that elicited a significant increase in the growth of LAB (data not shown). The LAB growth was noted at every 4 h interval at 37°C by measuring absorbance at 600 nm. The optical densities were measured using a spectrometer (Eppendorf Bio spectrometer®, Hamburg, Germany). The initial optical density value of the media was deducted from the final value for each test sample.

Tryptophan-producing ability of LAB

The tryptophan production by LAB was monitored as previously described (Vaitekūnas et al., 2020). In the prebiotic-supplemented LAB CFS samples, concentrations of tryptophan were determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS). First, the samples were mixed with an equal volume of acetonitrile and centrifuged for 10 min at 10,000 rpm. The samples were analyzed using the Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a photodiode array (PDA) detector (Shimadzu, Kyoto, Japan) and LCMS-2020 mass spectrometer (Shimadzu, Kyoto, Japan) with an electrospray ionization (ESI) source. The chromatographic separation was conducted using a YMC Pack Pro C18 column (3 × 150 mm; YMC, Kyoto, Japan) at 40°C and a mobile phase that consisted of 0.1% formic acid water solution (solvent A) and acetonitrile (solvent B) delivered in the 5–95% gradient elution mode. Mass scans were measured from m/z 50 up to m/z 2,000 at a 350°C interface temperature, 250°C desolvation line (DL) temperature, ±4,500 V interface voltage, and neutral DL/Qarray, using N_2 as nebulizing and drying gas. Mass spectrometry data were acquired in both positive and negative ionization modes. The data were analyzed using LabSolutions software (Shimadzu, Kyoto, Japan).

DNA extraction and molecular identification

Genomic DNA was isolated from sediment samples using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Seattle, United States) according to the manufacturer's recommendations. The concentration of extracted DNA was evaluated using an Eppendorf bio photometer (Eppendorf, Hamburg, Germany) (Lastauskienė et al., 2021). The molecular identification of LAB strains was conducted by 16S RNA and later confirmed by whole genome sequencing analysis. For 16S RNA sequencing, the strains were sent to Microgen, Netherlands, and for the whole genome sequencing the strains were sent to Cosmos, USA. Each sequence amplicon was BLAST® analyzed and aligned with the National Center for Biotechnology Information (NCBI) Sequence comparison database[1] to determine the sequence identity and GenBank accession number. A phylogenetic tree was constructed after para-wise alignment applying CLUSTAL W, using sequences obtained from the NCBI Gene Bank. The presentation of a neighbor-joining tree, which was further tested by bootstrap analysis with 1,000 replicates using MEGA 11.0 software, was performed to identify the LAB isolates.

1 www.ncbi.nlm.nih.gov

Safety analysis of LAB

Determination of antibiotic susceptibility

The susceptibility of the LAB to antibiotics was tested as reported previously (Wang et al., 2021). All the antibiotics were purchased from Carl Roth, Karlsruhe, Germany. The LAB were tested against 30 μg kanamycin (Kan), 25 μg streptomycin (Str), 10 μg gentamicin (Gen), 30 μg vancomycin (Van), 15 μg erythromycin (Ery), 30 μg chloramphenicol (Chl), 30 μg tetracycline (Tet), 2 μg clindamycin (Cli), 10 μg ampicillin (Amp), and 10 μg penicillin (Pen) using the disc diffusion method. The concentration of antibiotics was selected according to the EFSA guidelines (Aquilina et al., 2012). The agar plates were examined for the presence or absence of zones of inhibitions after incubation at 37°C for 24 h.

Hemolytic ability test

Hemolytic activity was performed as described in a previous study (Zhang et al., 2022). Overnight cultures of selected LAB were streaked on 5% defibrinated sheep blood agar plates and incubated at 37°C for 48 h. After incubation, the plates were observed for α-hemolysis (dark and greenish zones), β-hemolysis (lightened –yellow or transparent zones), and γ-hemolysis (no change or no zones).

Mucin degradation test

The mucin degradation ability of the LAB was assessed using a previously reported method with slight modification (Daliri et al., 2022) Briefly, the LAB strains were grown in MRS broth supplemented with both 0.5% (w/v) glucose and 0.5% (w/v) mucin. After inoculation, the cultures were incubated at 37°C for 48 h under aerobic conditions. The bacterial growth was estimated every 6 h by measuring absorbance at 600 nm. *E. coli* ATCC 25922 was used as positive control and grown in Tryptic soy broth (Carl Roth, Karlsruhe, Germany) containing 0.5% (w/v) glucose supplemented with or without 0.5% (w/v) mucin (Sigma- Aldrich, Poznań, Poland) and cultured at 37°C for 48 h under aerobic conditions. The optical densities were measured using a spectrometer (Eppendorf Bio spectrometer®, Hamburg, Germany). The initial optical density value of the media was deducted from the final value for each test sample.

Gelatin degradation test

The gelatin degradation ability of the LAB was investigated using MRS media containing 3% (w/v) gelatin (Sigma- Aldrich, Poznań, Poland) according to the method reported by Daliri et al. (2022). *Staphylococcus aureus* ATCC 6538 was used as a reference for quality control and was grown on tryptone soya broth (TSB) containing 3% (w/v) gelatin. Gelatin degrading ability was evaluated by the presence of a clear zone around the bacteria colony.

Search for antimicrobial resistance genes, virulence factors, and plasmid

The bacteria genomes were screened against two antimicrobial resistance gene databases: the ResFinder server 4.1 (https://cge.food.dtu.dk/services/ResFinder/ accessed on 27.02.2023) and ResFinderFG 2.0 server (https://cge.food.dtu.dk/services/ResFinderFG/ accessed on 27.02.2023). Search for virulent factors was performed using the VirulenceFinder-2.0 server (https://cge.food.dtu.dk/cgi-bin/webface.fcgi?jobid=63FCC7FC00005B2B4068D7E0;wait= assessed on 27.02.2023). Plasmids were searched from the genome data by screening the contigs against the PlasmidFinder server 2.1 (https://cge.food.dtu.dk/services/PlasmidFinder/ assessed on 27.02.2023).

Statistical analysis

All the experiments were performed in triplicate. Results were statistically analyzed by one-way ANOVA and two-way ANOVA and expressed as mean ± standard deviation calculated at a 95% confidence level. Tukey's test was employed to examine differences between means at $p < 0.05$. All statistical analyses were performed using GraphPad Prism version 8.4.3 (GraphPad Software Inc., Boston, United States).

Results

Isolation of LAB

In this study, 23 pure bacterial colonies were obtained from various fermented food samples from Hales Turgus Market (Vilnius, Lithuania). These colonies were isolated from fermented pear (6 isolates), fermented cherry tomato (5 isolates), fermented Lithuanian cucumber, (6 isolates), and fermented orange (6 isolates) (Supplementary Table S1). These isolates were stored in the probiotic library provided by the Department of Microbiology, Faculty of Life Sciences Centre, Vilnius University, Vilnius, Lithuania.

Screening of probiotic properties of isolated bacteria

Resistance to Low pH

During the process of digestion, the stomach lining produces gastric acid with a pH between 1 and 3, which is very acidic. This low pH plays a key role in the digestion of proteins by activating digestive enzymes, which together break down the long chains of amino acids of proteins. Hence in our study, we subjected the strains to pH 2 to observe their survival ability in *in vitro* conditions. Out of 23 isolates, only 18 strains showed survival abilities >50%. Among the strains that were tested, 55w, 18B, 2 T, and 9 s showed the highest survival abilities of 97.58, 96.02, 95.37, and 90.03%, respectively. The strains showing <50% survival abilities (LAB 57B, 42 T, and 29A) were excluded from further analysis since the drastic reduction in their survival ability could indicate fewer chances of surviving further harsh gastrointestinal conditions (Figure 1). However, the 18 strains showing resistance to low pH were tested for their tolerance to pepsin.

Tolerance to pepsin

Pepsin is a digestive enzyme that breaks down proteins into smaller peptides. It is produced in the stomach lining and is one of the main digestive enzymes present in the digestive systems of humans

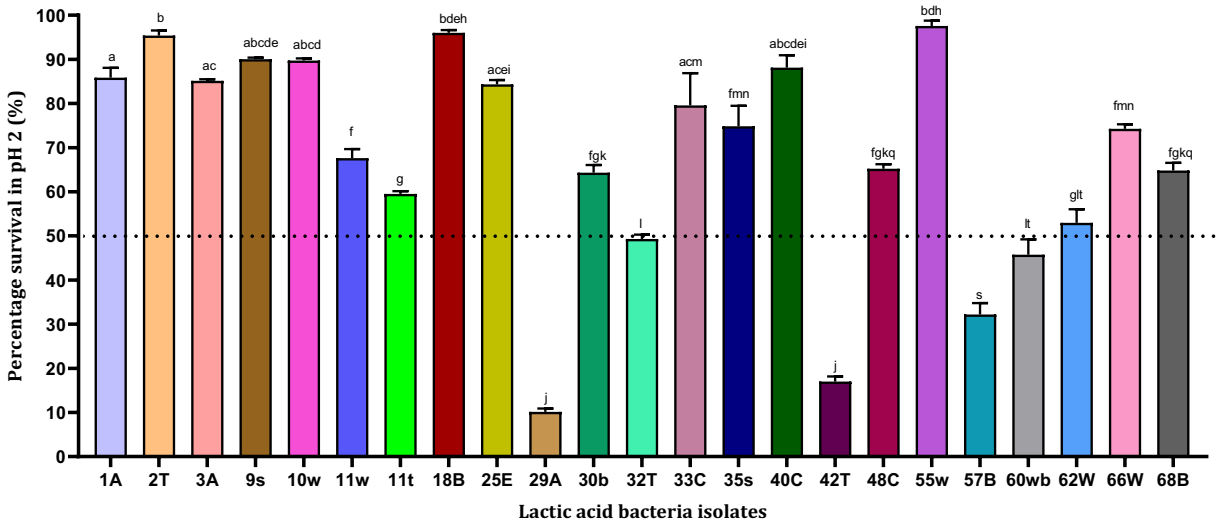

FIGURE 1
Acid resistance of lactic acid bacteria in phosphate-saline buffer (pH 2). Values are expressed as mean ± standard deviation ($n = 3$). Bars with the same lower-case letters are not significantly different, whereas those with different lower-case letters are significantly different ($p < 0.05$). The dotted line represents the minimum percentage survival requirement of the individual isolates.

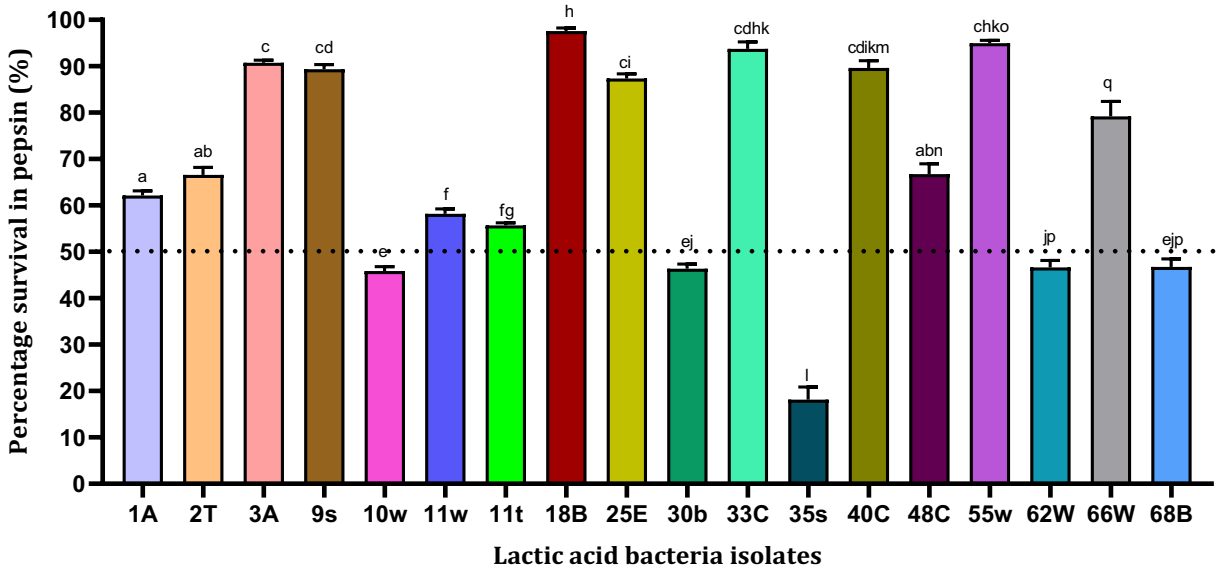

FIGURE 2
Resistance of lactic acid bacteria to pepsin (pH = 2). Values are expressed as mean ± standard deviation ($n = 3$). Bars with the same lower-case letters are not significant, whereas those with different lower-case letters are significantly different ($p < 0.05$). The dotted line represents the minimal requirement of survival of the individual isolates.

and many other animals. However, pepsin is activated at a low pH, and we tested the survival ability of isolates in the presence of pepsin at pH 2. In all, 18 LAB isolates were tested for their tolerance to pepsin. According to our results, four bacterial isolates showed pepsin resistance above 90% in the order 18B (97.58%) > 55w (94.99%) > 33E (93.74%) > 3A (90.75%), while 35 s (18.11%) showed the least survival ability (< 20%) (Figure 2). The least resistance to pepsin was observed by five strains, 35 s (18.11%) < 10w (45.83%) <30b (46.35%) <62 W (46.63%) < 68B (46.77%), with the least survival abilities (<50%) being therefore excluded from subsequent experiments.

Resistance to bile salts and pancreatin

Bile salts are steroid acids produced in the liver and stored in the gallbladder that help in the digestion of fats. The Pancreatin enzyme is produced by the pancreas and is important for digesting fats, proteins, and sugars. In this study, 13 LAB isolates showed varying levels of resistance to bile salts and 1 mg/mL pancreatin after 6 h of exposure (Figure 3). A total of six LAB strains, 25E (95.40%) > 9 s (95.37%) > 2 T (94.20%) > 3A (92.07%) > 55w (90.90%) > 40C (90.83%), were found to be highly tolerant (>90%) to simulated

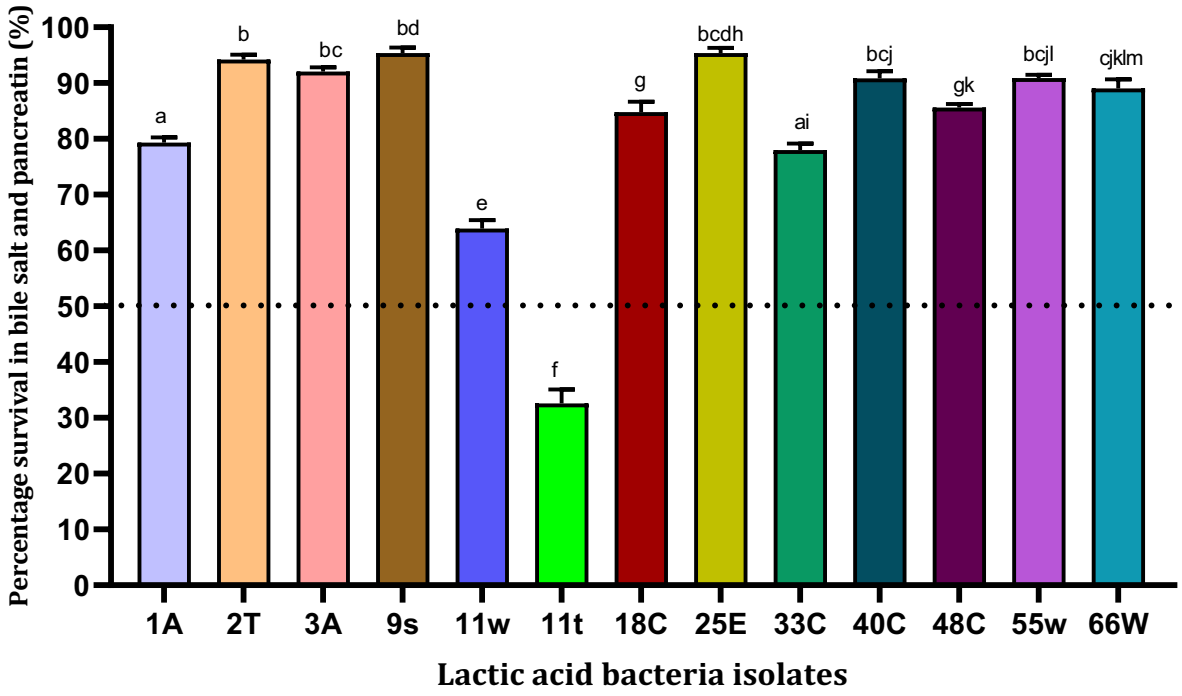

FIGURE 3
Viability of lactic acid bacteria in the presence of simulated intestinal fluid (0.3% (w/v) bile salt and 1 mg/mL pancreatin in peptone water, pH 7.2). Values are expressed in mean ± standard deviation ($n = 3$). Bars with the same lower-case letters are not significantly different, whereas those with different lower-case letters are significantly different ($p < 0.05$). The dotted line represents the minimal requirement of survival of the individual isolates.

intestinal fluid after 6 h of incubation (Figure 3). Strains 9 s and 25E showed high tolerance to bile salts and pancreatin and their percentage rates of survival were 95.37 and 95.40%, respectively. Meanwhile, strain 11 t showed the lowest survival ability of 32.60%. In all, 12 strains showed at least 50% resistance against bile salts and pancreatin (Figure 3). Hence, these 12 strains were subjected to functional characterization.

Functional characterization of selected probiotic candidates

Antimicrobial activity

Probiotics produce metabolites that can be useful for the host. Bacteriocin is a metabolite that hinders and/or suppresses the growth of pathogenic bacteria in the gut. To investigate their antimicrobial property, five pathogenic bacteria (*S. aureus* ATCC2913, *S. typhimurium* ATCC 14028, *S. pyogens* ATCC 12384, *S. pyogens* ATCC 19615, and *K. pneumoniae* ATCC 13883) were treated with the CFS of the selected LAB CFS. Only CFS from strains 18B, 25E, 48C, and 66 W inhibited these pathogens at varying degrees (Table 1). Strains 2 t, 11w, and 33E inhibited the growth of only two pathogenic microorganisms. Isolate 18B, 25E, and 48C hindered the growth of at least four pathogenic microorganisms, whereas 66 W showed an antagonistic ability to all the pathogens tested. Therefore, all 12 strains were subjected to further studies.

Trolox equivalent antioxidant concentration

The antioxidant property of the probiotic bacteria can have an important role in anti-aging functions and in scavenging free radicals from the body. For this reason, TEAC values were evaluated for LAB CFS. The highest TEAC was exhibited by isolates 3A and 55w with TEAC of 26.37 μg/mL and 26.06 μg/mL, respectively. The least TEAC was shown by isolate 40C with a TEAC of 9.57 μg/mL (Figure 4). Even though these isolates showed different antioxidant abilities, they were all tested for their potential gut colonization abilities.

Bacterial colonization ability

Auto-aggregation of probiotic strains appeared to be necessary for adhesion to intestinal epithelial cells. Among the LAB tested, 11w showed the highest auto-aggregation ability of 79.96% after 24 h. Compared to all the probiotic candidates 11w, 40C, and 55w showed aggregation of 79.96, 76.63, and 76.76%, respectively, after 24 h, whereas 3A (24.26%) showed the least <25% after 24 h. Four strains, 2 T, 3A, 9 s, and 25E, showed the least aggregative potential of 26.63, 24.26, 25.18, and 36.37% (<50%), respectively, after 24 h and were excluded. Eight strains that exhibited an aggregative property of over 50% were further evaluated for their adhesion ability on HCT116 cells (Figure 5).

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TABLE 1 The inhibitory ability of probiotic candidates against various pathogenic bacteria.

Culture	*Staphylococcus aureus* ATCC 29213	*Salmonella Typhimurium* ATCC 14028	*Streptococcus pyogenes* ATCC 12384	*Streptococcus pyogenes* ATCC 19615	*Klebsiella pneumoniae* ATCC 13883
1A	10.75 ± 0.5	12.00 ± 0.81	–	15.25 ± 0.56	–
2T	–	13.75 ± 0.5	–	–	18.00 ± 0.81
3A	12.75 ± 0.5	13.25 ± 0.5	12.75 ± 0.5	–	–
9s	11.75 ± 0.5	12.75 ± 0.5	11.75 ± 0.5	–	–
11w	14.25 ± 0.5	–	–	–	16.5 ± 1
18B	14.25 ± 0.5	–	15.62 ± 0.75	17.25 ± 1.25	18.75 ± 1.5
25E	16.75 ± 0.5	14.25 ± 0.5	15.25 ± 0.5	17.5 ± 0.57	17.5 ± 0.57
33E	–	–	–	12.75 ± 0.5	18.5 ± 1.29
40C	11.5 ± 0.57	11.5 ± 0.57	–	–	–
48C	14.75 ± 0.5	15.25 ± 0.5	–	18.00 ± 0.81	19.5 ± 0.57
55w	–	15.25 ± 0.5	12.25 ± 0.5	–	18.5 ± 0.57
66 W	11.25 ± 0.5	14.5 ± 0.057	11.25 ± 0.5	13.25 ± 0.95	19.5 ± 0.57

Results are expressed as the zone of inhibition in mm. The results are expressed as the means ± standard deviations of three independent replicates ($n = 3$). (–) indicates no zone of inhibition.

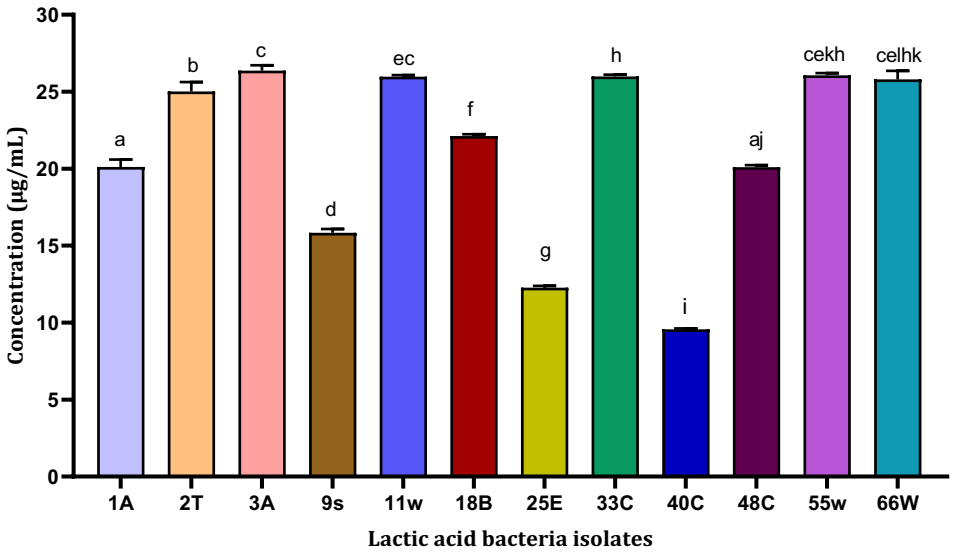

FIGURE 4
Trolox equivalent antioxidant concentration of 13 LAB CFS. Results are expressed as mean of triplicate values ± standard deviation ($n = 3$). Bars with the same lower-case letters are not significantly different, whereas those with different lower-case letters are significantly different ($p < 0.05$).

Bacterial adhesion to HCT116 cells

The HCT116 cell model (a human-cloned colon adenocarcinoma cell) structure and function are very similar to those of the highly differentiated intestinal epithelial cells. They possess the same microvilli, tight connection, and cell polarity, which can be utilized to simulate the function of intestinal epithelial cells *in vitro*. Therefore, we chose this cell model to identify the adhesion characteristics of LAB. Nine LAB isolates adhered to HCT116 cells. Adhesion levels of these LAB isolates to HCT116 cells varied from <10 to >25%. LAB 11w showed the best adhesion ability of 25.671 ± 0.43% compared to isolate 1A, which had the least adhesion ability of 9.26 ± 0.97% (Figure 6). Among the eight isolates, 1A, 18B, and 55w showed adhesion percentages <12%. For this reason, they were excluded from further studies.

Influence on growth in the presence of galactooligosaccharides

Prebiotic administration increases the growth of beneficial bacteria and promotes the growth of probiotics. GOS is a very well-known prebiotic that has been employed in therapeutic uses and administered with probiotics to improve the health of animals/humans. In our study, all the LAB strains showed significant growth in the presence of GOS. Increased growth was observed in a concentration-dependent manner. The OD values of each isolate differ from others, indicating that growth is strain specific. Isolates 11 w, 33E, 40C, 48C, and 66 W showed an increase in growth when supplemented with 2% GOS, and their growth escalated after the concentration was increased to 4% (Figure 7).

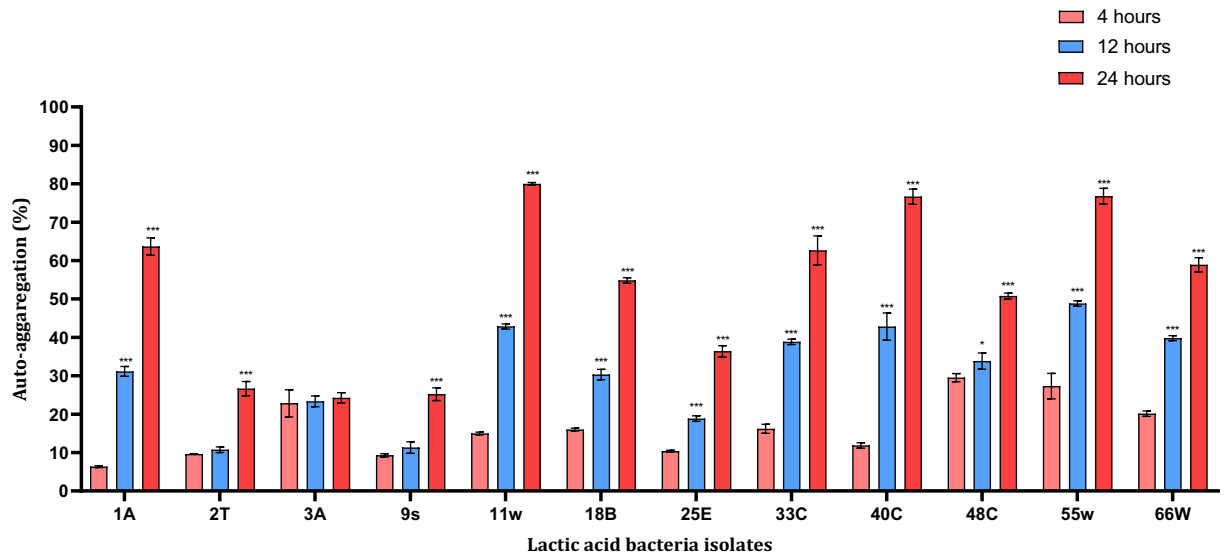

FIGURE 5
Auto-aggregation abilities of probiotic candidates after 20 h incubation at 37°C. Each value represents the mean ± standard deviation of three independent readings ($n = 3$). *Significant differences at $p < 0.05$, and ***significant difference at $p < 0.001$.

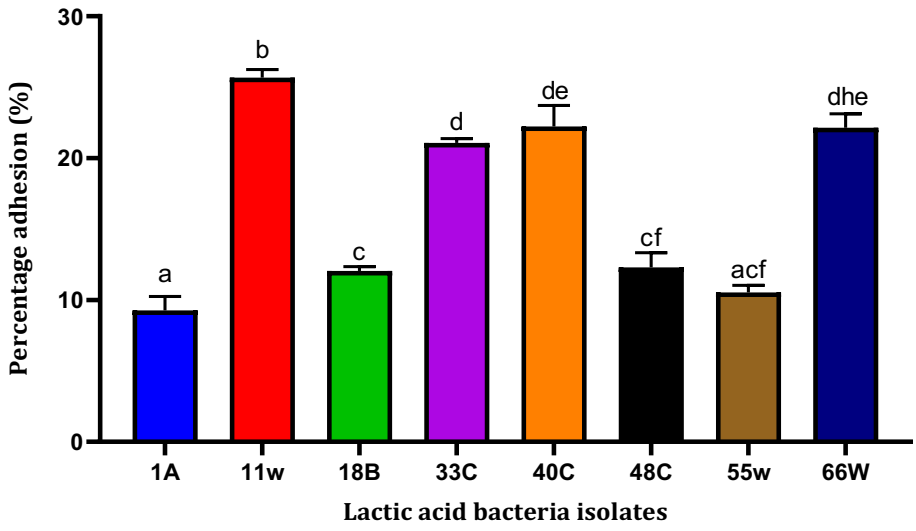

FIGURE 6
The ability of LAB to adhere to the HCT-116 cell lines. Results are expressed as mean of triplicate values ± standard deviation ($n = 3$). Bars with the same lower-case letters are not significant, whereas those with different lower-case letters are significantly different ($p < 0.05$).

Tryptophan production by LAB

Tryptophan is an essential amino acid that cannot be produced by humans but is produced by bacteria harboring in the gut. This metabolite is known to influence the psychology of mammals by inducing quality sleep, mood enhancement, strengthening pain tolerance, and having anti-depression and anti-anxiety effects. The bacteria were tested in the presence of glucose and 4% GOS. LAB isolate 11w produced 16.63 ± 2.25 μM of tryptophan and 40C produced 2.64 ± 0.5 μM when supplemented with 4% GOS, when compared to glucose in their media in 4% GOS supplementation (Table 2).

Molecular identification of probiotic candidates

The identification of LABs was done by 16S RNA sequencing and later confirmed by whole genome sequencing. Table 3 presents the identification and accession number belonging to the closest neighbor of the tested isolates. These isolates, which showed the closest match to the reference sequence in the NCBI GeneBank, were identified as *Lacticaseibacillus paracasei* (11w), *Lactiplantibacillus paraplantarum* (33E), *Lactiplantibacillus plantarum* (40C), *Lactiplantibacillus plantarum* (48C), and *Lactiplantibacillus paraplantarum* (66 W) (Figure 8).

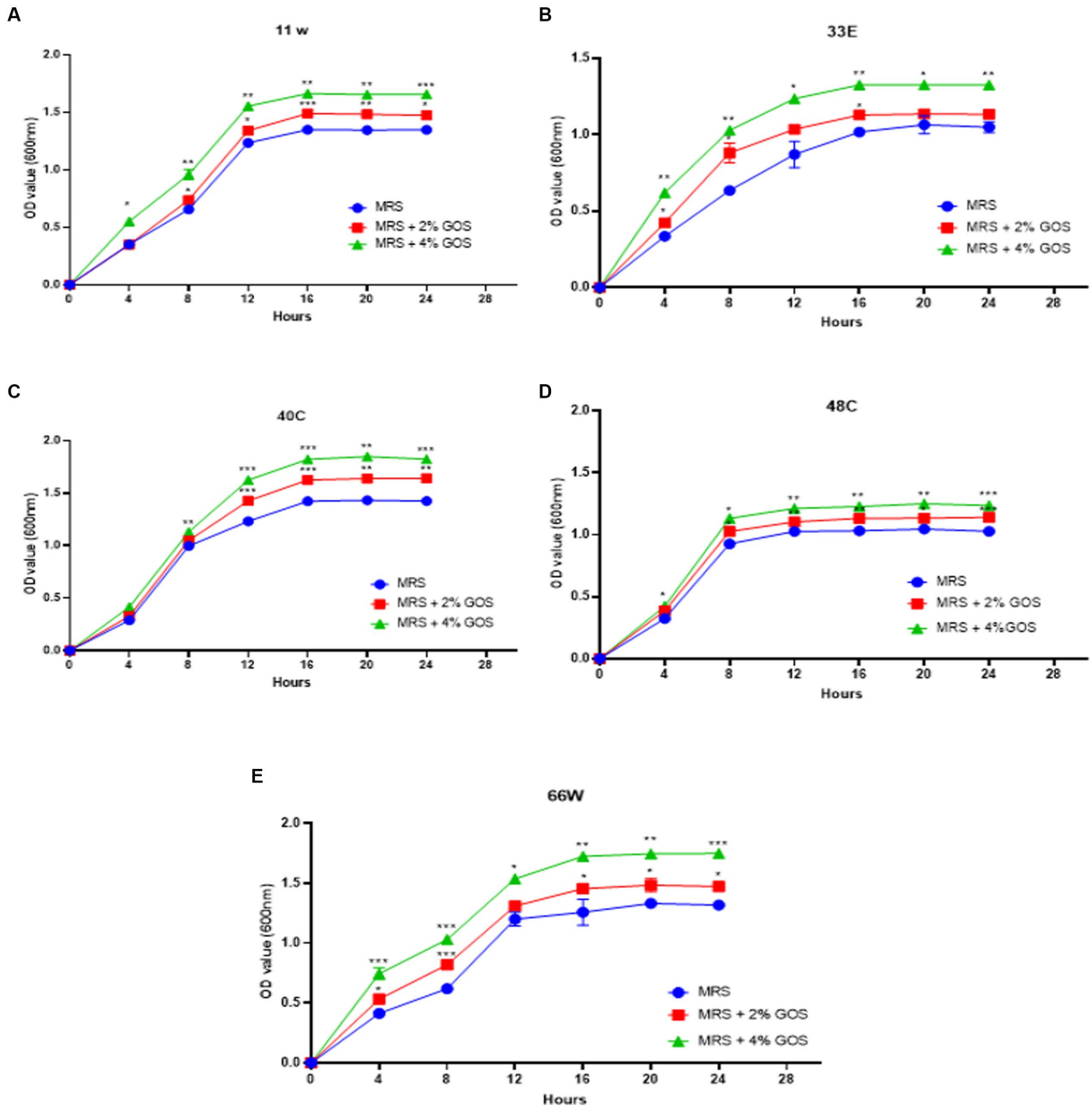

FIGURE 7
The growth curves of LAB isolates were measured at 600 nm with 2% GOS and 4% GOS. Growth curve of **(A)** 11w supplemented with 2% GOS compared with 11w with 4% GOS, **(B)** growth curve of 33E supplemented with 2% GOS compared with 33E 4% GOS, **(C)** growth curve of 40C supplemented with 2% GOS compared with 40C 4% GOS, **(D)** growth curve of 48C supplemented with 2% GOS compared with 4% GOS, and **(E)** the growth curve of 66 W supplemented with 2% GOS compared with 66 W 4% GOS. Each value represents the mean ± standard deviation of three independent readings ($n = 3 \pm$ SD). *Significant differences at $p < 0.05$, **significant difference at $p < 0.01$, and ***significant difference at $p < 0.001$.

TABLE 2 Tryptophan production by LAB CFS by HPLC-MS in 4% GOS.

LAB isolates	TSB + Glucose (μM)	TSB + 4% GOS(μM)
11w	9.95 ± 0.24	16.63 ± 2.25
33E	ND	ND
40C	ND	2.64 ± 0.56
48C	ND	ND
66 W	ND	ND

Values are expressed in mean ± standard deviation ($n = 2$). ND indicates no tryptophan production.

Safety analysis of probiotic candidates

Antibiotic susceptibility of probiotic candidates

All six LAB tested in this study were resistant to vancomycin, streptomycin, and kanamycin, whereas they were susceptible to chloramphenicol except for isolate 48C (Table 4). Most of the strains showed resistance to gentamycin except for isolate 48C (*L. plantarum* 48C). Of the eight antibiotics tested on *L. plantarum* 48C, it showed resistance to only three antibiotics and was susceptible to five. Due to its poor resistance to antibiotics, we did not include it in the subsequent safety steps.

TABLE 3 Species identification of LAB isolates by 16S rRNA sequencing.

Arbitrary name	Strain	Source	Closest Homolog	Similarity (%)	Closest homolog Gene Bank Accession Number (NCBI)
11w	*Lacticaseibacillus paracasei* 11w	Fermented pear	*Lacticaseibacillus paracasei* strain R094	99.71%	NR_025880.1
33	*Lactiplantibacillus paraplantarum* 33E	Fermented cherry tomato	*Lactiplantibacillus paraplantarum* strain DSM 10667	99.56%	NR_025447.1
40C	*Lactiplantibacillus plantarum* 40C	Fermented cherry tomato	*Lactiplantibacillus plantarum* strain JCM1149	99.56%	NR_117813.1
48C	*Lactiplantibacillus plantarum* 48C	Fermented cherry tomato	*Lactiplantibacillus plantarum* strain CIP 103151	99.71%	NR_104573.1
66 W	*Lactiplantibacillus paraplantarum* 66 W	Fermented cucumber	*Lactiplantibacillus paraplantarum* strain DSM 10667	99.85%	NR_025447.1

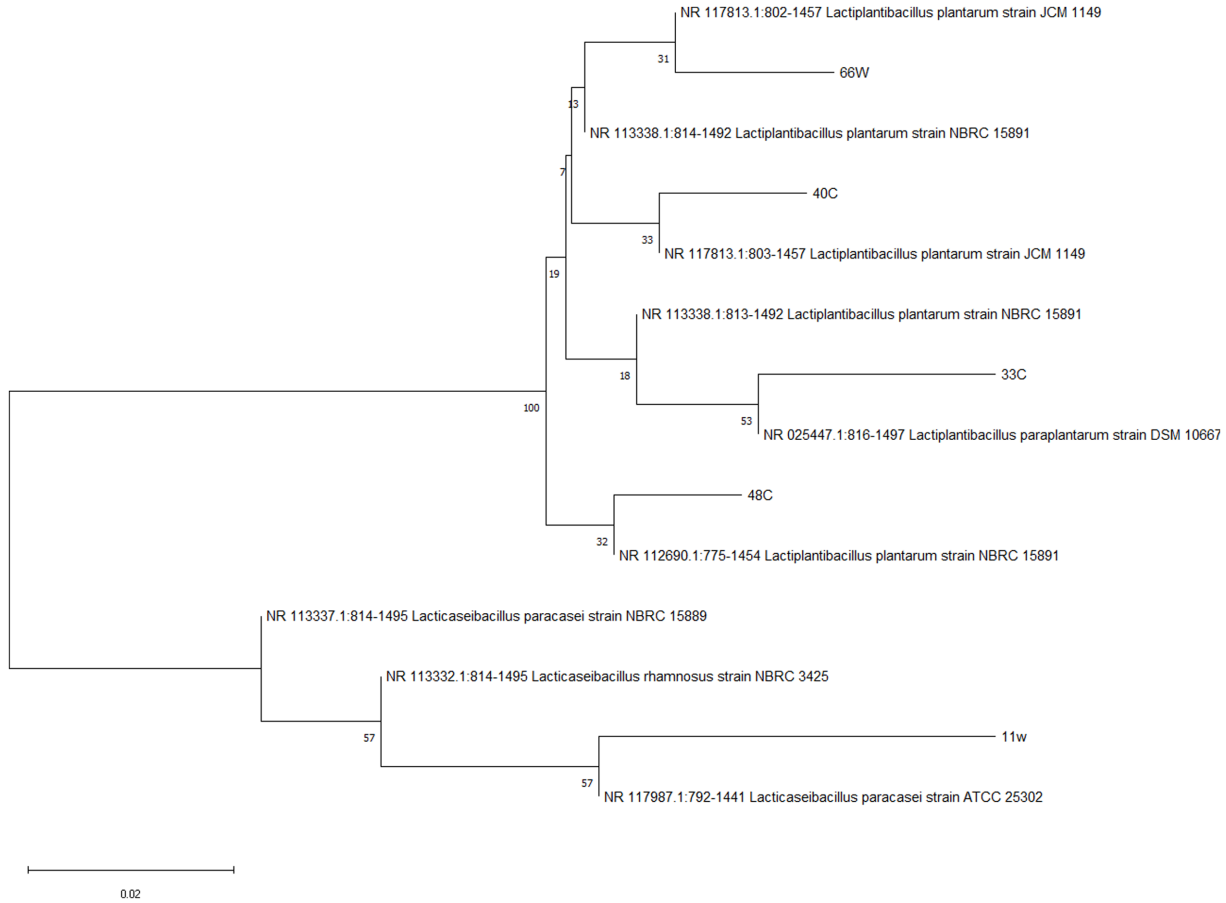

FIGURE 8
Neighbor-joining phylogenetic tree of LAB isolates based on 16S rRNA gene sequences. Bootstrap values based on 1,000 replications are listed as percentages at branch points. The scale bar represents a 0.02% divergence.

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TABLE 4 Susceptibility of LAB isolates to eight antibiotics (R, Resistant; S, Susceptible).

Cultures	Van	Strep	Kana	Gent	Novo	Ampi	Erth	Chlora
11w	R	R	R	R	S	S	S	S
33E	R	R	R	R	R	S	R	S
40C	R	R	R	R	R	S	R	R
48C	R	R	R	S	S	S	S	S
66 W	R	R	R	R	S	R	S	S

Van, vancomycin; Strep, streptomycin; Kana, kanamycin; Gent, gentamycin; Novo, Novobiocin; Ampi, Ampicillin; Erth, Erythromycin; Chlora, chloramphenicol.

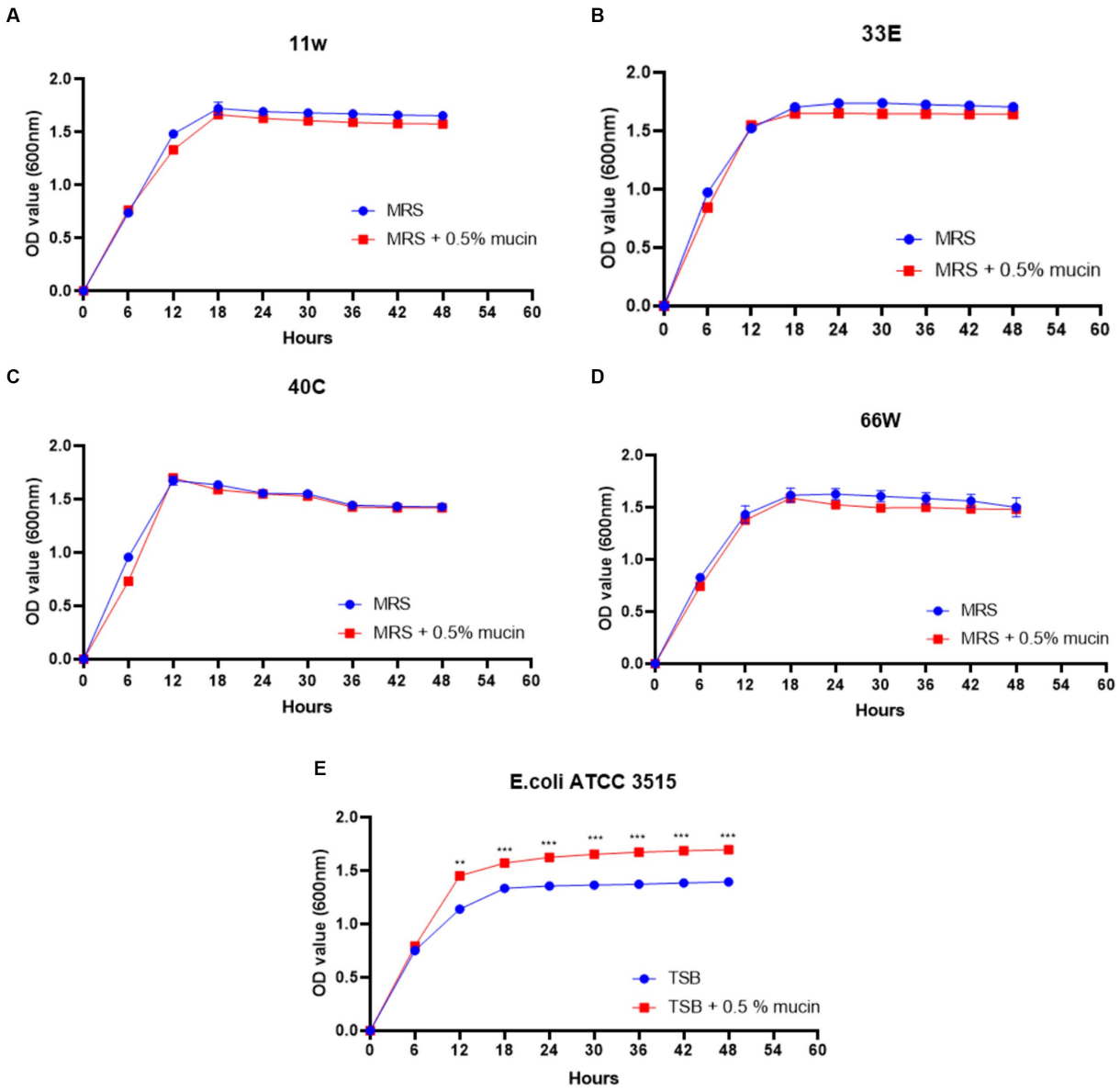

FIGURE 9
Mucin degradation ability of probiotic candidates. **(A)** growth curve of 11w (*Lacticaseibacillus paracasei* 11w) supplemented with 0.5% mucin compared with 11w without mucin, **(B)** growth curve of 33E (*Lactiplantibacillus paraplantarum* 33E) supplemented with 0.5% mucin compared with 33E without mucin, **(C)** growth curve of 40C (*Lactiplantibacillus plantarum* 40C) supplemented with 0.5% mucin compared with 40C without mucin, and **(D)** the growth curve of 66 W (*Lactiplantibacillus paraplantarum* 66 W) supplemented with 0.5% mucin compared with 66 W without mucin, **(E)** growth curve of *E. coli* ATCC 3515 supplemented with 0.5% mucin compared with *E. coli ATCC 3515* without mucin. *Significant differences at $p < 0.05$, **significant difference at $p < 0.01$, and ***significant difference at $p < 0.001$.

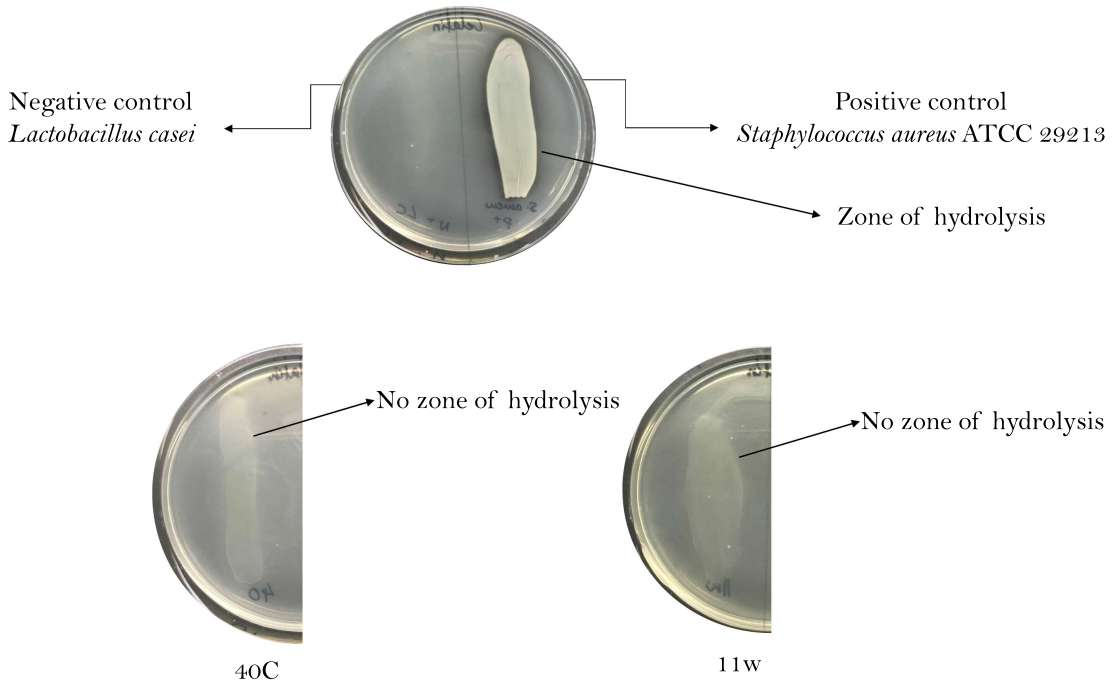

FIGURE 10
Gelatin degradation ability of LAB. Bacteria with gelatinase activity exhibited by positive control; *Staphylococcus aureus* ATCC 29213 shows clear zones around the bacteria colony, whereas the negative control *Lactobacillus casei* has no zone formation. 11w = *Lacticaseibacillus paracasei* 11w, and 40C = *Lactiplantibacillus plantarum* 40C. *Staphylococcus aureus* ATCC 6538 was used as a positive control. (+) indicates positive results and (–) indicates negative results.

Mucin degradation

As shown in Figure 9, the addition of mucin to TSB media extended the exponential phase of *E. coli* ATCC 35150 compared to when mucin was absent. Meanwhile, mucin supplementation did not increase the growth of any of the LAB strains.

Hemolytic activity

There was a hemolytic zone (β-hemolysis) around the *S. aureus* colony on blood agar. Two isolates, 33E (*L. paraplantarum* 33E) and 66 W (*L. paraplantarum* 66 W), showed mild hemolysis (α-hemolysis), i.e., greenish color due to surrounding the colonies. This indicates that 33E and 66 W exhibit subtle hemolysis of the erythrocytes. While the colonies of two isolates, 11w and 40C, had no zone effect (γ-hemolysis), indicating that they had no hemolytic activity. Only 11w and 40C proceeded to the subsequent safety steps of the study.

Gelatin hydrolysis

Gelatin is a natural biomacromolecule derived from collagen in animal skin, bones, and connective tissues. Gelatinase is an enzyme produced by several bacteria that is capable of degrading gelatin. Figure 10 shows that none of the LAB isolates degraded gelatin. Meanwhile, only *S. aureus* ATCC 29213 (positive control) showed gelatin degrading ability, as it showed a clear zone of inhibition around the colony. Three isolates did not have the property of breaking down gelatin, thus, they were considered safe.

Investigation for plasmids in LAB

Plasmids can be transferred from one bacterium to another through the conjugation process. The antibiotic resistance of one bacterium can be acquired by pathogenic bacteria. Therefore, for this reason, plasmids were searched from the genome data by screening the contigs against the PlasmidFinder server 2.1 (https://cge.food.dtu.dk/services/PlasmidFinder/ assessed on 27.02.2023). Isolate 11w showed intrinsic resistance to the antibiotics (Supplementary Figure S1), whereas 40C harbored plasmid (Supplementary Figure S2).

Discussion

The human gastrointestinal tract has several enzymes and pH conditions that may inhibit microbial survival (Smith, 2003; Singh et al., 2022). Hence, probiotics must therefore survive these harsh conditions to elicit their health potential. In the current study, different probiotic LAB strains were isolated from Lithuanian fermented foods, and 23 morphologically different isolates were screened *in vitro* for survival in gastrointestinal conditions. Interestingly, after consumption, probiotics first encounter lysozymes in saliva, following through the hostile environment of the stomach and then to the intestine (Conway et al., 1987). Since the pH of gastric juice is between 1.5 and 3.5, it is necessary to test the survival ability of probiotic candidates in low-pH environments and pepsin (Ruiz-Pulido and Medina, 2021). Low pH values in the gastric juice impair the cell membrane and the cell wall of the bacteria; influencing the membrane pathway, leading to undesirable metabolic processes,

energy depletion, and finally cell death (Sengupta et al., 2013). Survival under acid conditions is executed by adapting to low pH through a mechanism called acid tolerance response (Wendel, 2022). In this study, *L. paracasei* 11w showed a strong survival rate of 67.61% in low pH and this is similar to an earlier study that reported that *L. paracasei* T40 isolated from Tenate cheese has strong resistance to low pH because it has a system that automatically transports protons and lactic acid to the cell exterior (Falfán-Cortés et al., 2022). Indeed, the acid tolerance of the bacterium could be attributed to the high number of membrane-bound H+-ATPase they possess (Guan and Liu, 2020). These H+-ATPases play critical roles in maintaining intracellular pH and hence protect the cells from acid damage (Guan and Liu, 2020). The survival ability of different LAB at low pH depends on their phenotype characteristics and environmental conditions (Ko et al., 2022). The resistance to low pH of these LAB is very important as they can survive and proliferate in harsh gastrointestinal conditions.

In addition to the low pH, probiotics must survive the harsh proteolytic ability of pepsin in the stomach. In the present study, 13 out of 23 isolates showed strong tolerance towards pepsin and low pH. Earlier studies showed that LAB such as *L. paracasei* and *P. pentosaceus* are resistant to pepsin (Mantzourani et al., 2019). The ability of LAB to survive in GIT could be due to the developed mechanisms to survive in acidic conditions by producing alkaline compounds in the cell cytoplasm, altering their cell envelopes, inducing stimulation of H+-ATPase, and/ or ingestion of protons inside cells (Ayyash et al., 2021). After exiting the stomach, probiotics enter the duodenum where they encounter bile salts and pancreatin. Pancreatin is a combination of digestive enzymes that is essential for digesting fats, proteins, and sugars, whereas, bile is a digestive fluid that solubilizes lipid and lipid-soluble vitamins for digestion (Begley et al., 2005; Prete et al., 2020). A criterion for probiotic bacteria selection is the ability to tolerate the presence of pancreatic enzymes in the gut. In our study, isolate *L. plantarum* 40C showed tolerance to pancreatin and this result is in agreement with earlier studies, which demonstrated that *L. plantarum* SAM2 was resistant to pancreatin (EL-Sayed et al., 2022). A high bile acid concentration is injurious to the microbiota and studies have shown that *Lactobacillus plantarum* growth can be retarded by bile (Prete et al., 2020). In this study, however, 12 strains showed resistance to a mixture of bile salts and pancreatin after 6 h of exposure (Figure 3). Different LAB have particular genes, such as the *ula*A *and ula*B genes (found in *L. plantarum* S83), which help them to tolerate different bile salt conditions. However, resistance to bile salts is strain-specific (Ruiz et al., 2013).

The antibacterial property of LAB is complex and multifarious, and they do this mainly by exhibiting antagonism against pathogen growth and binding (Hu et al., 2019). It was observed that LAB 25E showed a strong inhibition against *Staphylococcus aureus* and *Streptococcus pyogenes* (Gram-positive bacteria), as well as *S. typhimurium* and *K. pneumoniae* ATCC 13883 (Gram-negative bacteria). It has been suggested that the inhibitory effects of potential probiotic strains against Gram-positive pathogenic bacteria are more promising than Gram-negative pathogenic bacteria (Shahverdi et al., 2023). Pathogen inhibition by probiotics is usually done via the production of antimicrobial compounds (Śliżewska et al., 2021) such as organic acids (mainly acetic acid and lactic acid), hydrogen peroxide, and/ or bacteriocin (Zare and Lashani, 2018). Furthermore, the suppression of pathogenic bacteria by LAB can also be influenced by numerous chemical, physical, and nutritional environmental factors (Hung et al., 2021). Our findings are similar to those of previous studies that showed that CFS of *L. plantatrum* (Leslie et al., 2021) had antimicrobial activity against Gram-positive and Gram-negative bacteria. Similarly, numerous studies propose health benefits postulated by the intake of viable LAB strains, which were correlated with their antimicrobial potential such as modulation of microbiota, suppression and prevention of pathogens (Liu et al., 2021), and immune modulation of the human host (Cristofori et al., 2021).

TEAC helps to evaluate the antioxidant capacity of food, beverage, and nutritional supplements using Trolox as standard (Maksimović and Dragišić Maksimović, 2017; Xiao et al., 2020). In a recent study on corn milk fermented by 20 isolates of *Limosilactobacillus fermentum*, *L. fermentum* L15 exhibited the strongest TEAC of 0.348 ± 0.005 mmol/L (Xu et al., 2022). In this study, the CFS of the LAB samples were analyzed, and the results of the TEAC confirm the free radical scavenging ability of each isolate (Figure 4). Two isolates, 3A and 55w, had the strongest antioxidant capacities of 26.37 μg/mL and 26.06 μg/mL, respectively, which indicates that the LAB released antioxidant compounds in their supernatants during growth (Lu et al., 2021).

The ability of LAB to auto-aggregate and adhere to the colon has been reported as a good indicator of gut colonization (Benítez-Cabello et al., 2019; Zawistowska-Rojek et al., 2022). The auto-aggregation capacity of LAB has been associated with their ability to adhere to epithelial cells (Botta et al., 2014). LAB aggregation can form a barrier to exclude pathogenic strains from adhering to the GIT (Klopper et al., 2018). In this study, the auto-aggregation of the LAB isolates increased with fermentation time (Figure 5). This was similar to reports from other studies where *L. plantarum* CCMA 0743 and *L. paracasei* CCMA 0504 showed, respectively, 38.62% ± 2.56 and 45.36% ± 6.30 aggregation after 5 h of incubation (Fonseca et al., 2021). Further analysis using HCT116 cells showed that *Lacticaseibacillus paracasei* 11w had the strongest attachment ability relative to the other strains (Figure 6). In agreement with a previous study, our results showed that the ability of LAB strains to adhere to the HCT116 cell line was strain-specific and varied even within the same species (Du et al., 2022).

Earlier studies have shown that prebiotics affect the growth and metabolism of probiotics (Slizewska and Chlebicz-Wójcik, 2020), and the growth may vary depending on the concentrations of the prebiotic used (Delgado-Fernández et al., 2019). In the present study, 2% and 4% GOS administration showed a proliferation in the growth of LAB isolates, which can be due to different concentration administration of prebiotics (Figure 7). An increase in LAB growth in the presence of GOS has a beneficial role in the mitigation of different diseases and influences the maturation and protection of the gut barrier as well as has an effect on the overall balance of the immune system (Manzoor et al., 2022; Megur et al., 2022). On the other hand, GOS administration has been shown to increase the production of essential amino acids, such as tryptophan and histidine, in *in vivo* studies (Purton et al., 2021; Saleh-Ghadimi et al., 2022). Tryptophan is an essential amino acid required for cellular energy, mood, immunity, and sleep regulation (Davidson et al., 2022). LAB-derived tryptophan metabolites are essential signals for maintaining gut homeostasis (Klaessens et al., 2022). In a study on *L. plantarum* ZJ316, this particular strain produced the tryptophan-derived metabolite indole-3-lactic acid, which resulted in the modulation of the gut by hindering the growth of pathogenic bacteria (Zhou et al., 2022). In the present study, *L. paracasei* 11w showed the

production of tryptophan in the presence of glucose (9.95 ± 1.23 μM) as well as 4% GOS (16.63 ± 2.25 μM), and *L. plantarum* 40C showed production of 2.64 ± 0.56 μM in the presence of 4% GOS.

LAB are generally recognized as safe, but safety properties should be evaluated prior to administration (Ayivi et al., 2020). In this study, isolates that passed the screening and characterization steps were tested for their antibiotic resistance, hemolytic analysis, mucin degradation, and gelatinase activity. Susceptibility to antibiotics is an important criterion in selecting probiotic candidates. This is because of the possibility of horizontal gene transfer of antibiotic-resistant genes from probiotic candidates to pathogenic bacteria (Lastauskienė et al., 2021; Daliri et al., 2023b). In this study, all the strains were resistant to vancomycin, streptomycin, and kanamycin, and susceptible to chloramphenicol except for strain *L. plantarum* 40C, which showed resistance to chloramphenicol (Table 4). All the strains except 11w harbored plasmid pR18, which contains linA (a linomycin and ampicillin resistance gene). Meanwhile, only strain 66 showed resistance to ampicillin, whereas the other five strains were susceptible to ampicillin. It is likely that only strain 66 has an active linA gene, which might have resulted in its resistance to ampicillin. Therefore, no other antimicrobial factors were detected in the bacteria genome. The strains were likely intrinsically resistant to most of the antibiotics tested.

The GIT mucus layer serves as a barrier to prevent bacteria translocation, which could result in sepsis (Haussner et al., 2019). Due to this reason, the mucolytic potentials of the selected LAB were assessed *in-vitro* using *E. coli* ATCC 35150 (a known mucin-degrading bacterium) as a positive control. It was observed that the presence of mucin in MRS media did not improve the growth of the tested LAB, and this implies that the bacteria could not metabolize mucin as a carbon source for their growth. Meanwhile, as expected, *E. coli* ATCC 35150 grew better in the presence of mucin than in TSB media containing limited glucose (Figure 9).

Some bacteria are known to produce enzymes that break down phospholipids and cause rupture of the cell membrane of red blood cells (Lee et al., 2023). *S. aureus* is known for its hemolytic abilities (Zhang et al., 2016). In this study, two LAB isolates showed alpha hemolysis. The two LAB isolates, *L. paracasei* 11w and *L. plantarum* 40C, did not cause any lysis of the erythrocytes of sheep blood, and thus they have no hemolytic activities. This investigation corroborates well with previous findings that showed that *L. paracasei* and *L. plantarum* (Sun et al., 2022) were non-hemolytic.

Gelatinase is a Zn metalloproteinase secreted by pathogenic bacteria. Gelatinase hydrolyses gelatin (a structural protein in connective tissues) and it can effectively attack the host by digesting the protein components of tissue, so as to facilitate the spread of bacteria. In addition, bacteria that produce gelatinase have been shown to have high chances of translocation from the gut to the liver, spleen, heart, and mesenteric lymph nodes (Zhang et al., 2015). In the present study, we tested whether any of the LAB could hydrolyze gelatin. Probiotics must not cause gelatin liquefaction in the host by producing a gelatinase enzyme. In prior studies it is demonstrated that *Lacticaseibacillus paracasei* (Martín et al., 2023) and *Lactiplantibacillus plantarum* (Kwon et al., 2021) are gelatinase-free and can be proceeded as potential probiotics. In comparison to our study, two isolates *Lacticaseibacillus paracasei* 11w and *Lactiplantibacillus plantarum* 40C did not demonstrate hemolysis and had no gelatinase activity (Figure 10). Furthermore, the *L. plantarum* 40C isolate harbored plasmid in its genome, whereas *L. paracasei* 11w did not harbor any plasmid, which makes it the only safe option among all the 23 strains isolates tested from different Lithuanian fermented foods.

Conclusion

Fermented foods are rich sources of LAB (probiotic bacteria), as demonstrated in the present study. However, it is imperative for their potential use that their functional potentials are studied as well as their safety assessments. We have demonstrated that most of the LAB strains isolated from Lithuanian fermented foods survived under simulated gastrointestinal conditions.

However, only five isolates, namely, *L. paracasei* 11w, *L. plantarum* 40C, *L. plantarum* 48C, *L. paraplantarum* 33C, and *L. plantarum* 66 W, displayed pathogenic bacteria inhibition, antioxidant potential, strong adhesion to gut epithelia, and high tryptophan production. After a safety assessment of the five isolates, only *L. paracasei* 11w met the safety requirements. Though *L. paracasei* 11w has demonstrated strong probiotic potential in this study, further studies are required to establish its health-promoting effects in animal models.

Results from the current study indicate that, though LAB isolated from fermented foods may have promising probiotic and functional potentials, they may pose some risks when consumed. They must, therefore, be subjected to strict safety assessments before use.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

AM, TB, and JS: investigation. AM, ED, and EL: management. AM and ED: formal analysis. AM, ED, and AB: supervision, validation, and writing—original draft. AM, ED, AB, TB, JS, and EL: writing—review & editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1213370/full#supplementary-material

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Application of multi-functional lactic acid bacteria strains in a pilot scale feta cheese production

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Feta cheese is the most recognized Greek Protected Designation of Origin (PDO) product in the world. The addition of selected autochthonous lactic acid bacteria (LAB) strains to cheese milk as adjunct cultures is gaining more attention, since they can impact the nutritional, technological and sensory properties of cheeses, as well as improve the safety of the product. The aim of this study was to produce Feta cheese with enhanced quality and safety, and distinctive organoleptic characteristics by applying autochthonous lactic acid bacteria (LAB) with multi-functional properties as adjunct cultures. Feta cheeses were produced with the commercial lactococcal starter culture and the addition of 9 LAB strains (*Lactococcus lactis* SMX2 and SMX16, *Levilactobacillus brevis* SRX20, *Lacticaseibacillus paracasei* SRX10, *Lactiplantibacillus plantarum* FRX20 and FB1, *Leuconostoc mesenteroides* FMX3, FMX11, and FRX4, isolated from artisanal Greek cheeses) in different combinations to produce 13 cheese trials (12 Feta trials with the adjunct LAB isolates and the control trial). In addition, Feta cheese manufactured with FMX3 and SMX2 and control Feta cheese were artificially inoculated (4 log CFU/g) with *Listeria monocytogenes* (a cocktail of 4 acid or non-acid adapted strains). Cheese samples were monitored by microbiological and physicochemical analyses during ripening, and microbiological, physicochemical, molecular and sensory analyses during storage at 4°C. The results showed that after manufacture, the LAB population was *ca.* 9.0 log CFU/g at all samples, whereas during storage, their population declined to 6.5–7.0 log CFU/g. In the *Listeria* inoculated samples, *Listeria* was absent after 60 days (end of ripening) and after 90 days in the adjunct culture, and in the control trials, respectively. Moreover, the addition of selected strains, especially *Lcb. paracasei* SRX10, led to cheeses with desirable and distinctive organoleptic characteristics. Furthermore, randomly amplified polymorphic PCR (RAPD-PCR) molecular analysis confirmed that the multi-functional LAB strains were viable by the end of storage. Overall, the results of this study are promising for the use of autochthonous strains in various combinations with the commercial starter culture to satisfy industry requirements and consumer demands for traditional and high added value fermented products.

KEYWORDS

feta cheese, autochthonous cultures, multi-functional LAB, *Listeria monocytogenes*, sensory characteristics, organic acids, RAPD-PCR

1. Introduction

Feta cheese, the "white gold" of Greece, is the most recognized traditional Greek food in the world. In 2002, Feta was approved as a Greek Protected Designation of Origin (PDO) product, according to Commission Regulation (EC) No. 1829/2002. Feta is a white soft cheese, ripened in brine and made exclusively of ewe's milk or from a mixture of ewe's and goat's milk (up to 30%) (Commission Regulation (EC) No. 1829/2002). Many researchers have examined the microbial "fingerprint" of Feta PDO cheese, and their results showed that LAB are the predominant bacterial community with great diversity. For instance, Michailidou et al. (2021) reported that the genera *Lactococcus, Lactobacillus, Streptococcus* and *Leuconostoc* were the most prevalent in Feta cheeses during their shelf life. In addition, in a variety of commercial Feta cheese samples, *Lactococcus lactis*, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (commonly used as starter cultures) were the most abundant species detected (Papadakis et al., 2021; Papadimitriou et al., 2022). Papadakis et al. (2021) also reported that although the starter cultures were found in the highest abundance, the nonstarter LAB species diversity was significant, revealing a high degree of polymorphism at the strain and species level inside the cheese microbiota.

A common practice for cheese production when using pasteurized milk is the addition of commercial starter cultures to standardize the fermentation process and/or improve the sensory characteristics. However, these cultures are responsible for a flat organoleptic pattern rather than a rich traditional taste (Leroy and De Vuyst, 2004; Psoni et al., 2006), so the addition of selected autochthonous LAB strains to cheese milk as adjunct cultures is gaining more attention, as it is reflected in the recent scientific literature (de Souza and Dias, 2017; Gaglio et al., 2021; Bettera et al., 2022). These added microorganisms can release intracellular enzymes during ripening and storage, which mainly impact the nutritional, technological and sensory properties of cheeses (Escobar-Zepeda et al., 2016; Fox et al., 2017). For instance, González and Zarate (2012) studied a local Tenerife cheese produced with commercial starter and with the addition of a mix of indigenous strains (*Lc. lactis* subsp. *lactis* TF53, *Lactiplantibacillus plantarum* TF191 and *Leuconostoc mesenteroides* subsp. *mesenteroides* TF756). Their results showed that the cheese made with the autochthonous LAB had enhanced sensory features compared to the cheese made with the CS. Bancalari et al. (2020) evaluated the effect of an indigenous *Lacticaseibacillus paracasei* strain to be used as an adjunct culture for the production of an Italian semi-hard cheese. A clear differentiation in terms of aromatic profile, color, texture and sensorial perception was observed in the experimental cheese (with the *Lcb. paracasei* 4321) in contrast to the control. Finally, many researchers have used indigenous LAB strains as adjunct cultures to produce Feta-type cheese, and they observed an improvement in the textural and sensory characteristics (Michaelidou et al., 2003; Manolaki et al., 2006; Dimitrellou et al., 2014; Mantzourani et al., 2018).

Cheese is considered a safe food product, however, contamination with foodborne pathogens can occur at various stages of the food processing environment (Kousta et al., 2010). The most common foodborne pathogens present in white brined cheeses (WBC) are *Listeria monocytogenes*, enteropathogenic *Escherichia coli* O157:H7, *Yersinia enterocolitica* and *Salmonella* spp. *Staphylococcus aureus* (Bintsis and Papademas, 2002; Papadimitriou et al., 2022). Among those microorganisms, *L. monocytogenes* can be a serious concern since there are known outbreaks with fatalities originating from a variety of soft and semi-hard cheeses (Campagnollo et al., 2018). The presence of *L. monocytogenes* in dairy products indicates inadequate pasteurization of milk (Cadavez et al., 2019) or post-pasteurization contamination due to poor hygienic conditions during cheese-making (Kapetanakou et al., 2017). *L. monocytogenes* has been detected in WBC in different countries including Jordan (Osaili et al., 2012), Turkey (Arslan and Özdemir, 2008) and Greece (RASFF, 2022; Papadimitriou et al., 2022). In Jordan, the overall prevalence of *Listeria* spp. in WBC was 27.1%, with *L. monocytogenes* possessing 11.1% in the samples, while other species shared lower percentages (*Listeria grayi, Listeria innocua, Listeria ivanovii*, *Listeria seeligeri*, and *Listeria welshimeri*) (Osaili et al., 2012). In Turkey, the incidence of *Listeria* spp. in a homemade WBC was 9.2% for *L. monocytogenes*, while the other aforementioned species shared lower percentages (Arslan and Özdemir, 2008). In Greece, the presence of *L. monocytogenes* in Feta cheese was confirmed through shotgun metagenomics (Papadimitriou et al., 2022), while Rapid Alert System for Food and Feed (RASFF) issued an alert notification about the presence of *L. monocytogenes* in Feta cheese (RASFF 2022). However, LAB cultures can improve the safety of cheeses by inhibiting the growth of several pathogens. In fact, a large number of LAB strains have proven anti-listerial activity and many of them have been applied as adjunct cultures in cheese fermentation to control *L. monocytogenes* (Silva et al., 2018). Papadopoulou et al. (2018) studied the fate of artificially inoculated *L. monocytogenes* during ripening and storage of Feta cheese without or with the addition of *Lpb. plantarum* T571 as an adjunct culture and noticed that the added LAB strain inactivated the pathogen in shorter time compared to the control samples. Based on their results, it was evident that the pathogen cannot survive well in the Feta environment, however, it can still be present during the early storage of this product, posing a threat to the consumers. Prezzi et al. (2020) evaluated the effect of *Lacticaseibacillus rhamnosus* GG on the growth of and *L. monocytogenes* inoculated on the surface of Minas Frescal cheese during storage at 7°C. Their results showed a decrease of 1.1–1.6 log CFU/g in the population of *L. monocytogenes* after 21 days of storage. Morandi et al. (2019) investigated the effect of LAB strains on the growth of artificially inoculated *L. monocytogenes* on Gorgonzola rinds during ripening and noticed that, *Lc. lactis* (FT27, N16 and SV77) inhibited the growth of *L. monocytogenes* after 50 days of ripening.

Organic acids are present in dairy products due to bacterial growth during the fermentation processes and as a result of normal biochemical metabolism (breakdown of milk components, i.e., proteins, fat, and lactose) (Izco et al., 2002). The most common organic acids in cheeses are lactic, acetic, and propionic acids (Hayaloglu, 2016). The level of each organic acid can be affected by the starters, the adjunct cultures and the non-starter lactic acid bacteria (NSLAB) present in the cheeses (Skeie et al., 2001; Manolaki et al., 2006). Overall, the presence of organic acids in dairy products is important for both flavor and preservation. In more detail, organic acids help to lower the pH of dairy products, which inhibits the growth of harmful bacteria and other spoilage microorganisms, thus extending their shelf life. They also contribute to the characteristic flavor and texture of dairy products (Jo et al., 2018; Pisano et al., 2020; Ahmed et al., 2021).

TABLE 1 Combinations of the adjunct LAB isolates and the commercial starter culture used for the manufacture of Feta cheese.

Feta case	Starter/adjunct culture combination	Ratio %
Feta 1	Commercial Starter culture *Lacticaseibacillus paracasei* SRX10	50:50
Feta 2	Commercial Starter culture *Lactiplantibacillus plantarum* FRX20	50:50
Feta 3	Commercial Starter culture *Leuconostoc mesenteroides* FRX4	50:50
Feta 4	Commercial Starter culture *Lactococcus lactis* SMX2	50:50
Feta 5	Commercial Starter culture *Leuconostoc mesenteroides* FMX3	50:50
Feta 6	Commercial Starter culture *Lactobacillus plantarum* FB1, *Levilactobacillus brevis* SRX20	50:25:25
Feta 7	Commercial Starter culture *Lacticaseibacillus paracasei* SRX10, *Levilactobacillus brevis* SRX20	50:25:25
Feta 8	Commercial Starter culture *Leuconostoc mesenteroides* FMX11, *Lactiplantibacillus plantarum* FB1, *Lacticaseibacillus paracasei* SRX10	50:17:17:17
Feta 9	Commercial Starter culture *Lactococcus lactis* SMX16, *Leuconostoc mesenteroides* FRX4	50:25:25
Feta 10	Commercial Starter culture *Lactiplantibacillus plantarum* FB1, *Lacticaseibacillus paracasei* SRX10	50:25:25
Feta 11	Commercial Starter culture *Leuconostoc mesenteroides* FMX11, *Levilactobacillus brevis* SRX20, *Lactiplantibacillus plantarum* FRX20	50:17:17:17
Feta 12	Commercial Starter culture *Leuconostoc mesenteroides* FMX3, *Lactococcus lactis* SMX2	50:25:25
Control	Commercial Starter culture	100

Based on the above, the aim of this study was to develop Feta cheese with enhanced quality and safety, as well as distinctive organoleptic characteristics. Thus, 9 indigenous LAB strains (in mono and mixed combinations) previously isolated from Greek cheeses and characterized with good technological (ability to produce β-galactosidase, partial proteolysis, no diacetyl production, and exopolysaccharides (EPS) production and functional characteristics (good survival rates at low pH and at bile salts) and/or attributed good organoleptic properties when used as adjunct cultures *in situ* (yogurt) (Kamarinou et al., 2022), were incorporated as adjunct cultures in the production of 12 different Feta trials, to evaluate their effect on the microbiological, physicochemical and sensory characteristics of the cheeses, during ripening and storage compared to the control trial. The strains that exhibited good technological and functional properties but did not attribute positive organoleptic properties to yogurt (Kamarinou et al., 2022) were applied only as mixed cultures. Furthermore, in selected cheese trials, the changes in organic acids were evaluated using high-performance liquid chromatography (HPLC), whereas the recovery rate of the added LAB strains was investigated using Random Amplified Polymorphic (RAPD-PCR). Finally, in order to monitor the survival of the pathogen *L. monocytogenes*, Feta cheese produced with a co-culture of *Leuconostoc mesenteroides* FMX3 and *Lactococcus lactis* SMX2 with *in vitro* anti-listerial activity (Kamarinou et al., 2022) was artificially inoculated with a four (4) strain cocktail of *L. monocytogenes*.

2. Materials and methods

2.1. Preparation of LAB adjunct cultures

Nine LAB strains (*Lc. lactis* SMX2, SMX16, *Levilactobacillus brevis* SRX20, *Lpb. plantarum* FRX20, FB1, *Ln. mesenteroides* FRX4, FMX3, FMX11, and *Lcb. paracasei* SRX10), which have been previously isolated from traditional Greek cheeses (Kamarinou et al., 2022), were used as adjunct cultures in Feta cheese production in various combinations as mono or co- cultures (Table 1). The different trials were selected by preliminary experiments producing yogurt with ewe's and goat's milk (in a ratio of 80:20) to mimic the consequent Feta cheese substrate and implementing sensory evaluation, according to the procedure described by Kamarinou et al., 2022. After this procedure, seven strains (SMX16, SRX20, FRX20, FB1, FRX4, FMX11, and SRX10) were selected to be applied as adjuncts in Feta monocultures in mono- or co-cultures (combinations of 2 or 3 strains), due to their contribution to the aforementioned yogurts sensory profiles (see Supplementary Figure S1). The co-cultures were

developed by combining only different microbial species. Additionally, two (2) strains (SMX2, FMX3) with anti-listerial activity *in vitro* (Kamarinou et al., 2022), were applied as adjuncts in Feta in mono-cultures or in combination. The strains were revived from a stock culture stored at −80°C and were grown aerobically in the appropriate culture media, i.e., de Man, Rogosa and Sharpe broth (MRS) (LAB094, LABM, Lancashire, United Kingdom) for lactobacilli and M17 broth (BK012HA, Biokar Diagnostics, Allonne, France) for lactococci, at 30°C and 37°C for 24 h, respectively. Fresh monocultures of the isolates were harvested by centrifugation (5,000 g, 10 min, 4°C), washed twice with ¼ strength Ringer's solution (LMNCM0191K, Neogen Corporation, Michigan, USA) and resuspended in 10 mL Ringer's solutions. To prepare the cocktail of the strains, the cells were mixed in the appropriate ratio (Table 1). Inoculum size was confirmed before use by the serial dilution method on MRS ISO agar (NCM0190, Neogen Corp.) and M17 Agar (4017194, Biolife, Milano, Italy).

2.2. Pilot-scale feta cheese production

Feta cheeses were produced at our industrial partner, a Greek dairy industry located in Trikala, Greece (Hellenic Dairies S.A, Trikala, Greece). The cheese production followed the typical industrial procedure. In brief, pasteurized ewe's and goat's milk (in a ratio of 80:20) was inoculated with the commercial starter culture (Choozit™ MA 11 LYO 250 Danisco Culture Unit - DCU, Danisco, Copenhagen, Denmark) containing *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* (control trial), or with the addition of the selected LAB strains in mono and co- cultures along with the commercial starter culture in a ratio of 1:1, as shown in Table 1. The inoculum of all added cultures in the pasteurized milk was approximately 7 log CFU/mL. After milk coagulation, the curd was cut and transferred to molds, where dry salt was added and the molds were turned upside down at appropriate times for 24 h to ensure even drainage. On the following day, the cheese pieces with pH 4.7 ± 0.04 (*ca.* 2 Kg from each mold) were placed in metal vessels of 16 kg, brine (7% w/v NaCl) was added and ripening followed. Ripening took place in two periods: (a) 7 days at 18°C (1st ripening), and (b) 53 days at 4°C (2nd ripening). After ripening, cheese of *ca.* 2 kg (pH 4.4 ± 0.1) was placed in plastic containers and fresh brine (7% w/v NaCl) was added. Samples were then stored at 4°C for 120 days (expiration date of commercial products). In total, 13 different trials were produced (12 Feta trials with the adjunct LAB isolates and the control trial-Table 1). The experiment was repeated twice, i.e., 2 different seasonal batches (September and January) with 3 replicates studied at every time point for each trial. The samples after production were coded and stored in the appropriate temperatures throughout ripening and storage. To ensure the randomization of the samples, an online computer software (Social Psychology Network, Middletown, CT, United States) was used, which is available at "https://www.randomizer.org/".

2.3. Preparation of acid and non-acid adapted *Listeria monocytogenes* cells

A cocktail of four *L. monocytogenes* strains was used to artificially inoculate Feta cheese, as described below. The strains FMCC-B-129 and FMCC-B-133 were provided by the laboratory of Food Microbiology and Biotechnology of the Agricultural University of Athens and the strains DSM15675 and DSM19094 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strains were revived from a stock culture stored at −80°C and grown aerobically in 10 mL Tryptic Soy Broth (TSB) (LAB004, Neogen Corp.) and incubated at 37°C for 18 h. A subculture of each strain was prepared in fresh 10 mL TSB supplemented with 0.6% yeast extract (MC001, Neogen Corp.) (TSB-YE) and incubated at 37°C for 18 h. 100 μL of each overnight culture of *L. monocytogenes* was inoculated in fresh 10 mL TSB-YE broth and incubated at 37°C, until reaching a 0.15 optical density (OD) (Cataldo et al., 2007). All the strains reached a population of *ca.* 6 log CFU/mL in the aforementioned OD. The OD was monitored using a microplate reader (SpectraMax Plus 384 Microplate Reader, Molecular Devices, Sunnyvale, VA, United States) by measuring the absorbance at 600 nm. Then, the cells were harvested by centrifugation (5,000 g, 10 min, 4°C) and each pellet were resuspended in fresh TSB-YE broths with pH adjusted to 5.0 using 85% lactic acid (acid adapted) or in TSB-YE broths with pH adjusted to 7.0 (non-acid adapted/control) and subsequently incubated at 37°C for 1 and 3 h (Cataldo et al., 2007). In order to determine the acid tolerance response (ATR), the cells were harvested by centrifugation (5,000 g, 10 min, 4°C), resuspended in fresh TSB-YE broths with pH adjusted to 3.5, and subsequently incubated for 2 h at 37°C, while their survival was determined by enumeration on Tryptic Soy Agar (TSA) (BK047HA, Biokar Diagnostics) plates (24–48 h at 37°C). Acid response (AR) was determined by centrifuging each overnight culture of *L. monocytogenes* in TSB-YE (5,000 g, 10 min, 4°C). Then bacteria were resuspended in TSB-YE acidified to pH 3.5 for 2 h at 37°C and their survival was determined by enumeration on TSA plates (Cataldo et al., 2007).

The *Listeria* strains (acid or non-acid adapted) were mixed in equal volumes to achieve a cocktail culture with a final population of 4 log CFU/g in the cheese. Each of the *Listeria* strains (acid or non-acid adapted) population, as well as the inoculum size of the cocktail culture were confirmed before use by the serial dilution method on Palcam Agar (BK145HA, Biokar Diagnostics) with a Palcam selective supplement (BS00408, Biokar Diagnostics).

2.4. Feta cheese inoculation with *Listeria monocytogenes* strains

The day after Feta cheese production (day 1), samples without (control samples) or with the addition of *Ln. mesenteroides* FMX3 and *Lc. lactis* SMX2 (Feta L12, Table 2), were artificially inoculated with the pathogen in the Institute of Technology of Agricultural Products Laboratory (Hellenic Agricultural Organization - DIMITRA, Lycovrissi, Greece). Each cheese piece of 2 kg (22 × 10 × 10 cm) was inoculated with *L. monocytogenes* (cocktail of four strains, acid or non-acid adapted, Table 2) using sterilized syringes, to reach a final population of 4 log CFU/g in the cheese. The inoculum was injected in each of the four 22 × 10 sides of the cheese pieces. In this respect, the syringe was slowly immerged in every 10 cm^2 of each side in a depth of 3.5 cm, releasing 250 μL of inoculum (6 log CFU/mL). Consequently, the exact procedure of cheese ripening as with the non-inoculated (without *L. monocytogenes*) cheeses was followed.

TABLE 2 Feta cheese trials artificially inoculated with acid or non-acid adapted *Listeria monocytogenes* (4 strain cocktail).

Feta case (Code names)		Starter/adjunct culture combination	Ratio (%)	*Listeria monocytogenes* Inoculum
Batch 1	Batch 2			
FLA1	FLA2	Commercial Starter culture *Ln. mesenteroides* FMX3 *Lc. lactis* SMX2 (Feta L12)	50:25:25	Acid adapted cells Cocktail of 4 strains FMCC-B129 FMCC-B133 DSMZ15675 DSMZ19094
CLA1	CLA2	Commercial Starter culture	100	
FLN1	FLN2	Commercial Starter culture *Ln. mesenteroides* FMX3 *Lc. lactis* SMX2 (Feta L12)	50:25:25	Non-acid adapted cells Cocktail of 4 strains FMCC-B129 FMCC-B133 DSMZ15675 DSMZ19094
CLN1	CLN2	Commercial Starter culture	100	

2.5. Microbial enumeration

The microbial analyses of the Feta cheese samples (samples without the pathogen) were carried out at six time points (1, 60, 90, 120, 150 and 180 days after cheese production). The samples inoculated with *L. monocytogenes* (Feta L12 and the control) were examined at 12 time points (1, 5, 8, 15, 22, 36, 50, 60, 90, 120, 150 and 180 days after cheese production). In brief, 25 g of triplicate Feta cheese samples (from each batch) were aseptically added to 225 mL of sterilized ¼ Ringer's solution and subjected to serial dilutions in the same diluent. 0.1 or 1 mL samples were spread or poured on the following agar media; Plate Count Agar (4021452, Biolife) for Total Aerobic Viable Counts (TVC), incubated at 30°C for 48–72 h, MRS ISO Agar overlaid with the same medium and incubated at 30°C for 48–72 h for the enumeration of mesophilic LAB, M17 Agar overlaid with the same medium incubated at 37°C and 42°C for 48–72 h for the enumeration of lactococci, Baird Parker Agar (LAB285, LABM) with Egg Yolk Tellurite (X085, LABM) for coagulase-positive staphylococci incubated at 37°C for 48 h, Violet Red Bile Glucose Agar (402.85, Biolife) for *Enterobacteriaceae* counts overlaid with the same medium and incubated at 37°C for 18–24 h, Xylose Lysine Deoxycholate (CM0469, Oxoid) for *Salmonella* spp. incubated at 37°C for 16–18 h, Palcam Agar with Palcam selective supplement for the enumeration of *L. monocytogenes* incubated at 37°C for 24 and 48 h (to lower the detection limit of the pathogen to 1.0 log CFU/g, 1 mL of the samples' first decimal dilution was spread onto three Petri dishes). To ensure the absence of *Salmonella* spp. and *L. monocytogenes* enrichment was applied in the samples according to ISO 6579-1:2017 and ISO 11290-1:2017, respectively. (International Organizational for Standardization, 2017a,b).

2.6. Physicochemical analysis

The pH of cheese samples was measured at the same time intervals as with the microbiological analysis by immersing the penetrating electrode (FC200B, HANNA instruments, Woonsocket, RI, USA) in the cheese samples. pH measurements were carried out using a digital pH-meter (HI2211, pH/ORPMeter, HANNA instruments).

The cheeses (1st batch) were also analyzed in the premises of Hellenic Dairies S.A. for their physicochemical characteristics, i.e., protein, fat, moisture and sodium chloride, during storage using FoodScan™ Lab (Routine Analysis Software, FOSS, Hillerød, Denmark), with three measurements per sample being obtained each time.

2.7. Sensory evaluation

A group of 10 people, previously trained in evaluating dairy products (staff from the Institute of Technology of Agricultural Products of Hellenic Agricultural Organization - DIMITRA and the industry), were selected to form the sensory panel. The selection criteria for the sensory panel are thoroughly explained in the previous work by Papadopoulou et al. (2018). The evaluation was carried out under artificial lighting conditions in individual booths in the sensory analysis room located in the Institute of Technology of Agricultural Products. All panelists provided consent prior to their participation in the study. For sensory evaluation, non-pathogen inoculated cheeses from all Feta trials were used. Initially, the overall quality in terms of like or dislike was evaluated (total appearance, total aroma, total taste, and total texture) and then the specific sensory attributes were examined. The specific indicators of each sensorial attribute included appearance, i.e., white color, cracks, and holes on cheese; aroma; i.e., butter, and acid, taste; i.e., bitter, acid, salty, sweet, and texture; i.e., consistency, graininess, and hardness. Data were obtained through a 10-cm hedonic scale, where the scale direction was from left to right with increasing intensities, i.e., weak to strong, little to much, etc. (Papetti and Carelli, 2013).

2.8. Determination of organic acids by high-performance liquid chromatography

A high-performance liquid chromatography (Agilent 1200 series, Agilent Technologies, Palo Alto, CA, USA) was performed to determine the concentration of organic acids in six selected cheese trials. Duplicate samples (36) of the aforenoted trials were examined

at different time points (0, 60, 90, 120, 150 and 180 days). To prepare the samples for HPLC analysis, the method of Argyri et al. (2011) was followed. In brief, 2 g of each cheese sample was homogenized with 4 mL sterile deionized water for 2 min, centrifuged (9,000 × g, 5 min, 4°C) and then 20 μL of trifluoroacetic acid (Trifluoroacetic Acid for UV, PanReac AppliChem, Darmstadt, Germany) was added at 2 mL of the supernatant. Samples were again centrifuged at the same conditions and the collected supernatant was filtered through a 0.22 filter (Millipore PVDF Syringe filter/L0.22 μm). For HPLC analysis, 20 μL of the sample was injected into the injection port. Elution was carried out using a 0.009 N H_2SO_4 solution as the mobile phase, with a flow rate of 0.7 mL/min and an oven temperature of 65°C (Skandamis and Nychas, 2001). The separation was performed isocratically using a cation exchange Repromer H column (9 μm 300 × 7.8 mm, Dr. Maisch GmbH, Germany). The HPLC system was equipped with a G1314A Variable Wavelength Detector and a Rheodyne HPLC manual injector (Model 7,010). The UV Absorbance was monitored at a wavelength of 210 nm and the Agilent ChemStation software (Agilent ChemStation Software B.02.01.SR1 Revision, Agilent Technologies, Inc., Santa Clara, CA, USA) was used for data acquisition and processing. As reference substances, solutions (1 to 0.001% w/v) of citric, lactic, tartaric, acetic, malic, propionic, oxalic, succinic and formic acids (HPLC grade) were used.

2.9. LAB survival and strain differentiation using RAPD -PCR

At the end of storage (180 day), a total of 58 LAB strains were randomly collected from the highest countable dilution (≥6 log CFU/g) of MRS or M17 agar plates of 5 Feta cheese trials (Feta 1, 9, 10, 11 and 12) according to Papadopoulou et al. (2018). All the recovered isolates were stored at −80°C in MRS or M17 broths supplemented with 30% (v/v) glycerol until use. DNA was extracted according to Cocolin et al. (2007). RAPD-PCR was performed using the universal oligonucleotide primer M13 (5′-GAGGGTGGCGGTTCT-3) according to Giraffa et al. (2000). PCR amplicons were stained with GelRed (6X GelRed® Prestain Loading Buffer with Tracking Dye, Biotium, California, USA) and visualized on agarose gel (2%) using a Gel Doc System (Gel Doc Go Imaging System, Bio-Rad, Hercules, CA, USA). As a DNA molecular weight marker, a 1 kbp plus DNA Ladder (New England Biolabs, Ipswich, MA, USA) was included. The obtained profiles of the recovered LAB strains were compared with the known LAB profiles (commercial cheese starters and adjunct LAB strains) (Supplementary Figure S2).

2.10. Statistical analysis

Data were indicated as means; mean ± SD of three replicates × two different batches. Microbiological, physicochemical, sensory and HPLC results were analyzed for statistical significance with one-way analysis of variance (ANOVA) (StataMP17, StataCorp LLC, Texas, United States). Significance was established at $p < 0.05$. *Post hoc* analysis- Tukey's HSD test was performed to determine significant differences among results. Additionally, data mining and interpretation of the sensory data and the HPLC data were performed using the online platform MetaboAnalyst v3.0″ (McGill University-Xia Lab, Montreal, QC, Canada, www.metaboanalyst.ca, access date: 20/10/2022). Two datasets were created (i) using the sensory scores (X variables) and the different storage days (60–180 day) (Y variables) of the 13 cheese trials and (ii) the organic acid compounds after HPLC analysis (X variables) and the different storage days (1–180 day) (Y variables) of the six cheese trials. The data sets were uploaded to the online platform and first transformed via scaling (autoscale) and then data analytics (partial least squares–discriminant analysis [PLS-DA] and hierarchical cluster analysis, i.e., heatmaps and dendrograms) were performed on the datasets.

3. Results and discussion

3.1. Microbial enumeration of the 13 feta trials

The changes in the populations of mesophilic LAB and lactococci in the 13 Feta trials during ripening and storage are presented in Figures 1A,B, respectively. The population of mesophilic LAB on the 1st day after cheese production was found between 8.5–9.2 log CFU/g

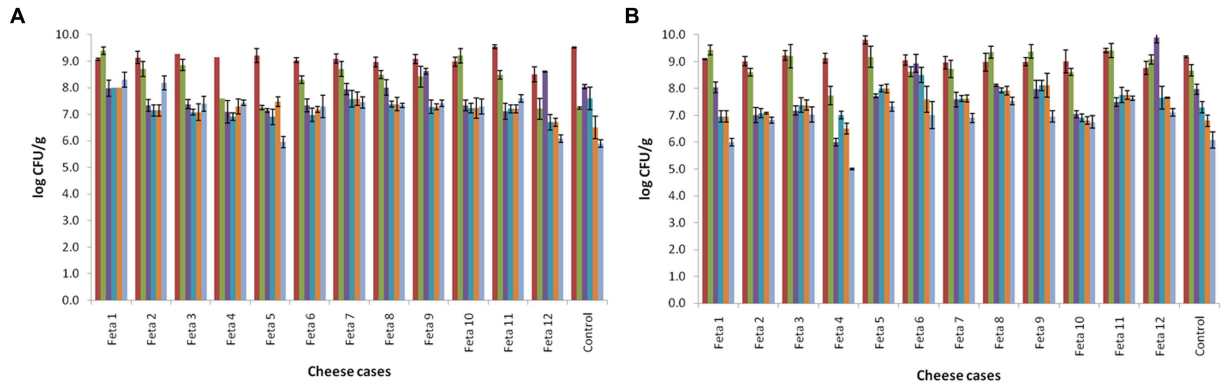

FIGURE 1
Population of mesophilic lactobacilli **(A)** and lactococci **(B)** of Feta cheese trials (according to Table 1), on the 1st (1d), 60th (60d), 90th (90d), 120th (120d), 150th (150d) and 180th day (180d) after cheese production. The bars represent the mean values ± standard deviations.

in the trials produced with the adjunct cultures (Feta 1–12), while for the control, the population was found at 8.3 ± 0.02 log CFU/g ($p < 0.05$). At the end of ripening (60th day), the population in all trials decreased by approximately 0.5 log CFU/g. During storage, an additional decrease was observed in the population of mesophilic LAB in all Feta trials ($p < 0.05$). In more details, Feta trials with the adjunct cultures displayed a population of 6.0 to 8.3 log CFU/g, while the control showed a population of 5.9 ± 0.14 log CFU/g at the end of storage ($p < 0.05$). Regarding the lactococci, their population in all Feta trials the 1st day after cheese production was found between 8.7–9.0 log CFU/g ($p < 0.05$). During ripening, their population decreased to levels of 7.5–8.9 log CFU/g, depending on the trial ($p < 0.05$) (Figure 2). During storage, an additional decrease was observed, where the population levels in Feta trials 1–12 decline to final population of 6.5 to 8.1 log CFU/g (depending on the trial), while in the control, a lower population was observed (6.1 ± 0.31 log CFU/g) ($p < 0.05$). TVC followed the population of the dominant microbiota for each trial, i.e., mesophilic LAB and/or lactococci and thus presented similar counts (data not shown). Finally, the rest of the examined microbiota, i.e., *Enterobacteriaceae*, staphylococci, yeasts and molds, were always below the detection limit of the enumeration method. Pathogenic microorganisms, such as *L. monocytogenes* and *Salmonella* spp. were absent after enrichment.

The reduction trend of LAB (lactococci and lactobacilli) during ripening and storage is in accordance with previous studies on pilot Feta cheese production. For instance, Papadopoulou et al. (2018) studied the microbiota of an industrial Feta produced with the addition of *Lpb. plantarum* T571 and observed that during cold storage of Feta, the population of mesophilic lactobacilli and lactococci decreased in all samples. In the same study, it was also observed that the addition of the adjunct culture did not influence the level of lactobacilli and lactococcci counts throughout ripening and storage, a result that has also been found in other studies concerning Feta cheese (Dimitrellou et al., 2014; Papadopoulou et al., 2018). On the contrary, other researchers noted that in Feta-type cheeses that were produced with the use of *Lcb. casei* ATCC 393 as adjunct culture, the population of lactobacilli was significantly increased after 120 days of storage, while the population of lactococci did not significantly differ among the samples (Terpou et al., 2018a). The absence of *Enterobacteriaceae*, staphylococci, *Listeria* spp., *E. coli* and yeasts and molds in the present study verifies the high hygienic conditions during the production of cheese.

3.2. Feta inoculated with *Listeria monocytogenes*

Before Feta inoculation, the survival of the *Listeria* strains after acid adaptation (pH 5) for 1 and 3 h and subsequent incubation at lethal pH (3.5) for 2 h was evaluated. Among the 4 tested strains, the survival of strain FMCC-B-129 showed a strong ATR for both 1 and 3 h of incubation at moderate pH (pH 5), which subsequently resulted in elevated survival (89.9 and 91.2% of the bacterial population, respectively) after exposure to lethal pH. However, the other 3 strains exhibited a better survival rate (81.5–84.9%) only after 1 h of exposure to moderate pH, so the 1 h of acid adaptation was selected for this study. Regarding the AR, results demonstrated that the survival of the strains was very low (<30%), except for the strain FMCC-B-129 which showed a moderate survival (52%). The changes in the population of *L. monocytogenes* and TVC during ripening and storage in *Listeria* inoculated cheeses (Feta L12 and the control samples) are presented in Figure 2.

After the inoculation of the fresh cheese (day 1) with the acid and non-acid adapted *Listeria* strains, the population of the pathogen was estimated at approximately 3.30–3.65 log CFU/g, depending on the trial ($p < 0.05$). During ripening, it was observed that the *Listeria* population had a decreasing trend at all samples (the *p*-values for the 1st, 5th, 36th, 50th, and 60th days were found to be less than 0.05, however, for the 8th, 15th, and 22nd days after production, the p-values were $p \geq 0.05$), but this decrease was more intense at Feta trial 12 inoculated with both

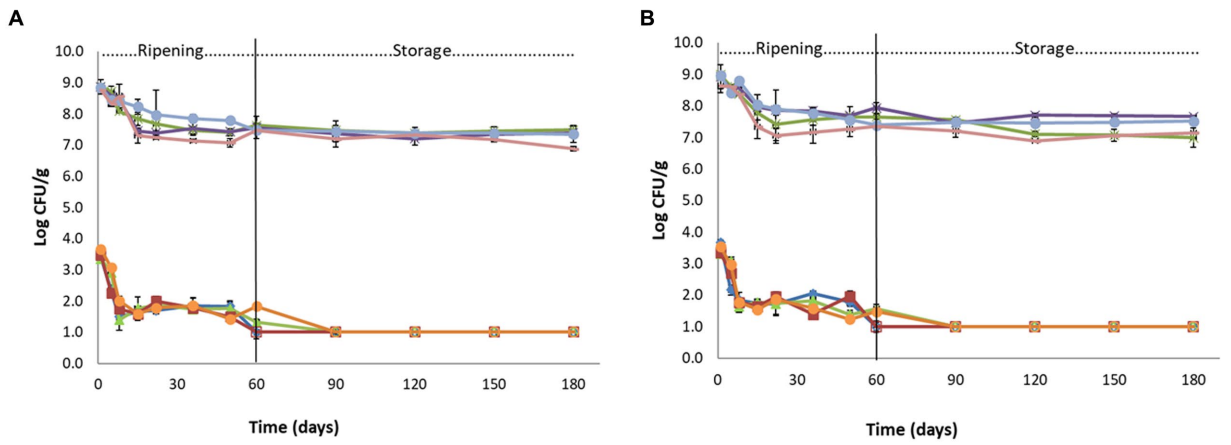

FIGURE 2
Population of *L. monocytogenes* (♦): FLA1, (■): FLN1, (▲): CLA1, (●): CLN1 and population of TVC: (✱): FLA1, (●): FLN1, (X): CLA1, (–): CLN1 in Feta cheeses (Feta case L12 and the control) during ripening and storage (Batch 1 - **A**, Batch 2 - **B**). Open symbols indicate absence of *L. monocytogenes* after applying enrichment method. The bars represent the mean values ± standard deviations. FLA: Feta L12 inoculated with acid adapted *Listeria* strains, FLN: Feta L12 inoculated with non-acid adapted *Listeria* strains, CLA: Control Feta inoculated with acid adapted *Listeria* strains, CLN: Control Feta inoculated with non-acid adapted *Listeria* strains.

acid and non-acid adapted *Listeria* strains at both batches (Table 2). In more detail, until the 50th day of ripening, the population of *L. monocytogenes* decreased in all Feta L12 trials (acid and non-acid adapted strains) by 1.4–2.3 log CFU/g, depending on the trial. At the 60th day (end of ripening), the pathogen was not detected after enrichment in all trials of Feta L12, while the population of the pathogen in the control trials was estimated at 1.3–1.8 log CFU/g (Figures 2A,B).

During storage at 4°C, the pathogen was not detected after enrichment in Feta L12 trials (inoculated with acid and non-acid *Listeria* strains), as reported above, whereas in corresponding control trials, the pathogen was not detected after enrichment from the 90th day (storage) onwards.

The initial population of mesophilic lactobacilli and lactococci in Feta L12 and the control samples (day 1) inoculated with *Listeria* was 8.5–9.2 log CFU/g, in both batches. The results showed that both populations gradually decreased during ripening and storage in both trials, a result that was also observed in the non-inoculated Feta trials. More specifically, the population of mesophilic lactobacilli was reduced by 1.2–1.5 log CFU/g by the end of storage, while the population of lactococci was reduced by 1.0–1.9 log CFU/g in all trials (data not shown). The TVC population was found to be 8.6–9.0 log CFU/g, in both batches at day 1, while during ripening and storage the population gradually decreased (Figures 2A,B). The other examined microbiota (i.e., *Enterobacteriaceae*, staphylococci, yeasts and molds) was found below the detection limit of the enumeration method (data not shown). The initial pH values of the cheeses were 4.6–4.7 on the 1st day of production in all samples, while during ripening and storage the pH values of all samples showed a slight decrease and obtained pH values of 4.2–4.4 (data not shown).

To prevent the growth and spread of *Listeria monocytogenes* in cheese, it is important to implement good manufacturing practices and food safety measures (Kapetanakou et al., 2017). Additionally, natural antimicrobial ingredients such as bacteriocins or probiotics are becoming increasingly popular as a way to prevent the growth of *L. monocytogenes* in food products, including cheese (Reis et al., 2012). Up to date, many studies have confirmed the anti-listerial activity of selected autochthonous LAB strains in cheeses (Dal Bello et al., 2012; Coelho et al., 2014; Morandi et al., 2019; Ivanovic et al., 2021). The use of such strains can lead to an effective reduction of the pathogen below the limit imposed by Commission Regulation (EC) No. 1441/2007 (<2.0 log CFU/g). Papadopoulou et al. (2018) studied the effect of *Lpb. plantarum* T571 on the fate of *L. monocytogenes* strains during ripening and storage of Feta. Their results showed that the added strain inactivated the pathogen during ripening and in a shorter time compared to the control samples. Pisano et al. (2022) investigated the use of three *Lc. lactis* strains and two *Lpb. plantarum* strains in limiting the growth of *L. monocytogenes* on laboratory fresh cheeses. These researchers reported that the examined strains could be used as protective cultures since they reduced the pathogen population by 3–4 log CFU/g compared to the control. Finally, in the present study, it deemed necessary to study the fate of *L. monocytogenes* strains after acid adaptation due to the high acid concentrations present in the Feta matrix (pH 4.6). However, the results of this study demonstrated that during ripening, significant differences ($p < 0.05$) were found between the treatments in the survival of the pathogen, while during storage, no significant differences ($p \geq 0.05$) were found. Similar results were found in an Italian-style soft cheese, in which the acid adaptation of *Listeria* cells provided the same population levels at the examined cheeses as with the non-acid adapted strains (Cataldo et al., 2007).

3.3. Physicochemical analysis

pH values decreased in all trials from a range of 4.6–4.8 after production to 4.2–4.6 at the end of ripening, while a post-acidification was observed during storage, reaching values of 4.1 to 4.4 depending on the trial (data not shown). In more detail, until the end of ripening, Feta 12 demonstrated a pH of *ca.* 4.6, Feta 2, 6 and 10 had pH 4.4, Feta 1 and 8 had pH 4.3 while Feta 3, 4, 5, 7, 9, 11 had a similar pH value to the control trial (*ca.* 4.5). Until the end of storage, a reduction in pH values was observed in all Feta trials. More specifically, Feta 4, 5 and 9 had a pH of ca 4.4, Feta 1, 2, 8 and 10 had a pH value of 4.2, while Feta 3, 6, 7, 11 and 12 had a similar pH value to the control trial (*ca.* 4.3). The results obtained by FoodScan in the food industry at the beginning and end of storage for the 13 Feta trials, revealed minor differentiations between the trials (Supplementary Table S1).

According to Abd El-Salam and Alichanidis (2004) pH of Feta cheese reaches 4.4–4.6 during ripening at 16–18°C and its moisture decreases to <56% before release to the market. According to Kourkoutas et al. (2006), Dimitrellou et al. (2014) and Papadopoulou et al. (2018), who studied Feta and WBC produced with the addition of adjunct LAB strains, a post-acidification is usually apparent with pH reaching values of 4.4 or lower, a result that was also observed in the current study.

3.4. Sensory evaluation

The results of the sensory evaluation of the 13 Feta trials (without the pathogen) are presented in Figure 3. All the results are presented in comparison to the control trial. In general, the nine selected LAB strains mixed in 12 different combinations produced cheese products with sensorial characteristics that differentiated them from the control.

From the evaluation of the total appearance, it was observed that all the Feta trials were considered acceptable until the end of storage. More specifically, until the 90th day all Feta trials were evaluated with high scores (approximately 9/10). However, from 120th up to the 180th day, there was a variation in scores. Feta trials 1, 2, 7, 8, 9, 10, 11, and 12 received scores between 7 and 8/10, while Feta trials 3, 4, 5, and 6, along with the control, were rated with 6/10. At the end of storage, the Feta trials 4 and 12 were scored higher (8/10) than the control (7/10) and the Feta trials 2, 3, 5, 6, and 7 were scored lower (approximately 5/10) than the control. This decline in scores was attributed to the presence of irregular holes and cracks, as opposed to the small mechanical openings found in the control ($p \geq 0.05$).

The evaluation of the overall aroma indicated that all the Feta trials were acceptable until the 90th day, with the majority of them receiving higher scores (ranging from 7 to 8/10) compared to the control (rated at 6/10). On 120th day, the Feta trials 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, and 12, received higher scores (ranging from 6 to 8 out of 10) compared to the control (rated at 5/10), while the Feta trial 4 was considered unacceptable (score 4/10). On 150th day, Feta trials 1, 6, 7, 9, 10 and 11 were evaluated with scores of up to 7/10. Feta trials 8, 12 and control received scores of 5/10, while the Feta trials 2, 3, 4 and 5 were considered unacceptable with scores ranging from 1 to 4/10. At the end

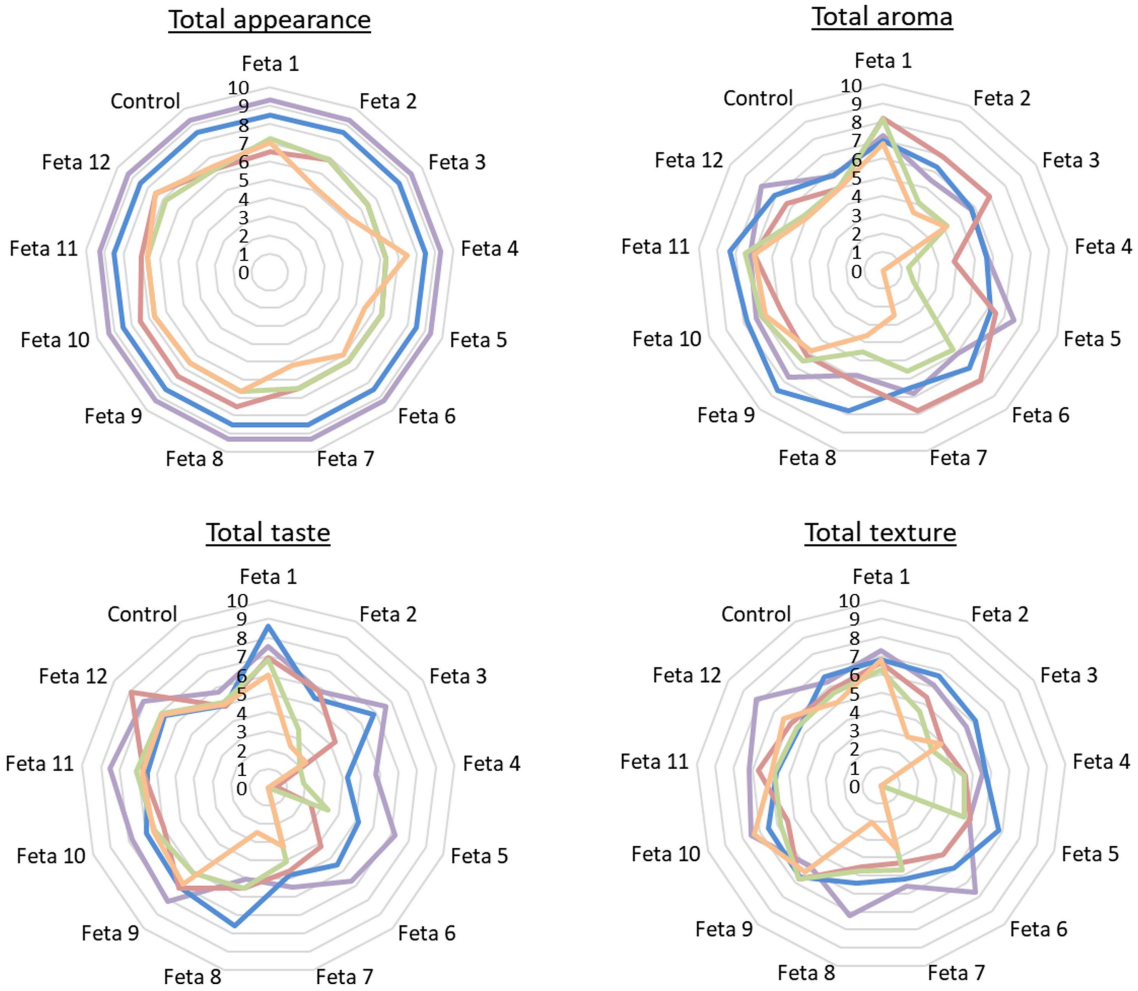

FIGURE 3
Sensory analysis of all Feta cheese trials on 60th day (60d), 90th day (90d), 120th day (120d), 150th day (150d) and 180th day (180d) after cheese production regarding total appearance, total aroma, total taste and total texture.

of storage period (180th day), Feta trials 1, 9, 10 and 11 were evaluated with scores of 6–7/10. Feta trials 12 and control received scores of 5/10, while the remaining trials were considered unacceptable ($p < 0.05$). The presence of the intense acid aroma in several trials during the later stages of storage had significantly impacted their overall aroma scores.

Regarding the evaluation of the overall taste, the majority of Feta trials were scored higher than the control until the 120th day, while later the Feta trials showed variability in their scores. In more detail, at the end of ripening the Feta trials 1, 3, 5, 6, 9, 10, 11, and 12 received scores from 7 to 9/10, Feta trials 2, 4 and control 6/10, while the Feta trials 7 and 8 receive scores 5/10. On 90th day Feta trials 1, 3, 6, 7, 8, 9, 10, 11, and 12, received scores ranging from 7 to 9/10, Feta trials 2, 5, 7 and control rated at 5/10, while the Feta trial 4 was consider unacceptable (score 4/10). On 120th day, Feta trials 1, 2, 8, 7, 9, 10, 11 and 12 were evaluated with scores from 7 to 9/10. Feta trials 7 and control received scores of 5/10, while the Feta trials 3, 4, 5 and 6 were considered unacceptable with scores ranging from 0 to 4/10. On 150th day Feta trials 1, 9, 10 and 11 were evaluated with higher scores (6–7/10) compared to the control (5/10), while the others scored lower than the control rated from 0 to 4/10. At the end of storage (180th day), Feta trials 1, 9, 10, 11 and 12 were evaluated with higher scores (from 6 to 7/10) compared to the control (5/10), while the remaining trials were considered unacceptable (scores from 0 to 3/10) ($p < 0.05$). The Feta trials that were found unacceptable displayed intense increase of characteristics "bitter" and "acid."

Finally, regarding the overall texture, trials 3, 4, 7 and 8 were considered unacceptable from the 120th day, while the Feta trials 2, 5, and 6 were considered unacceptable from the 150th day and beyond. Their exclusion was attributed to their bad consistency and granular texture ($p < 0.05$). The trials with high scores (from 7 to 8/10) in the feature "overall texture" were Feta 1, 9, 10, 11, 12. From the above results, it is apparent that the Feta trials 1, 9, 10, 11 and 12 were acceptable until the end of the storage and were graded with better scores in the evaluation of all sensorial characteristics compared to the control. Most of these trials included cheeses produced with mixed cultures (i.e., Feta 9, 10, 11 and 12), while Feta 1 was the only cheese trial that was produced with the monoculture of *Lcb. paracasei* SRX10. A detailed description of the selected Feta trials sensory characteristics after ripening and at the end of storage is presented in Supplementary Figure S3.

With regards to sensory discrimination, the results from hierarchical cluster analysis (Supplementary Figure S4) showed that the cheeses were

classified into three (3) main clusters. One cluster contained all cheese trials from early storage (60 and 90 days after production), while the other two (2) clusters contained samples from middle (120 and 150 day) and final (180 day) storage days. Trials from 180 days were mostly classified in one cluster except for 4 trials, while cheese trials from 120 and 150 days were rather mixed. No discrimination between different cheese trials were found, yet the cheeses were mostly classified between early and middle/final storage times.

Feta has pleasant organoleptic properties that has worldwide acceptance. Some of the organoleptic characteristics of this cheese are the salty and slightly acidic taste, smoothy, creamy and firm texture, which make the cheese easy to cut into slices (Sarantinopoulos et al., 2002). As regards texture, no gas holes should be present, but irregular small mechanical openings are desirable (Anifantakis, 1991; Abd El-Salam et al., 1993). In this study, the presence of uneven holes and cracks in many Feta trials can be attributed to the heterofermentative nature of the added LAB strains (i.e., *Ln. mesenteroides* FRX4, FMX3 and *Lvb.* brevis SRX20), which are able to produce gas, a property that was previously studied in Kamarinou et al. (2022). Several LAB strains have been used as adjunct cultures in the manufacture of different types of cheese and their addition may affect the organoleptic characteristics of the products thus, a sensory assessment is crucial. Ahmed et al. (2021) used *Lcb. casei* LBC, *B. bifidum BB* and *Lc. lactis* subsp. *lactis* LL as adjunct cultures in the manufacture of low-fat Feta-type cheese. Their results showed that the cheese with the added strains presented a good appearance and color throughout storage (Ahmed et al., 2021). Mantzourani et al. (2018) demonstrated that the addition of *Lcb. paracasei* SP3 in a Feta-type cheese, improved the organoleptic characteristics of the product. In the study of Papadopoulou et al. (2018), it was shown that the addition of *Lpb. plantarum* T571 as an adjunct culture in a pilot Feta cheese production influenced positively the overall texture of the cheese during storage at 4°C. In contrast, the study of Yousefi et al. (2021) showed that no significant differences were found between the sensory characteristics of ultrafiltered Feta-type cheese containing the adjunct culture *Lvb. brevis* KX572376 and the control. Many of the aforementioned studies indicated that selected LAB strains can lead to the production of cheeses with desirable organoleptic characteristics, a result that is in accordance with the current study at selected Feta trials (1, 9, 10, 11 and 12). As regards the differentiation between the mono and mixed cultures, it was observed that the addition of *Ln. mesenteroides* FMX3 as a monoculture (Feta 5) affected the attributes of Feta cheese negatively, while the addition of *Ln. mesenteroides* FMX3 as a mixed culture (Feta 12) improved the organoleptic characteristics of the cheese. Due to the heterofermentative metabolism of this genus, Server-Busson et al. (1999) suggested that *Leuconostoc* isolates should be used in combination with acid-producing lactococci, as starters or adjuncts, and not as monocultures. Finally, it should be highlighted that the use of *Lcb. paracasei* SRX10 as an adjunct monoculture in the present study, produced Feta cheese with high scores (>6) in all of the examined organoleptic characteristics (appearance, aroma, taste and texture), This result was also confirmed by other studies, where it was shown that the addition of several *Lcb. paracasei* strains as adjunct cultures enhanced the flavor formation and increased the overall quality of cheeses, i.e., in a short-ripened Italian semi-hard cheese (Bancalari et al., 2020), in a Feta-type cheese (Bintsis and Robinson, 2004) and in a WBC (Terpou et al., 2018b).

3.5. HPLC analysis of organic acids

Six (6) cheese trials (Feta 1, 9, 10, 11, 12 and control) were selected after the sensory analysis (scores above 5 in the hedonic scale) to undergo HPLC analysis. The analysis of the chromatograms from HPLC resulted in the discrimination of three organic acids, i.e., citric acid, lactic acid and acetic acid. The other examined acids, i.e., tartaric, succinic, formic, and propionic, were not detected in any of the samples. In details, citric acid concentrations were estimated to be between 2.14–3.33 mg/g after cheese production (day 1) and reduced during storage in trials 1, 9, 10 and 12 to 0.1–0.7 mg/g, except in trial 9, where the acid was not detected during storage ($p < 0.05$). Lactic acid concentration displayed the highest levels in all cheese trials and was estimated between 16.3–22.0 mg/g after cheese production, depending on the different trials ($p < 0.05$). During storage, a small decline was observed in all trials, where at the end of storage, the concentration of lactic acid was estimated between 9.94–14.82 mg/g. Acetic acid concentration was found in traces throughout ripening and storage ($p \geq 0.05$). After ANOVA, no significant difference was found between the concentrations of organic acids in the six Feta trials ($p \geq 0.05$). As regards storage discrimination, the results from PLS-DA showed that citric and lactic acids were highly correlated (VIP scores>1) with fresh samples (day 1) (data not shown). The obtained dendrogram (Supplementary Figure S5) showed that the samples were classified into two main clusters. One cluster contained samples from days 1 and 60, and the second cluster contained samples from days 90, 120, 150 and 180 days. Figure 4 represents the heatmap obtained from the combination of storage days and the organic acid concentration. It can be observed that all acids were highly correlated with the ripening period rather than the storage period. To conclude, it was observed that the changes were more evident due to the different storage days than the different adjunct mono and mixed cultures used in the six examined trials, a trend that was also evident for the sensory changes in the 13 cheese trials, as described previously.

According to previous studies, the organic acids that are present in Feta and Feta-type cheeses during ripening are mainly citric, acetic and lactic acids (Papadakis and Polychroniadou, 2005; Manolaki et al., 2006). Lactic acid, the major product of sugar fermentation by LAB, is the most abundant organic acid present in cheeses, where its concentration can vary from 1.94 to 17.4 mg/g in aged cheeses (Kaminarides et al., 2007). Among the other detected acids, citric acid is usually lost in the whey during cheese production, however, the retained citric acid may be metabolized to volatile compounds (i.e., acetic acid, diacetyl, etc.) by some LAB, such as *Lc. lactis* subsp. *diacetylactis* and *Leuconostoc* spp. (Izco et al., 2002; Kaminarides et al., 2007; Jo et al., 2018). Consequently, acetic acid can contribute to cheese flavor (Jo et al., 2018). As shown above, Feta trials showed a high concentration of lactic acid, in contrast to the citric and acetic acids that were detected at lower levels. Comparable results were observed by Akalin et al. (2002), who studied the organic acids in WBC and demonstrated that lactic acid accounted for about 95% of the total organic acid content. In addition, Papadopoulou et al. (2022) investigated the changes in organic acids during storage of a semi-hard bovine sliced cheese supplement with a cocktail of 3 LAB strains and their results showed that lactic acid concentration displayed the highest levels. In accordance with the above, in the current study, it was also observed that the concentration of citric and lactic acids was found to be higher after cheese production (day 1) and declined during storage.

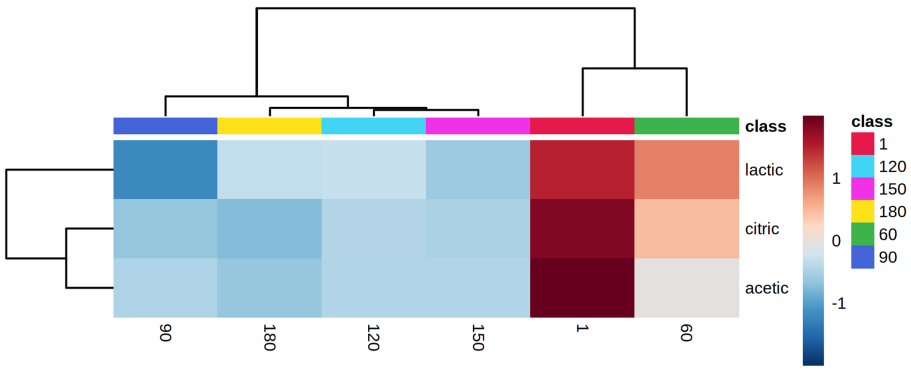

FIGURE 4
Hierarchical clustering result shown as a heatmap of organic acids associated with the different days (ripening and storage) of the 6 Feta cheese trials (1, 9, 10, 11, 12 and the control). Ward-linkage clustering was based on the Euclidean correlation coefficients of the identified organic acids in the different cheese trials. The color scale represents the scaled abundance of each variable, with red indicating high abundance and blue indicating low abundance.

Finally, Sabbagh et al. (2010) observed that during the ripening of Iranian low-fat white cheese, the concentrations of lactic and acetic acids decreased, a result that was in accordance with the current study.

3.6. RAPD-PCR for monitoring LAB survival and strain differentiation

The population of LAB was maintained at over 6.0 log CFU/g until the end of storage in the 5 selected cheese trials (Feta 1, 9, 10, 11 and 12; received scores over 5 in the hedonic scale), as shown previously in Figure 1. So, it was crucial to verify the presence and survival of the added multi-functional LAB strains in a harsh environment, such as the Feta cheese. The observation of RAPD-PCR profiles revealed that the total of the recovered isolates in Feta cheese trials belonged to the adjunct and CS cultures, but in different percentages, depending on the different Feta trials (Figure 5). In brief, in Feta 1, *Lcb. paracasei* SRX10, which was used as an adjunct monoculture, survived well until the end of storage and showed a high recovery percentage (59%) compared to the CS culture (41%). Similarly, in Feta 9, the adjunct mixed culture presented a high recovery percentage, i.e., 31% for *Ln. mesenteroides* FRX4 and 57% for *Lc. lactis* SMX16. In Feta 10, the strain *Lpb. plantarum* FB1 exhibited a higher recovery percentage (46%) than the strain *Lcb. paracasei* SRX10 and the CS culture, which shared similar recovery percentages (25 and 29%, respectively). In Feta 11, the strain distribution was *Lvb. brevis* SRX20 42%, *Lpb. plantarum* FRX20 25%, *Ln. mesenteroides* FMX11 12% and CS culture 21%. Finally, in Feta 12, the strain *Lc. lactis* SMX2 was recovered in a high percentage (50%), followed by *Ln. mesenteroides* FMX3 (29%) and the CS culture (21%).

Feta cheese is a dynamic ecosystem, and its microbiota is constantly changing during ripening and storage, where salting (*ca.* 7%) and/or the low pH (4.4–4.6) can affect the survival of selected species or strains. From the RAPD analysis results, it was evident that the autochthonous added strains dominated the LAB community in all the examined Feta trials. It is known that lactobacilli can grow and survive in fermented food products with low pH values (3.7–4.3) (Tripathi and Giri, 2014), where *Leuconostoc* spp. as NSLAB are naturally occurring in various stages during cheese ripening and storage (Seixas et al., 2018). Angelopoulou et al. (2017) and Papadopoulou et al. (2018) have also confirmed the survival of the adjunct cultures used for Feta or Feta-type production during ripening and/or storage of the cheeses by using molecular tools. However, limited research is available for Feta cheese produced with a cocktail of autochthonous LAB strains as well as for their survival in the final product. The aforementioned studies have dealt with the addition of one autochthonous strain for cheese production, whereas in the present study, multiple combinations were made. For that reason, it was essential to study the prevailing microbiota to confirm the survival of the added LAB strains by using a molecular tool such as RAPD-PCR.

4. Conclusion

The findings of the present study revealed that the incorporation of many of the examined multifunctional LAB strains led to products with desirable sensory properties (salty and slightly acidic taste, smoothy, creamy and firm texture with small mechanical openings and high scores in the "overall taste" and "overall aroma" attributes), without altering but enhancing the traditional character of Feta cheese. Furthermore, as regards the safety of the novel products, the 2 strains with anti-listerial activity *in vitro* (*Ln. mesenteroides* FMX3 and *Lc. lactis* SMX2) provided encouraging results, since the pathogen was eliminated in a shorter time in contrast to the CS culture and before the product release in the market (i.e., 60 days). In addition, many of the added strains were able to survive and thrive in the harsh Feta environment in adequate populations to consider the product a functional food.

Although further research is needed, the results of the present study are promising for the production of new dairy products with enhanced quality, safety and high added value, by using selected LAB strains. In this respect, *Lcb. paracasei* SRX10 and the anti-listerial *Ln. mesenteroides* FMX3 and *Lc. lactis* SMX2 strains are promising adjunct candidates to develop functional Feta cheese with distinctive sensory character (SRX10) and enhanced safety (FMX3 and SMX2). In addition, bioinformatic analysis of these indigenous multi-functional strains may reveal valuable information about specific genes with safety aspects, technological properties and/or functional properties. Furthermore, the use of molecular tools for the

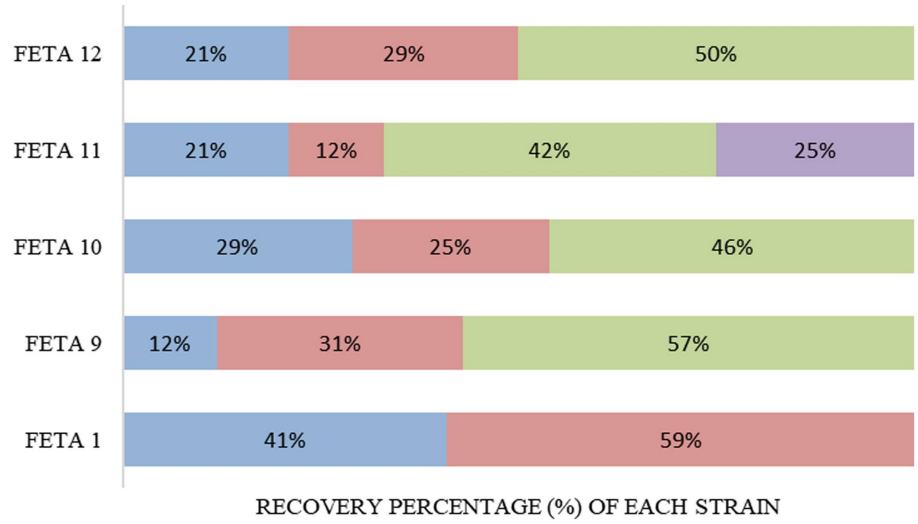

FIGURE 5
Recovery rate of the adjunct functional LAB strains at 180 days (end of storage). Commercial Starter culture (■), Feta 1: *Lcb. casei* SRX10 (■), Feta 9: *Ln. mesenteroides* FRX4 (■), *Lc. lactis* SMX16 (■), Feta 10: *Lcb. paracasei* SRX10 (■), *Lpb. plantarum* FB1 (■), Feta 11: *Ln. mesenteroides* FMX11 (■), *Lvb. brevis* SRX20 (■), *Lpb. plantarum* FRX20 (■), Feta 12: *Ln. mesenteroides* FMX3 (■), *Lc. lactis* SMX2 (■).

investigation of the relationship between the cheese environment and the bacterial functionality, will be a desired aspect, leading to fermented products with exceptional properties.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

CK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. OP: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Visualization, Writing – review & editing. AD: Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing – review & editing. CT: Conceptualization, Visualization, Writing – review & editing. AG: Methodology, Supervision, Visualization, Writing – review & editing. NC: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – review & editing. AA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1254598/full#supplementary-material

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Genetic and technological diversity of *Streptococcus thermophilus* isolated from the Saint-Nectaire PDO cheese-producing area

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Streptococcus thermophilus is of major importance for cheese manufacturing to ensure rapid acidification; however, studies indicate that intensive use of commercial strains leads to the loss of typical characteristics of the products. To strengthen the link between the product and its geographical area and improve the sensory qualities of cheeses, cheese-producing protected designations of origin (PDO) are increasingly interested in the development of specific autochthonous starter cultures. The present study is therefore investigating the genetic and functional diversity of *S. thermophilus* strains isolated from a local cheese-producing PDO area. Putative *S. thermophilus* isolates were isolated and identified from milk collected in the Saint-Nectaire cheese-producing PDO area and from commercial starters. Whole genomes of isolates were sequenced, and a comparative analysis based on their pan-genome was carried out. Important functional properties were studied, including acidifying and proteolytic activities. Twenty-two isolates representative of the diversity of the geographical area and four commercial strains were selected for comparison. The resulting phylogenetic trees do not correspond to the geographical distribution of isolates. The clustering based on the pan-genome analysis indicates that isolates are divided into five distinct groups. A Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotation of the accessory genes indicates that the accessory gene contents of isolates are involved in different functional categories. High variability in acidifying activities and less diversity in proteolytic activities were also observed. These results indicate that high genetic and functional variabilities of the species *S. thermophilus* may arise from a small (1,800 km^2) geographical area and may be exploited to meet demand for use as autochthonous starters.

KEYWORDS

***Streptococcus thermophilus*, diversity, genome comparison, functional properties, autochthonous starter, cheese**

1. Introduction

Streptococcus thermophilus is a thermophilic lactic acid bacteria (LAB) with major economic importance for the dairy industry. Due to its ability to rapidly acidify the milk, it is extensively used for the manufacture of several important fermented dairy products and is considered the second most important species among LAB after *Lactococcus lactis* (Harnett et al., 2022). *Streptococcus thermophilus* is a clonal species that has only recently emerged from a commensal ancestor of the *salivarius* group (Delorme, 2008). This species is part of the genus *Streptococcus* and is the only one that obtained the Generally Recognized as Safe (GRAS) status and the Qualified Presumption of Safety (QPS) status within this genus. Comparative genomic studies on *Streptococcus thermophilus* genomes highlight that this species has lost many virulence-related functions common among pathogenic streptococci. These studies suggest that the *Streptococcus thermophilus* genome has followed a regressive evolution process toward a specialized bacterium dedicated to growth in milk. Comparative genomics also revealed that the dairy *Streptococcus* genome may have been shaped mainly through loss-of-function events and horizontal gene transfer (Bolotin et al., 2004; Hols et al., 2005; Goh et al., 2011).

In the dairy industry, *Streptococcus thermophilus* is used as a starter culture for its capability to rapidly acidify the milk, which is a technological characteristic of major importance to guarantee a good outcome of the dairy process and to enhance food safety by preventing the development of pathogenic bacteria (Rakhmanova et al., 2018). Proteolysis is considered one of the most important enzymatic pathways in the manufacture of many fermented dairy products. In cheese production, proteolysis plays a crucial role in the flavor and texture of cheese by releasing peptides and free amino acids that could undergo secondary reactions (Fox, 1989; Smit et al., 2005).

Over the past few years, changes in the way of life of consumers have imposed requirements for uniformity in the quality of traditional dairy products with high typicality (Settanni and Moschetti, 2014). Due to this growing interest in preserving the typical sensorial properties of traditional cheeses, there is an increasing demand for autochthonous (also called local, indigenous, or wild) and specific strains to replace the well-defined industrial starter strains largely widespread and extensively used in dairy industries (Munekata et al., 2022). It is now well known that a careful selection and use of autochthonous microbial strains can contribute to obtaining high-quality, functional, and safe dairy products (Gómez et al., 2016; Campagnollo et al., 2018; Fusco et al., 2019; Ait Chait et al., 2021; Huang et al., 2021). Such strains are mostly isolated from traditionally made cheeses or raw milk (Carafa et al., 2015; Terzić-Vidojević et al., 2015; Frau et al., 2016; Fusco et al., 2019; Özkan et al., 2021).

Many studies have focused on the genetic and functional diversity of *Streptococcus thermophilus* strains (Giraffa et al., 2001; Morandi and Brasca, 2012; Vendramin et al., 2017; Alexandraki et al., 2019), but few have studied the genetic diversity of wild strains of this species (Andrighetto et al., 2002; Erkus et al., 2014). Few studies have been conducted on the biodiversity analysis of *Streptococcus thermophilus* strains using whole genome sequencing (Hu et al., 2020). All these studies reported high genetic and technological diversity within *S. thermophilus* strains isolated from dairy products.

Protected designation of origin (PDO) cheese producers from Massif Central (France) mostly use commercial starter cultures for the manufacture of cheeses. The use of commercial starters in cheese production results in the loss of typical characteristics of the products (Hansen, 2002). To strengthen the link with the area and improve the sensorial qualities and typicality of cheeses, PDO cheese producers are increasingly interested in the development of specific autochthonous starter cultures. To meet this demand, a first step would be to isolate new wild strains from the producing areas and investigate the genetic and functional diversity of these strains within these reservoirs. Saint-Nectaire is an uncooked pressed cheese that was awarded the PDO label in 1955. Made in one of the smallest PDO areas in Europe, between Puy-de-Dôme and Cantal (France), this is the third French PDO cheese with cow's milk.

The present study explores the genetic and functional diversity of wild strains of *Streptococcus thermophilus* from natural habitats at a local scale. This diversity was explored in the Saint-Nectaire cheese-producing PDO area, one of the smallest PDO areas in Europe with only 1,800 km^2. The comparative analysis of the whole genome sequences of 26 wild strains of *Streptococcus thermophilus* was carried out. Important functional properties of the isolates were investigated, including milk acidification and proteolytic activities.

2. Materials and methods

2.1. Isolation and identification of *Streptococcus thermophilus* strains

2.1.1. Isolation of strains

Presumptive *Streptococcus thermophilus* strains were isolated from (i) 62 milk samples (heated for 6 h at 42°C) collected on 31 different farms in the Saint-Nectaire cheese-producing PDO area (France) in summer and in winter to study the seasonal effect on biodiversity and (ii) commercial starter cultures. Isolation was carried out on M17 agar medium (Biokar Diagnostics, Beauvais, France) incubated at 42°C for 48 h. Representative colonies were picked out of this medium, purified twice, and maintained frozen at −80°C in an M17 broth medium containing 20% (v/v) glycerol. One *Streptococcus thermophilus* isolate was obtained from a bacterial collection of LAB isolated in 1998 from Saint-Nectaire cheeses (INRAE-ARILAIT collection). The Saint-Nectaire PDO area spreads over 69 municipalities between Puy-de-Dôme and Cantal (France), which are referenced in Supplementary Table S1. The specifications for the PDO of Saint-Nectaire cheese describe the conditions for cow breeding, milk production, cheese manufacturing, and ripening of cheeses.[1]

2.1.2. 16S rDNA identification

16S rDNA of Saint-Nectaire isolates was amplified with the universal primers WO2 (5′-100 GNTACCTTGTTACGACTT-3′) and W18 (5′-GAGTTTGATCMTGGCTCAG-3′), as described previously by Callon et al. (2004). Polymerase chain reaction (PCR) amplification was carried out in a final volume of 50 µL containing 1X PCR buffer with $MgCl_2$, 1 colony of isolates, 0.25 mM each primer, 200 mM each dNTP, and 1 U Taq DNA polymerase. The thermal cycling conditions were 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 50°C for

1 https://www.inao.gouv.fr/show_texte/4840

30 s, 72°C for 1 min 30 s, and a final extension step of 72°C for 10 min. The 16S rDNA gene sequencing was performed by LGC Biosearch Technologies (Berlin, Germany).

16S rDNA of commercial strains was amplified with the universal primers A (5′-AGAGTTTGATCCTGGCTCAG-3′) and H (5′-AAGGAGGTGATCCAGCCGCA-3′; Feurer et al., 2004). PCR amplification was carried out as described above for the Saint-Nectaire isolates. The 16S rDNA gene sequencing was performed by Eurofins Genomics (Konstantz, Germany).

To identify the partial 16S rDNA sequences obtained, a search of the NCBI GenBank DNA database was conducted using the BLAST algorithm. The percentage of similarity with DNA sequences deposited in this bank was determined.

16S rDNA sequences were deposited in the NCBI GenBank database under accession numbers OR350537-OR350561.

2.1.3. Selection of representative isolates

In total, 58 isolates and 4 commercial strains were identified as *Streptococcus thermophilus* and were screened for their acidifying and proteolytic activity and for their growth characteristics in sterilized skimmed milk. Notably, 200 mL of sterilized skimmed milk (LACTALIS Ingredients, Bourgbarré, France) was inoculated with isolates at 10^6 cfu.mL^{-1} and incubated for 24 h in a temperature-controlled batch reactor programmed to simulate the decrease in temperature during the manufacture of Saint-Nectaire cheese type (decrease from 33°C to 9°C over 24 h). The acidification kinetics were studied by continuous pH recording (iCINAC system). Proteolysis abilities of isolates were measured using the OPA (o-phtalaldehyde) method described by Church et al. (1983). Growth characteristics of isolates were determined by enumeration of isolates on M17 agar medium every hour for 24 h.

Three groups of isolates were formed based on these results. A total of 22 isolates representative of these three groups were selected for further characterization in this study. All selected isolates collected from the farms were isolated from dairy farms with milk production and cheese manufacturing directly on the farm.

TABLE 1 *Streptococcus thermophilus* isolation sources with genomes assembly accession numbers analyzed in this study.

Isolates	Isolation source	Season of isolation	EBI accession ID project
19M1a	Farm 19	Summer	GCA_950101965
19bM2	Farm 19	Summer	GCA_950102045
21bM2	Farm 21	Summer	GCA_950101935
26bM1	Farm 26	Summer	GCA_950102035
26bM3	Farm 26	Summer	GCA_950101995
29bM1	Farm 29	Summer	GCA_950101945
29bM2	Farm 29	Summer	GCA_950102105
H10bM5	Farm 10	Winter	GCA_950101905
H11M1a	Farm 11	Winter	GCA_950102095
H11M1b	Farm 11	Winter	GCA_950102065
H11M1c	Farm 11	Winter	GCA_950102075
H11bM1	Farm 11	Winter	GCA_950102085
H11bM2	Farm 11	Winter	GCA_950102055
H14bM5	Farm 14	Winter	GCA_950102025
H20bM1	Farm 20	Winter	GCA_950101925
H23bM1	Farm 23	Winter	GCA_950101975
H23bM2	Farm 23	Winter	GCA_950101915
H23bM3	Farm 23	Winter	GCA_950101895
H26bM2	Farm 26	Winter	GCA_950102015
H26bM3	Farm 26	Winter	GCA_950101955
H26M3c	Farm 26	Winter	GCA_950102005
M134	ARILAIT collection	–	GCA_950101985
CS1	Commercial starter	–	PRJEB61322
CS3	Commercial starter	–	PRJEB61322
CS4	Commercial starter	–	PRJEB61322
CS2	Commercial starter	–	PRJEB61322

2.2. Genetic characterization of strains

2.2.1. DNA extraction and sequencing

Genomic DNA was extracted from cell cultures with Nuclospin® Tissue from Macherey Nagel according to the manufacturer's instructions. Final concentrations were measured with a Qubit™ fluorometer using the dsDNA Broad Range Kit (Thermo Fisher Scientific). The extracted DNA was further sequenced using Illumina technology. Library preparation and sequencing were handled by Eurofins genomics (Konstantz, Germany) using a Novaseq 6000 sequencing system (Illumina, San Diego, CA, United States).

2.2.2. Genome assembly and annotation

Sequencing reads from raw fastq files were filtered at Q30 with a minimal length of 110 bp with prinSeq (Cantu et al., 2019). Reads with remaining sequencing adapters were excluded with cutadapt V4.1 (Martin, 2011). Each genome was assembled using Spades V3.13 (Bankevich et al., 2012) with the careful option and annotated with Bakta V1.5 (Schwengers et al., 2021). EBI accession numbers are referenced in Table 1.

2.2.3. Pan-genome assembly and visualization

Annotated GFF3 files of 22 *Streptococcus thermophilus* isolated from the Saint-Nectaire cheese-producing PDO area and 4 strains of *Streptococcus thermophilus* isolated from commercial starter cultures genomes were submitted to Roary (Page et al., 2015) for pan-genome analysis using default parameters. A gene presence–absence data matrix was derived and visualized using Phandango (Hadfield et al., 2018).

2.2.4. Phylogenetic reconstruction

Nucleotide sequences of 1,350 core genes (excluding duplicated genes) were extracted from Roary results. They have been aligned with Mafft (Katoh and Standley, 2013), and distance computing and tree sampling were realized with BEAST (Suchard et al., 2018). A strict molecular clock was chosen with the HKY substitution model. BEAST (Suchard et al., 2018) is a program for Bayesian analysis of molecular sequences using MCMC (Markov chain Monte Carlo). It is entirely orientated toward rooted, time-measured phylogenies inferred using strict or relaxed molecular clock models. BEAST uses MCMC to average over tree space, so that each tree is weighted proportional to

its posterior probability. We chose the strict molecular clock model with the HKY substitution model because this assumes that all branches on the tree have the same rate of evolution. This appeared reasonable considering the absence of knowledge on those rates for our new isolates.

Core-genome single nuclear polymorphism (SNP) tree was created with Parsnp (Treangen et al., 2014) on the Galaxy platform (The Galaxy Community, 2022). The resulting phylogenetic tree was visualized using iTOL.[2] Parsnp is a genome multi-alignment tool designed to align genome sequences. It aligns and provides the output as the multiple sequence alignment of given sequences, SNP variations, and the core genome phylogeny. For this reconstruction, LMD-9 was used as the reference genome. The genome sequencing of *Streptococcus thermophilus* LMD-9 was described previously (Makarova et al., 2006), and the complete genome sequence can be accessed at GenBank under accession number CP000419.

Kyoto Encyclopedia of Genes and Genomes (KEGG) numbers (Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2023) for accessory genes were obtained using the eggnog-mapper v2 web tool (Iyer et al., 2010). Partial least squares discriminant analysis (PLS-DA) was performed using the mixOmics package of R software V 4.2.3.[3]

2.3. Technological characterization of isolates

2.3.1. Preparation of model cheese curd

The model cheese curds (MCC) were prepared according to Callon et al. (2016) with some modifications. In total, 40 mL of pasteurized milk (Ferme des Peupliers, Normandie, France) was incubated at 33°C and inoculated with isolates at 10^6 cfu.mL^{-1}. The milk was coagulated with 12 µL of calf rennet for 45 min at 33°C and then centrifuged for 20 min at 20,000 *g* at 33°C. The supernatant (lactoserum) was discarded, and the curd was incubated for 24 h in a temperature-controlled batch reactor programmed to simulate the decrease in temperature during the manufacture of Saint-Nectaire cheese type (decrease from 33°C to 9°C over 24 h). Each strain was tested three times.

2.3.2. Growth characteristics

To determine the growth ability of isolates, MCC samples were collected at 0, 6, and 24 h. MCC samples were blended two times for 2 min in a phosphate buffer of pH 7.5 using a Stomacher blender (Interscience, St Nom la Bretèche, France) for dissociating all the cell aggregates. After appropriate dilution in Ringer's solution, *S. thermophilus* isolates were enumerated on M17 agar and incubated at 30°C for 48 h.

2.3.3. Acidifying activity

To analyze the acidifying activities of isolates, MCC samples were collected at 0, 6, and 24 h, and pH was measured in the core of the MCC samples using a WTW pH 526 pH meter with a LOT406-M6-DXK electrode (Mettler-Toledo S.A, Viroflay, France). A classification of isolates based on their acidifying activities was constructed according to that previously reported by Morandi and Brasca (2012), with some modifications. The pH unit target values were modified because we worked on a model cheese curd with a temperature decrease mimicking Saint-Nectaire cheesemaking (33°C to 9°C), while precedent studies worked on fermented milk at a constant temperature of 37°C.

2.3.4. Proteolytic activity

The extraction of water-soluble nitrogen from the MCC samples was carried out according to the method described by Myagkonosov et al. (2021) with some modifications. Five grams of MCC samples were mixed with 5 cm^3 of deionized water and homogenized with a stomacher for 4 min. The resulting mixture was transferred to a volumetric flask, and the volume was made up to 100 mL with deionized water. The mixture was kept at 40°C for 1 h with continuous shaking. The samples were centrifuged at 3,000 *g* for 30 min. After centrifugation, the samples were cooled to 4°C, and the upper fat layer was removed. The supernatant was separated and filtered with a pore size of 0.45 µm. The resulting filtrate was mixed with deionized water at a ratio of 1:5. Next, 3 mL of OPA (o-phtalaldehyde) reagent prepared according to Church et al. (1983) was added to 300 µL of the solution, and after 2 min, absorbance was measured at 340 nm with a 7,200 spectrophotometer Jenway (Dutscher, France). The results have been expressed in mmol.L^{-1} of glycine based on a calibration curve. The proteolytic activity of isolates was determined at 24 h of fermentation by subtracting the results at 0 h of fermentation.

2.3.5. Statistical analysis

Statistical analysis of biochemical data was performed using the XLSTAT software (Addinsoft, Paris, France). The results are reported as means ± standard deviation. The normality of the data was checked using a Shapiro–Wilk test ($p < 0.05$). The test rejected the hypothesis Ha of normality when the value of p was ≤ 0.05. A large number of variables did not have a normal distribution, and therefore a non-parametric test (Kruskal-Wallis) and *post-hoc* comparison (Conover-Iman procedure) were used to compare the concentrations obtained. Differences between the mean values were considered significant at $p < 0.05$. Pearson's correlations at the 5% significance level ($p < 0.05$) were used to explain the relationship between functional properties.

3. Results and discussion

3.1. Isolation and identification

In this study, strains belonging to the *Streptococcus thermophilus* species were isolated from the Saint-Nectaire cheese-producing PDO area (France).

All isolates were identified on the basis of 16S rDNA sequence alignment using the NCBI blast algorithm. Isolates showing a percentage of similarity equal to or higher than 99% with *Streptococcus thermophilus* DNA sequences available in this database were considered to be *Streptococcus thermophilus*. In total, 58 isolates were identified as *Streptococcus thermophilus*.

Twenty-two isolates collected throughout the geographical producing area and representative of diversity, together with four commercial strains, were selected and characterized for their genetic

2 https://itol.embl.de/

3 http://mixomics.org/

and technological properties. Conditions of isolation and the EBI accession number are referenced in Table 1. The genome sequencing and assembly-related information are shown in Supplementary Table S2.

3.2. Genetic diversity

3.2.1. Phylogenetic reconstruction

Two phylogenetic analyses were performed (Figure 1). The first one was based on core-gene alignment using the Bayesian method and allowed to identify seven clades (Figure 1A), namely, A–G. The other was based on single nucleotide polymorphisms (SNPs) detected (Figure 1B) and allowed the distinction of seven clades, namely, 1–7.

From the two resulting trees, it could be noticed that SN-isolates from the same farms were distributed in various clades. For example, in Figure 1A, isolates H11M1a, H11bM1, and H11M1b were all isolated on farm 26, but were not located in the nearby clade. A similar situation is observed in Figure 1B for isolates 26bM1, 26bM3, and H26M3c. Moreover, strains from different farms may be clustered together.

These results indicate that the phylogenetic reconstruction of isolates did not correspond to their geographical distribution, despite a small isolation area (1,800 km^2) and closed milk production. Vendramin et al. (2017) and Hu et al. (2020) reported similar results with strains isolated from China and Italy, respectively, compared to strains isolated from other continents. This distinction indicated that the isolation source did not exert an important impact on the evolution of *Streptococcus thermophilus*, although the farms are quite close to one another. It is also notable that isolates from summer and winter milk were distributed into different clades and not clustered together, suggesting that the genetic variability of the isolates did not depend on the season of isolation.

3.2.2. Pan-genome analysis

The pan-genome of the 22 Saint-Nectaire isolates and the 4 commercial strains was performed by clustering the genes encoding complete protein sequences into core and accessory genomes using Roary (Page et al., 2015).

The core genome is the set of genes shared by all the genomes studied and can be divided into hard-core genes, which are defined to be present in >99% of the genomes, and soft-core genes, which are present in 95%–99% of the genomes. The accessory genome is shared by a subset of the genomes tested and is subdivided into shell genes, which are present in 15%–95% of genomes, and cloud genes, which are found in less than 15% of genomes. From the 3,269 genes constituting the pan-genome of the 26 *Streptococcus thermophilus* isolates studied here, we found 1,450 (44%) core genes (present in 24/26 genomes), including 1,355 (41%) hard core genes (present in 25/26 genomes) and 95 (3%) soft core genes (present in 24/25 genomes), and 1,819 (56%) accessory genes (present in less than 24 genomes), which might be responsible for the fundamental differences in phenotypic characteristics between the isolates (Daughtry et al., 2018), including 776 (24%) shell genes (present in 3–24 genomes) and 1,053 (32%) cloud genes (present in less than 3 genomes; Figure 2). These results are similar to those reported by Alexandraki et al. (2019), who analyzed the pan-genome of 23 strains of *S. thermophilus* and identified 1,089 core genes (52%) and 997 accessory genes (48%).

The generated clustering based on the pan-genome was concordant with the phylogenetic reconstruction based on SNPs (Figure 1B) in defining the relationships among isolates. It showed five main clusters with obvious differences in accessory genes for each of them (Figure 2). The first cluster consisted of SN-isolate H10bM5 and commercial strains CS4 and CS2. Cluster 2 included SN-isolates H11bM1, M134, and H23bM1. SN-isolates 21bM2, 29bM2, H20bM1, H26M3c, H14bM5, and commercial strain CS3 were clustered together. The fourth cluster comprised SN-isolates H11M1b, H11M1a, 26bM1, 29bM1, H11M1c, and H11bM2. Finally, the fifth cluster consisted of SN-isolates 19M1a, H23bM2, 19bM2, H26bM2, H23bM3, H26bM3, 26bM3, and commercial strain CS1. These results reveal a high genetic variation level probably related to the accessory gene content across isolates, which are involved in different functional categories. These findings are in accordance with Giraffa et al. (2001),

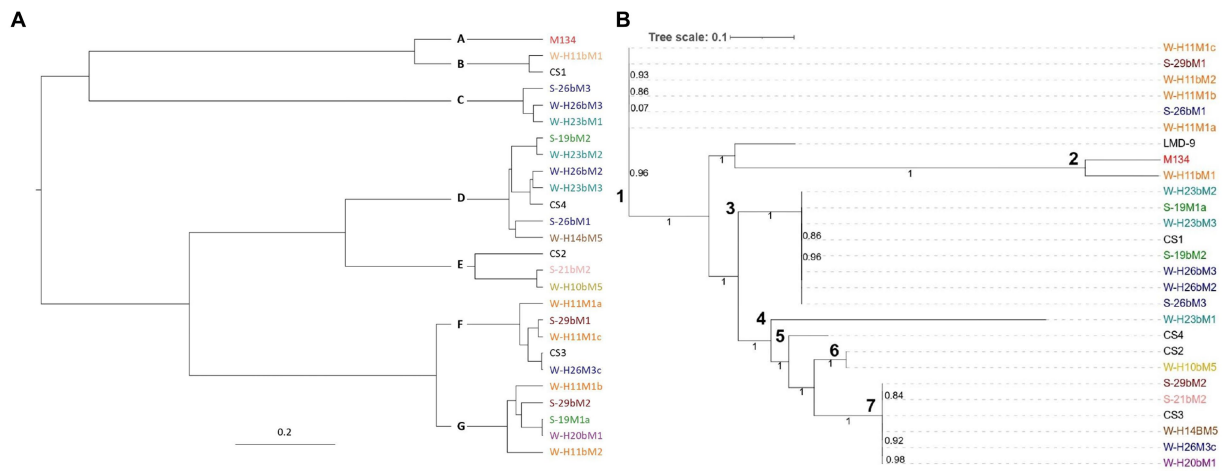

FIGURE 1
Phylogenetic trees of the 26 *Streptococcus thermophilus* isolates based on **(A)** core genome and **(B)** single nucleotide polymorphisms. Isolates from summer milks are annotated with an "S" and isolates from winter milks are annotated with a "W." Isolate names with the same colors were isolated from the same farm.

Andrighetto et al. (2002), and Morandi and Brasca (2012), who also reported a high genetic variability of *Streptococcus thermophilus* with the RAPD-PCR method.

3.2.3. KEGG functional analysis

A hierarchical clustering of the isolates was performed on the accessory genes annotated with the KEGG pathway (Figure 3). Almost 77% of the accessory genes were positively annotated with a KEGG ko number. These genes were divided into 23 functional KEGG pathways, the most important being "Protein families: signaling and cellular processes" (18% of the total annotated genes), "Unclassified: genetic information processing" (16%), "Membrane transport" (10%), "Carbohydrate metabolism" (7%), "Protein families: genetic information processing" (7%), "Protein families: metabolism" (6%), and amino acid metabolism (6%). These results are in accordance with Vendramin et al. (2017), who performed a genome comparison of eight *Streptococcus thermophilus* strains of dairy origin isolated in Italy. They investigated the strain-specific features by assigning functional categories of the SEED subsystem to the gene content of the strains. Among the functional categories identified, four accounted for a large part of the strain diversity, including "amino acids and derivatives," "carbohydrates," "DNA metabolism," and "membrane transport," covering almost 50% of the specific genes. Although the annotations are different due to the databases used, their results are similar to the present study.

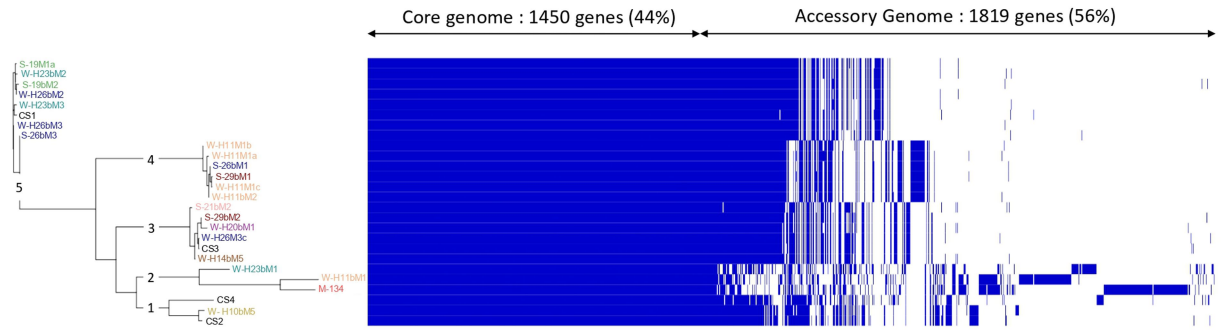

FIGURE 2
Clustering of isolates associated with the visualization of the *Streptococcus thermophilus* isolate pan-genome. The pan-genome was visualized based on the Phandandgo software (Hadfield et al., 2018). In the Roary matrix, genomes are shown as rows, and homologous gene clusters are depicted as columns. The presence of a gene cluster in a genome is indicated by blue. Core gene clusters that are found in all genomes are shown on the left side of the matrix. Isolates from summer milks are annotated with an "S" and isolates from winter milks are annotated with a "W." Isolate names with the same colors were isolated from the same farm.

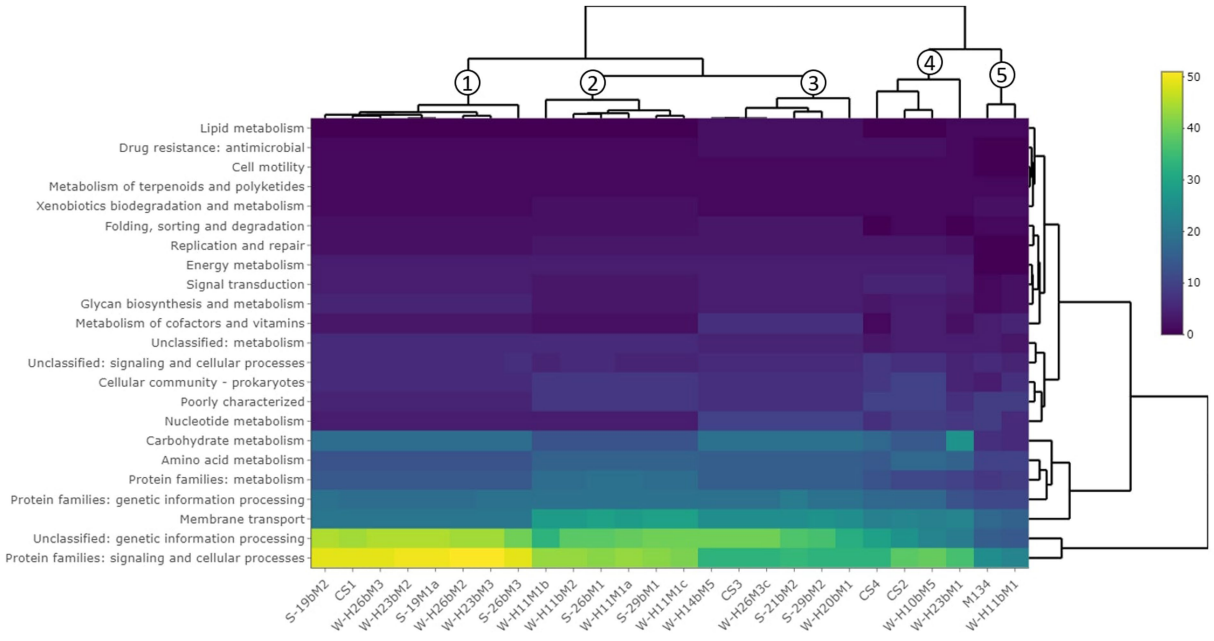

FIGURE 3
Hierarchical clustering of isolates based on the gene content summarized by KEGG pathways. The clustering on top of the figure shows similarities between isolates.

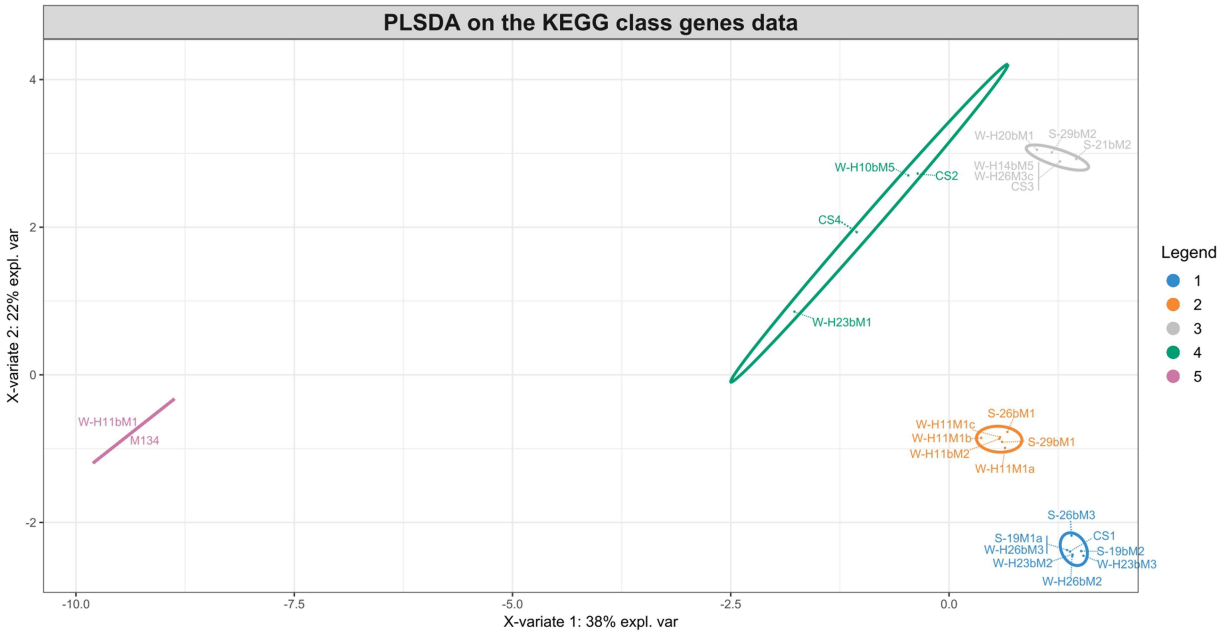

FIGURE 4
Sample plots from the PLS-DA analysis performed on accessory gene numbers of the KEGG class for groups 1 (blue), 2 (orange), 3 (gray), 4 (green), and 5 (pink).

This hierarchical clustering (Figure 3) allowed an evaluation of the similarity between isolates based on the number of accessory genes associated with a functional KEGG pathway. Five groups were identified and are similar to those observed in the phylogenetic tree based on the pan-genome with a difference (Figure 2).

The first group is composed of isolates H23bM2, 19bM2, 19M1a, H26bM3, 26bM3, and CS1. The second group includes isolates H11M1c, 29bM1, H11M1a, 26bM1, H11bM2, and H11M1b. Isolates 21bM2, H14bM5, H26M3c, 29bM2, H20bM1, and CS3 formed the third group. Isolates H10bM5, H23bM1, CS4, and CS2 were clustered in the fourth group, and the fifth group consisted of isolates M134 and H11bM1.

The different groups stand out for their functional KEGG class. A PLS-DA analysis was performed (Figure 4) to identify KEGG classes that differentiate isolates into these groups. PLS-DA results showed that the groups were easily recognized based on the accessory gene content of each KEGG class. These KEGG classes may be the indicators to distinguish these three clades. Figure 5 shows the variable's contribution from components 1 (A) and 2 (B) of the PLS-DA analysis, and Figure 6 represents the clustered image map (CIM) associated with it. These figures showed the functional KEGG pathways overrepresented in each of the clades that allowed their differentiation. Heatmaps, representing the lowest level of the KEGG pathway database (gene ko level), were constructed for each KEGG pathway identified as characteristic of a group (Supplementary Figures S1–S4).

The first group was characterized by three KEGG pathways, namely, "Protein families: signaling and cellular process," "Unclassified: genetic information processing," and "Glycan biosynthesis and metabolism." More accessory genes encoded for transposases and putative transposases were identified in the "Unclassified: genetic information processing" KEGG pathway for the first group in comparison with others (Supplementary Figure S1). Transposases are typically present within bacterial genomes as small, mobile elements solely comprising their own transposition genes; they are important factors for horizontal gene transfer between strain genomes and are therefore important for the safety evaluation of strains for their use as starter cultures. However, many of the accessory genes of the first group, grouping in the "Protein families: signaling and cellular processes," were involved in the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) proteins immune defense system in comparison to other groups (Supplementary Figure S2). It is now well known that the CRISPR/Cas system confers resistance against foreign genetic elements, such as phages (Bolotin et al., 2004; Mojica et al., 2005; Lillestøl et al., 2006). As phage infection of starters is the most common cause of incomplete fermentation in the dairy industry, such genes could represent a great advantage for the use of these isolates as starter cultures.

Then, the KEGG pathways "Protein families: metabolism" and "Membrane transport" allowed the differentiation of the second group, which might confer benefits to isolates during the manufacture of cheeses since accessory genes associated with these functional KEGG pathways encoded for dipeptidases (pepDA, pepDB; Supplementary Figure S3) and peptide transport systems (oligopeptide transport system Opp; Supplementary Figure S4), among other things, which are involved in proteolysis and thus are important for aromatic and textural properties of cheeses (Savijoki et al., 2006; Rodríguez-Serrano et al., 2018).

The third group stands out for the KEGG pathways "Protein families: genetic information processing," "Nucleotide metabolism," "Metabolism of cofactors and vitamins," "Drug resistance: antimicrobial," "Folding, sorting, and degradation," and important functional categories for cheese manufacturing, including "carbohydrate metabolism" and "Lipid metabolism." Carbohydrate

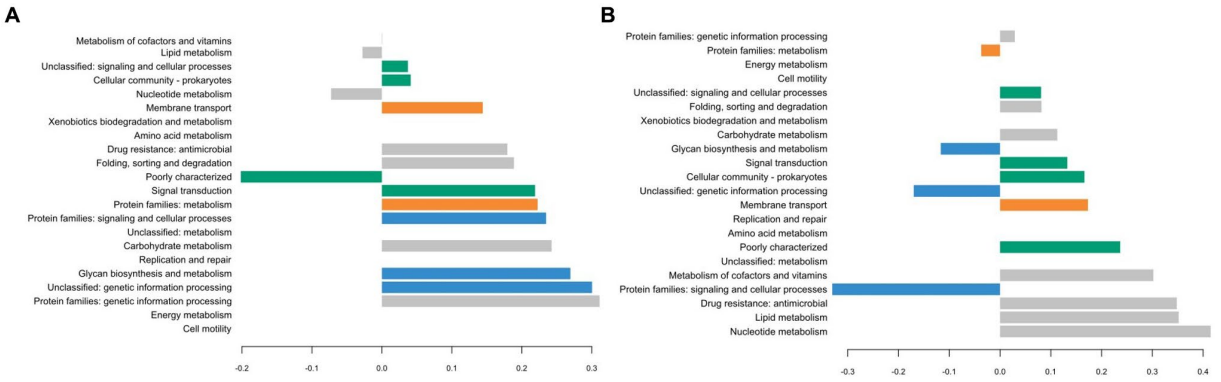

FIGURE 5
Plot loadings representing the variable's contribution on component 1 **(A)** and on component 2 **(B)** of the PLS-DA analysis. Group 1 is colored in blue, 2 in orange, 3 in gray, 4 in green, and 5 in pink.

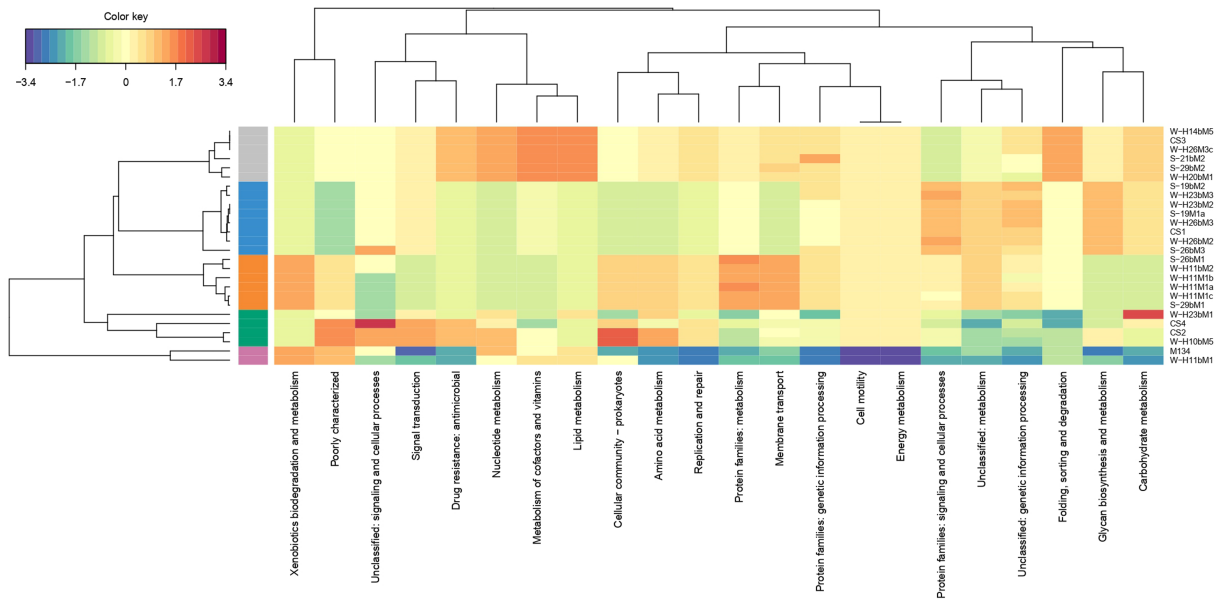

FIGURE 6
Clustered image map (CIM) associated with PLS-DA analysis. Groups are distinguished by colors on the left side of the map. Group 1 is colored in blue, 2 in orange, 3 in gray, 4 in green, and 5 in pink.

metabolism is the most important metabolism pathway for starter cultures because of their primary role in producing lactic acid from lactose (Hutkins and Goh, 2014). Lipid metabolism is also of high interest because lipolysis plays an essential role in the development of flavors in cheeses (McSweeney and Sousa, 2000).

The KEGG pathways "Cellular community: prokaryotes," "Signal transduction," and "Unclassified: signaling and cellular process" characterized the fourth group and might facilitate the survival of cells under stressful environmental conditions. These functional traits may be of great advantage as the manufacture of cheeses exposes starters to various environmental stresses such as low pH, osmotic stress, and high pressure (Zotta et al., 2008).

The PLS-DA analysis did not identify the characteristic KEGG functional class of the fifth group, probably due to their lower content of accessory genes in comparison with other isolates, with only 210 accessory genes for isolate M134 and 207 for isolate H11bM1. However, these isolates were distantly separated from commercial strains based on the phylogenetic reconstruction based on core genes (Figure 1) and on the clustering associated with the pan-genome (Figure 2), which could be an added value for potential use as autochthonous starter cultures to strengthen the typical characteristics of cheeses.

Interestingly, commercial strains CS1, CS2, and CS3 were not significantly distantly separated from the centroid of their respective clusters, unlike the CS4 strain (Figure 2), indicating that commercial strains are representative of *Streptococcus thermophilus* genetic diversity. According to the PLS-DA analysis (Figures 4–6), the main differences between CS4 and SN-isolates H10bM5 and CS2 were the number of genes implicated in several KEGG categories, including "Protein families: metabolism" and "Carbohydrate metabolism." In

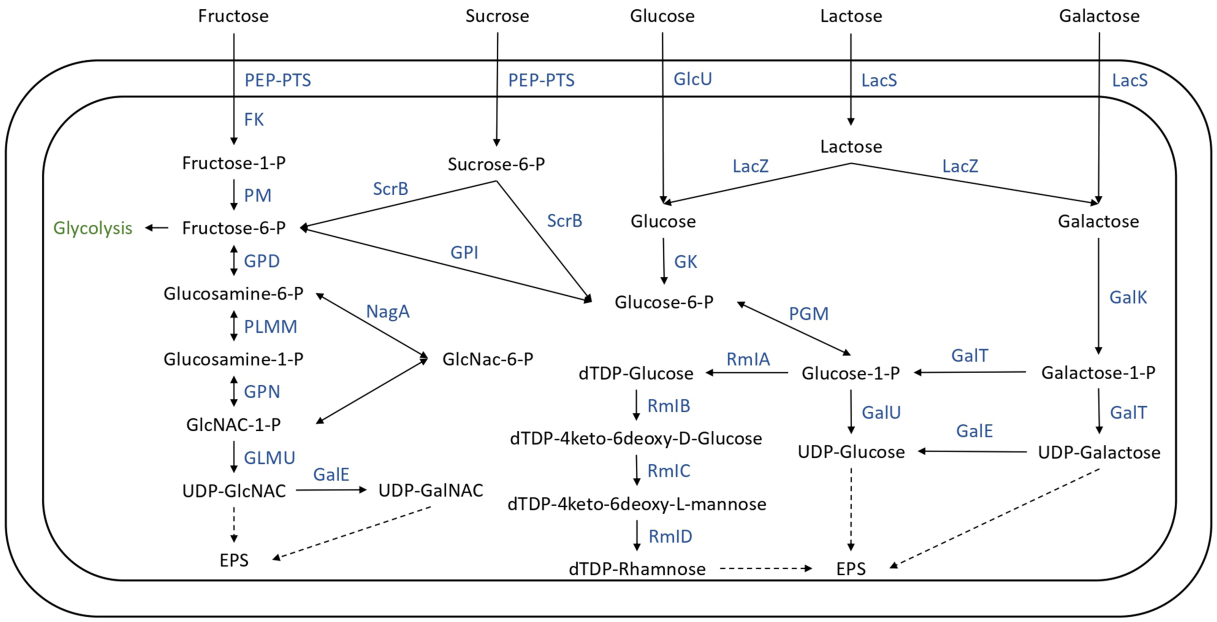

FIGURE 7
Carbohydrate transport and metabolism in the 26 *Streptococcus thermophilus* isolates. FK, fructokinase; GalE, UDP-galactose-4-epimerase; GalK, galactokinase; GalT, UTP-galactose-1-phosphate uridyltransferase; GalU, UDP-glucose pyrophosphorylase; GK, glucokinase; GlcU, glucose permease; GLMU, *N*-acetylglucosamine-1-phosphate uridyltransferase; GPD, glucosamine-6-phosphate deaminase; GPI, glucose-6-phosphate isomerase; GPN, glucosamine-1-phosphate *N*-acetyltransferase; LacS, lactose permease; LacZ, beta-glucosidase; Nag, *N*-acetyl-alpha-D-galactosaminidase; PEP-PTS, phosphoenolpyruvate-sugar phosphotransferase system; PGM, α-phosphoglucomutase; PLMM, phosphoglucosamine mutase; PM, phosphomutase; RMla, glucose-1-phosphate thimidyltransferase; Rmlb, dTDP-glucose 4, 6 dehydratase; RmlC, dTDP-4-deshydrorhamnose 3,5-epimerase; RmlD, DTDP-4-keto-L-rhamnose reductase; ScrB, sucrose-6-phosphate hydrolase.

these categories, isolate CS4 had a higher number of accessory genes encoding dipeptidases (Supplementary Figure S3) and a higher content of accessory genes implicated in carbohydrate transport and metabolism (Supplementary Figure S5) than CS2 and H10bM5 isolates. However, these results focused only on accessory genes. To avoid this bias and study the carbohydrate metabolism of each isolate, a search for specific genes in their whole genomes was performed.

3.2.4. Carbohydrate metabolism and sugar transport system

The sugar utilization ability of *S. thermophilus* strains is of major importance in dairy fermentation as it directly affects the rate of milk acidification. Various studies suggest that the utilization of glucose, lactose, and fructose by *S. thermophilus* is consistently observed, whereas the utilization of sucrose, galactose, mannose, and maltose exhibits variable profiles (O'Leary and Woychik, 1976; Thomas et al., 2011; Erkus et al., 2014).

The lactose, glucose, galactose, sucrose, and fructose transport and utilization systems were analyzed in the genomes of the 22 SN-isolates and the 4 commercial strains (Figure 7), and the genes of each isolate implicated in these systems are listed in Supplementary Table S3.

Lactose represents the principal carbohydrate in milk and is the preferred carbon and energy source of *Streptococcus thermophilus* (Xiong et al., 2019), probably due to the adaptation of the species to the milk (Goh et al., 2011; Alexandraki et al., 2019). In this species, lactose transport and hydrolysis are controlled by the lac operon. It encodes a lactose permease (LacS) and a cytoplasmic β-galactosidase (LacZ) which cleave the lactose into glucose and galactose (Xiong et al., 2019). The 26 genomes all harbored the LacZ and LacS genes (Figure 7; Supplementary Table S3). The resulting glucose moiety is phosphorylated to glucose-6-P by glucokinase and is further utilized through the glycolysis pathway (Figure 7). The Leloir pathway is the most common route for galactose utilization in *S. thermophilus*. Galactose is converted to glucose-1-phosphate by the galRKTEM gene cluster, which consists of the regulator GalR, galactokinase (GalK), galactose-1-phosphate uridyltransferase (GalT), UDP-glucose-4-epimerase (GalE), and galactosemutarotase (GalM; Xiong et al., 2019; Figure 7). However, most strains of *S. thermophilus* are unable to ferment galactose despite having intact galRKTEM gene clusters for the Leloir pathway (Vaughan et al., 2001; de Vin et al., 2005). The most probable explanation for this observed phenotype is a deficient promoter leading to insufficient transcription (Vaughan et al., 2001).

Gal-positive strains are of technological importance principally for their ability to completely ferment lactose, which results in a reduced amount of galactose being present, which cannot serve as a carbon source for spoilage or pathogenic bacteria (Hutkins and Morris, 1987).

The DNA fragments of the *galR-galK* intergenic region of the 26 isolates were picked out from their genome sequences (Supplementary Figure S6). Four different types of fragments were detected in the 26 genomes analyzed. Isolates 19M1a, H23bM2, 19bM2, 26bM3, H23bM3, H26bM2, H26bM3, and CS1 are type A; 21bM2, 29bM2, H20bM1, H26M3c, CS3, H14bM5, H10bM5, CS2, CS4, and H23bM1 are type B; H11M1a, H11M1b, H11M1c, 26bM1, 29bM1, and H11bM2 are type C; and isolates M134 and H11bM1 belong to type D.

It is now well documented that a single point of mutation with a G-to-A substitution in the −9 position of the −10 box in the *galK* promoter results in improved *galK*-promoter activity and could be responsible for the ability to ferment galactose (Vaughan et al., 2001; van den Bogaard et al., 2004; de Vin et al., 2005; Giaretta et al., 2018; Xiong et al., 2019). The presence of this mutation was examined in the 26 genomes studied here. Consequently, among the isolates, only M134 and H11bM1 appeared capable of galactose utilization as they owned the relevant mutation related to the Gal-positive phenotype. However, de Vin et al. (2005) and Hu et al. (2020) demonstrated that the gal promoter does not exclusively determine the gal-positive phenotype of *Streptococcus thermophilus* strains. Experimental verification is therefore required to validate this prediction.

The utilization of sucrose and fructose by *S. thermophilus* required a phosphoenolyruvate-phosphotransferase system (PEP-PTS), consisting of a PEP-dependent phosphotransferase (enzyme I, EI), a histidine-containing phosphocarrier protein (HPr), and a sugar-specific permease (enzyme II, EII). The 26 genomes investigated all harbored a PEP-dependent phosphotransferase and the HPr, as well as genes responsible for sucrose and fructose PTS transporter sugar-specific permease enzymes (Figure 7; Supplementary Table S3). Furthermore, genes responsible for sucrose and fructose utilization detailed in Figure 7 were detected in these genomes (Supplementary Table S3). However, experimental procedures are required to identify the ability of these isolates to ferment these carbohydrates.

3.3. Technological diversity

The acidifying abilities of 22 *Streptococcus thermophilus* isolated from the Saint-Nectaire cheese-producing PDO area (SN-isolates) and 4 strains of *Streptococcus thermophilus* isolated from commercial starter cultures were evaluated in model cheese curd. From the pH values obtained, ΔpH6h and ΔpH24h were calculated (Figure 8).

Half of the 22 SN-isolates lowered the pH by more than one pH unit after 6 h of fermentation, suggesting fast acidifying ability. As expected, all commercial strains presented fast acidifying abilities, which is one of the most important criteria for cheese manufacturing (Tidona et al., 2020).

Concerning the ability to reduce the pH of the model cheese curds at 24 h, the following three main groups of isolates were distinguished: (i) low acidifying isolates with a pH decrease below 1.30 pH units, including 32% of the SN-isolates (19bM2, H10bM5, H11M1b, H11M1c, H23bM2, H23bM3, and 29bM1); (ii) medium acidifying isolates, showing a pH decrease between 1.30 and 1.50 pH units, comprising 27% of total SN-isolates and 100% of the commercial strains (CS1, 19M1a, 21bM2, H11bM2, H14bM5, H26bM3, M134, CS3, CS4, and CS2); (iii) high acidifying isolates, causing a pH decrease higher than 1.50 pH units, regrouping 41% of the SN-isolates (H11M1a, H26bM2, H26M3c, 26bM1, 26bM3, 29bM2, H11bM1, H20bM1, and H23bM1). It could be noticed that SN-isolates from the same farms and SN-isolates from summer and winter milk are distributed into the three acidifying activity categories, and that the four commercial strains were clustered in the group of medium

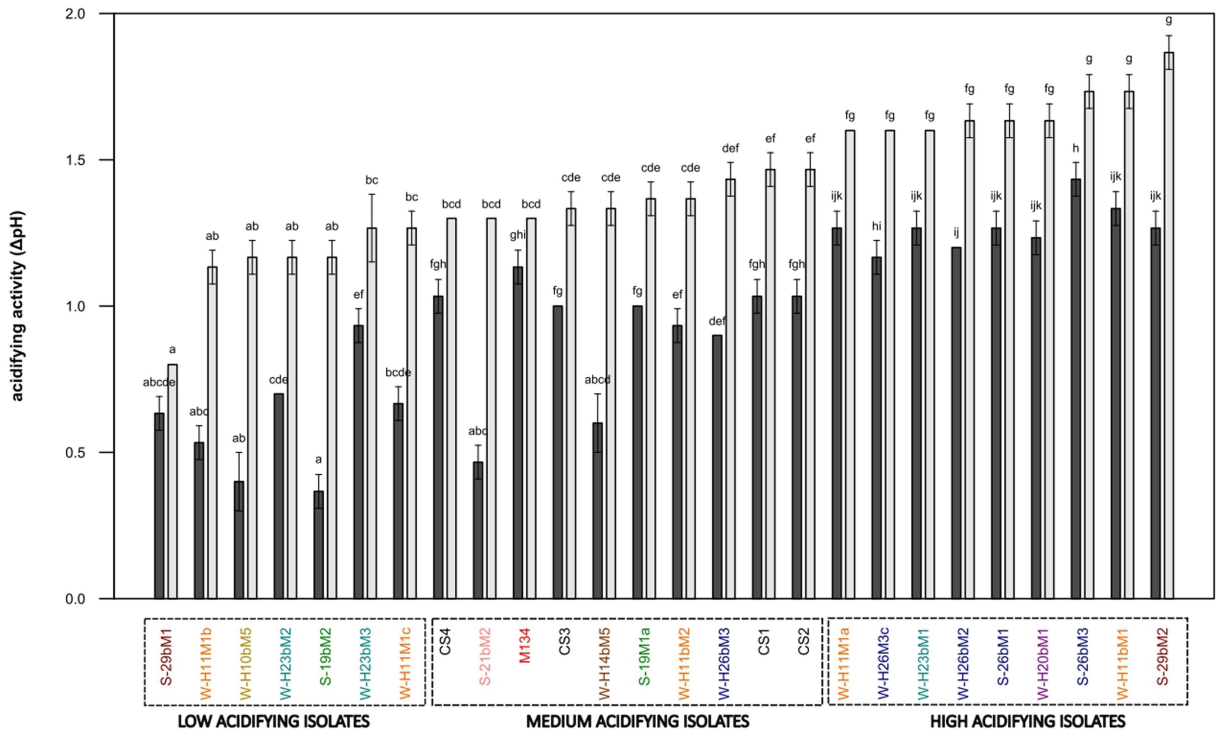

FIGURE 8
Acidifying activity of *Streptococcus thermophilus* isolates (a–k): mean values of the same time of fermentation without a common superscript are significantly different ($p < 0.05$) according to the Conover-Iman test. Isolates from summer milks are annotated with an "S" and isolates from winter milks are annotated with a "W." Isolate names with the same colors were isolated from the same farm.

acidifying activity, while the autochthonous SN-isolates showed various acidifying activities.

This clustering reveals a high variability in acidifying activity observed within *Streptococcus thermophilus* SN-isolates, suggesting that this feature is strain-dependent, in accordance with Giraffa et al. (2001). This diversity of acidifying activities of the SN-isolates does not depend on the season or the farms. In contrast, commercial strains showed homogeneous acidifying activity. This homogeneity is probably due to the specific selection of the strains based on their functional characteristics to be used as starter cultures (Gibbons and Rinker, 2015).

The proteolytic activities of the 22 SN-isolates and the 4 commercial strains of *Streptococcus thermophilus* were evaluated in a model cheese curd at 24 h of fermentation, and the results are presented in Figure 9. The proteolytic activities of *Streptococcus thermophilus* SN-isolates and commercial strains varied slightly, from 0.046 to 0.077 $mmol_{eqGlycine}.L^{-1}$ for 29bM1 SN-isolates and CS2 commercial strains, respectively.

Galia et al. (2009) and Dandoy et al. (2011) have highlighted a link between high acidifying activities and the presence of an efficient proteolytic system, whereas our results showed low proteolytic activities and a limited diversity on this criteria in comparison with other studies on the characterization of *Streptococcus thermophilus* strains (Urshev et al., 2014; Hu et al., 2020; Tidona et al., 2020) or other species (Zaaraoui et al., 2021; Abarquero et al., 2022; Sugajski et al., 2022). These findings are in accordance with Harnett et al. (2022), who reported *Streptococcus thermophilus* as a LAB species generally considered poorly proteolytic. To ensure sufficient proteolytic activity in dairy products, *Streptococcus thermophilus* is often associated with other species for the development of starter cultures, such as *Lactobacillus delbrueckii* subsp. *bulgaricus*. The symbiotic growth of both species leads to protocooperation, inducing higher proteolytic and acidifying rates (Hervé-Jiménez, 2008).

In addition, a PLS-DA analysis was performed on the KEGG annotation of accessory genes with acidification groups (Supplementary Figure S7) and indicated that the KEGG annotations of accessory genes did not allow for distinguishing the acidification groups. The lack of diversity in proteolytic activities also indicates that no link could be found between these functional properties and groups obtained by hierarchical clustering based on the KEGG annotation of the accessory genes.

The literature does not often discuss the gene expression of LAB in relation to its acidifying capabilities. The variations in acidifying potential among different strains, besides the presence/absence of genes, could be due to differential expression of multiple genes related to metabolism. For instance, in a study conducted by Galia et al. (2016), two strains harboring the same allele of the prtS gene, encoding a cell-wall-anchored proteinase linked to fast acidifying capabilities, presented different acidification rates. The authors demonstrated that this difference could be due to the differential expression of several metabolic-related genes (prtS, codY, ilvE, livJ, and relA) during growth in milk, with an over-expression of these genes in the most acidifying strain compared to the other. They also reported that the transcriptional regulator CodY could also play an

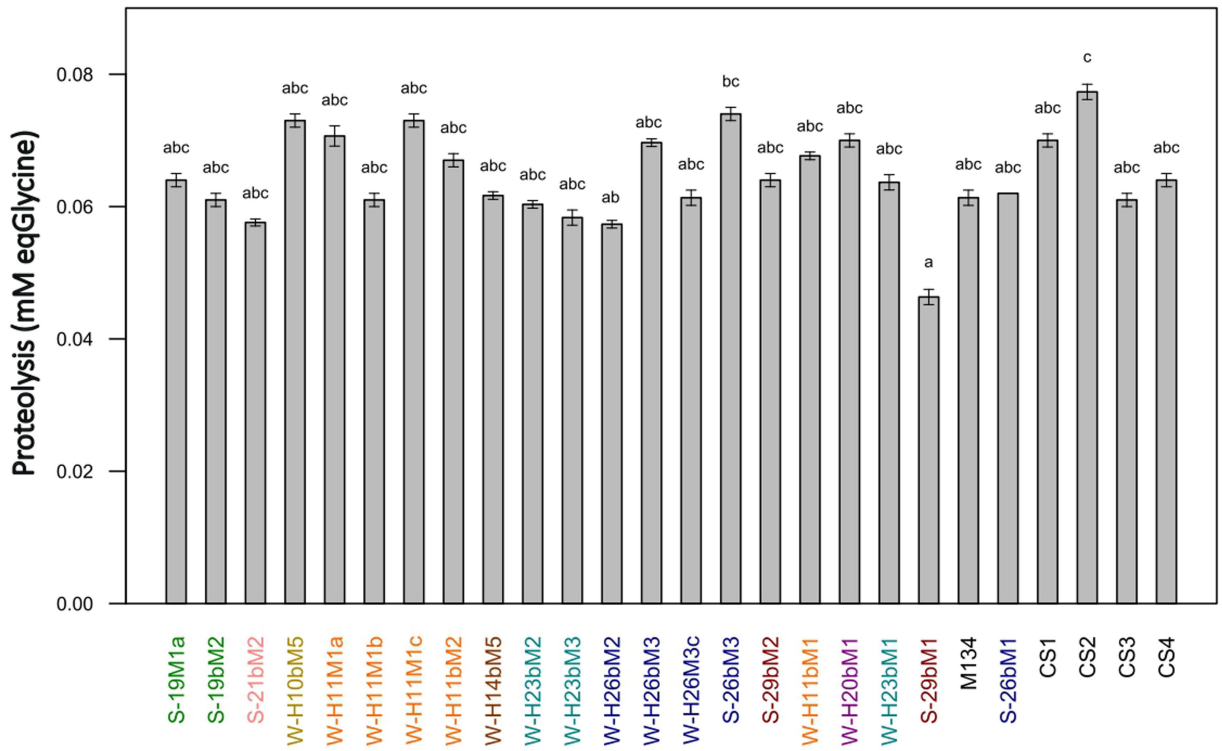

FIGURE 9
Proteolytic activities of *Streptococcus thermophilus* isolates (a–c): mean values without a common superscript are significantly different ($p < 0.05$) according to the Conover-Iman test. Isolates from summer milks are annotated with an "S" and isolates from winter milks are annotated with a "W." Isolate names with the same colors were isolated from the same farm.

important role in the acidifying capacity of *Streptococcus thermophilus* through the regulation of nitrogen and carbon metabolism. These conclusions support the results of Lu et al. (2015), who reported that CodY plays an important role in the regulation of cellular processes in *Streptococcus thermophilus* and showed that the global regulator CodY controlled amino acid metabolism and lactose utilization processes. To better understand the phenotype differences of our genetically closed isolates, a thorough study of the expression and regulation of genes involved in carbon and nitrogen metabolism should be conducted.

The lack of a link between the technological capacities and the presence/absence of the genes of the 26 isolates could also simply be due to the low number of studied isolates. Indeed, when it comes to analyzing correlations between phenotypic and genotypic traits of bacteria, the number of samples (or strains) sequenced plays a crucial role in the power to detect correlations. By increasing the number of samples, it would be possible to significantly improve the ability to identify statistically significant correlations.

4. Conclusion

Wild strains of *Streptococcus thermophilus* could represent a source of genetic and functional variability from which novel strains or properties might be selected for cheese production. This diversity exists within *Streptococcus thermophilus* isolated at the same location and is not dependent on the season of isolation. A specific technological characterization and safety evaluation of isolates should be carried out to validate the use of this diversity as an autochthonous starter culture.

Data availability statement

The data presented in this study are deposited in online repositories. Genomes can be found in the EBI repository under the project accession number PRJEB61322. All input and output files of the bioinformatic analysis are available at https://doi.org/10.57745/MSGWXC.

Author contributions

AG conceived the study, carried out experiments, analyzed the data, performed genome analysis, and wrote the manuscript. ST conceived the study, performed genome analysis, analyzed the data, and participated in the writing of the manuscript. CCa designed the experiments and conceived the project. SH and ED-B supervised the experiments and provided guidance. PG conceived the project and supervised the study. PB and CCh conceived the project, participated in the writing of the manuscript, and supervised the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1245510/full#supplementary-material

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Developing a novel selection method for alcoholic fermentation starters by exploring wine yeast microbiota from Greece

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The selection of native yeast for alcoholic fermentation in wine focuses on ensuring the success of the process and promoting the quality of the final product. The purpose of this study was firstly to create a large collection of new yeast isolates and categorize them based on their oenological potential. Additionally, the geographical distribution of the most dominant species, *Saccharomyces cerevisiae*, was further explored. Towards this direction, fourteen spontaneously fermented wines from different regions of Greece were collected for yeast typing. The yeast isolates were subjected in molecular analyses and identification at species level. RAPD (Random Amplified Polymorphic DNA) genomic fingerprinting with the oligo-nucleotide primer M13 was used, combined with Matrix Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) technique. All yeast isolates were scrutinized for their sensitivity to killer toxin, production of non-desirable metabolites such as acetic acid and H_2S, β-glucosidase production and resistance to the antimicrobial agent; SO_2. In parallel, *S. cerevisiae* isolates were typed at strain level by interdelta – PCR genomic fingerprinting. *S. cerevisiae* strains were examined for their fermentative capacity in laboratory scale fermentation on pasteurized grape must. Glucose and fructose consumption was monitored daily and at the final point a free sorting task was conducted to categorize the samples according to their organoleptic profile. According to our results, among the 190 isolates, *S. cerevisiae* was the most dominant species while some less common non-Saccharomyces species such as *Trigonopsis californica, Priceomyces carsonii*, *Zygosaccharomyces bailii, Brettanomyces bruxellensis* and *Pichia manshurica* were identified in minor abundancies. According to phenotypic typing, most isolates were neutral to killer toxin test and exhibited low acetic acid production. Hierarchical Cluster Analysis revealed the presence of four yeast groups based on phenotypic fingerprinting. Strain level typing reported 20 different *S. cerevisiae* strains from which 65% indicated fermentative capacity and led to dry wines. Sensory evaluation results clearly discriminated the produced wines and consequently, the proposed yeast categorization was confirmed. A novel approach that employs biostatistical tools for a rapid screening and classification of indigenous wine yeasts with oenological potential, allowing a more efficient preliminary selection or rejection of isolates is proposed.

KEYWORDS

Greek terroir, phenotypic diversity, indigenous yeast, wine yeast selection, biostatistics tools

Introduction

The principal metabolic process in wine production is alcoholic fermentation (AF), wherein grape sugars are transformed into ethanol, carbon dioxide through the action of yeast and in parallel a plethora of secondary metabolites are produced (Ribéreau-Gayon et al., 2006; Fleet, 2008; Querol et al., 2018). Although the traditional function of wine yeasts is carrying out alcoholic fermentation, the advent of modern wine microbiology targets to unravel the yeasts properties, in order not only to improve fermentation performance but also wine quality (Barata et al., 2012; Suárez-Lepe and Morata, 2012; Cordero-Bueso et al., 2013; Maicas, 2020).

During fermentation process, the consortium of yeasts is rapidly evolving and shaped by biotic and abiotic factors (Ciani et al., 2004; Jolly et al., 2014; Belda et al., 2016; Sha et al., 2018; Comitini et al., 2021; Dimopoulou et al., 2022). Non-Saccharomyces (NS) yeasts dominate the early stages of fermentation, but the gradual production of ethanol allows the prevalence of the more adaptable species with the most dominant; *Saccharomyces cerevisae* (Lambrechts and Pretorius, 2000; Soden et al., 2000; Clemente-Jimenez et al., 2005; Sadoudi et al., 2012; Liu et al., 2016; Gobert et al., 2017). Other factors besides ethanol, which create a stressful environment, are high sugar concentration (osmotic stress), low pH (acid stress), decreasing oxygen (hypoxia), presence of numerous microorganisms that compete for nutrients or produce inhibitory compounds and also presence of sulfur dioxide (Mateo et al., 2001; Benito et al., 2015; Roudil et al., 2020; Reiter et al., 2021). Nowadays grapes during harvest contain even higher concentrations of sugars due to climate change, rendering the role of yeast even harder and increasing the possibility of stuck or delayed fermentation. Yeast inoculation in wine industry is the key to ensure fermentation flow and sugar depletion (Bely et al., 2008; Benito et al., 2015; Ciani and Comitini, 2015; Dimopoulou et al., 2020). The commercialization of selected autochthonous strains of *S. cerevisiae* to drive alcoholic fermentation is justified by their remarkable adaptability to wine stressors (Fleet, 2008; Rossouw et al., 2012; Reiter et al., 2021). Commercial *S. cerevisiae* strains assure fermentation completion and enhance the standardization and reproducibility of the final product. However, they often lack some unique characteristics linked to biodiversity parameters, and therefore the final wines may lack complexity and typicity (Comitini et al., 2017; Parapouli et al., 2020; Sidari et al., 2021; Christofi et al., 2022).

Targeting the success of alcoholic fermentation and the production of high value wines, producers have focused on the selection of indigenous *S. cerevisiae* strains which have been previously evaluated for their oenological properties to drive AF (Caridi et al., 2002; Le Jeune et al., 2006; Pulcini et al., 2022). Numerous studies focus on the selection of "novel" *S. cerevisiae* with main concern, their improved technological and organoleptic properties; such as high yields of productivity, stress tolerance, unique aromatic characteristics and positive sensory attributes (Capece et al., 2010, 2019; Suárez-Lepe and Morata, 2012; Basa et al., 2022; Tronchoni et al., 2022). Some beneficial oenological traits are alcohol tolerance, lower production of acetic acid and H_2S, SO_2 tolerance, neutral killer character and resistance to high concentrations of sugars (de Ullivarri et al., 2011; Comitini et al., 2017; Pulcini et al., 2022). Furthermore, some yeasts possess the enzymes of β-glucosidases, whose activity results in releasing aglycones and this procedure directly affects beneficially the produced aroma (Mansfield et al., 2002). All the abovementioned characteristics are criteria for the selection of starter cultures, resulting in wines with controlled quality and attractive organoleptic profile (Christofi et al., 2022; Pulcini et al., 2022).

The autochthonous yeast strains, which drive and survive until the end of alcoholic fermentation, are usually characterized by high fermentation rate and alcohol tolerance (Suárez-Lepe and Morata, 2012; Gutiérrez et al., 2013; Garofalo et al., 2018). Numerous researchers have previously isolated a large collection of native yeasts and by means of molecular biology, culture-based methods and mini-vinifications have concluded in some strains with oenological perspective (Caridi et al., 2002; Mestre Furlani et al., 2017; Garofalo et al., 2018; Binati et al., 2019). However, this procedure demands time, advanced analysis and special equipment. The rapid elimination of some isolates from a big yeast collection can result in a more practical and cost-efficient way to select new autochthonous strains with oenological interest. The main objective of this research was to classify a large collection of yeast isolates from spontaneously fermented wines produced from various cultivars and regions in Greece, based on their technological properties with oenological interest. A simple and applicable phenotypic-based methodology for rapid preselection of wine autochthonous yeast with oenological potential is proposed. The qualitative data were transformed accordingly and treated by various biostatistical tools in order to achieve a classification method. The proposed HCA on selected phenotypic tests was validated by wine micro-fermentation trials of the 20 isolated *S. cerevisiae* strains and their corresponding sensory attributes.

Materials and methods

Origin of the samples

Fourteen samples of spontaneously fermented wines were obtained, from four geographical areas in Greece, namely Goumenissa in northern Greece, Pelion in central Greece, Nemea in southern Peloponnese and the island of Santorini (Table 1). The varieties and the vintage of the wines are noted in Table 1. All samples were collected from dry wines (before SO_2 addition), with an alcohol level from 12.5% vol to 14% vol. The majority of the wineries have never used commercial *S. cerevisiae* strains to drive alcoholic fermentation, whatsoever for the wineries that do use, the profile of the commercial strains (Supplementary Table S1) has been compared with the isolated strains of the present study.

All wines were collected from the wineries at the end of the fermentation process.

Molecular characterization and identification of microorganisms

Colonies isolation and purification

For yeast isolation, 100 μL of wine was directly and aseptically spreaded on WL agar plates (Condalab, Madrid Spain). Plates were incubated at 28°C for 48 h. Each sample was analyzed in duplicate. When there were noted more than 20 colonies by plate, a representative

TABLE 1 Sample coding and geographical origin of the wine samples.

Sample ID	Origin	Variety	Type of wine	Vintage	Isolates
GB	Santorini	Assyrtiko	White	2020	5
A6	Santorini	Assyrtiko	White	2020	15
A26	Pelion	Assyrtiko	White	2020	21
K21	Pelion	Xinomavro	Red	2019	9
K29	Pelion	Xinomavro	Red	2020	18
K23	Pelion	Xinomavro	Red	2020	24
K24	Pelion	Xinomavro	Red	2018	21
A30	Nemea	Assyrtiko	White	2020	12
K32	Nemea	Agiorgitiko	Red	2019	18
K33	Nemea	Agiorgitiko	Red	2019	15
K34	Nemea	Agiorgitiko	Red	2019	17
A9	Nemea	Roditis	White	2019	5
A19	Goumenissa	50 Malagouzia/50 Muscat	White	2020	5
K16	Goumenissa	Xinomavro	Red	2020	5

selection of yeast colonies was made from WL plates in accordance with the method described by Harrigan and McCance (1976). Colonies were purified by streaking on YPD agar plates [(g/L): Yeast extract 10, Bacteriological peptone 20, Dextrose (D-Glucose) 20, Agar 20]. Plates were incubated at 28°C for 48 h. Each sample was analyzed in duplicate. Additionally, the cultures were maintained at −20°C in YPD broth supplemented with 30% (v/v) glycerol (Serva, Heidelberg, Germany). Before experimental use each isolate was subcultured twice in YPD broth (at 28°C) for 48 h.

Genomic DNA extraction

Total genomic DNA from the yeast isolates was extracted according to the protocol described by Ercolini et al. (2001) modified by adding lyticase at 2.5 U/mL (Lyticase from Arthrobacter luteus, Sigma–Aldrich, Germany) for yeast cell lysis (Bonatsou et al., 2018). Moreover, quantification and quality control of DNA extract was performed by spectrophotometer (Epoch, Biotek, USA) at wavelengths of 260, 280, and 230 nm.

PCR fingerprinting

RAPD-PCR analysis was initially used for clustering the isolates, employing the primer M13 (5′-GAGGGTGGCGGTTCT-3′), according to the protocol of Lieckfeldt et al. (1993). PCR amplification was conducted in 20 μL final reaction volumes, containing 5 μL of One Taq Quick-Load Reaction Buffer (New England Biolabs, USA), 1 U of One Taq Quick-Load DNA Polymerase (New England Biolabs, USA), 100 μM of dNTP's (10 mM), 10 μM of M13 oligonucleotide primer and 20 ng of template DNA. The amplification program consisted of: 30 s of initial denaturation at 94°C, 3 cycles of 30 s at 94°C, 5 min at 35°C, 5 min at 68°C and then 32 cycles of 30 s at 95°C, 2 min at 53°C, 3 min at 68°C, concluding with 3 min at 68°C.

Genetic diversity within *S. cerevisiae* isolates was assessed by interdelta analysis proposed by Legras and Karst (2003) with minor modifications. Briefly, PCR amplifications were carried out in 20 μL reaction containing 2.5 μL of Buffer A 10 X, 0.25 μL of Taq DNA Polymerase (5 U/μL, Kapa Biosystems, USA), 100 μM of each dNTP, 10 μM of each oligonucleotide primer [delta 12 (5′- TCAACAATGGA ATCCCAAC-3′) and delta 21 (5′-CATCTTAACACCGTATATGA-3′)]. Amplification reactions were performed with the following conditions: 4 min at 95°C followed by 40 cycles of 30s at 95°C, 30 s at 46°C and 90s at 72°C and a finishing step of 10 min at 72°C. In addition to the indigenous *S. cerevisiae* strains, the commercial *S. cerevisiae* strains (Supplementary Table S1) were also examined. The commercial strains were treated as all the other isolates subjected in the fingerprint analysis. All amplifications were carried out in a thermocycler (T100, Biorad, United States).

The products were run on a 1.5% (w/v) agarose gel in 1 × TAE buffer, stained with ethidium bromide (20 min) at 110 V for 140 min and scanned under ultraviolet light (MiniBIS, DNr, Israel). A 100 bp and 1Kb DNA ladder (Nippon Genetics, Germany) served as size standard in RAPD-M13 and interdelta PCR products, respectively. The resulting fingerprints were digitally captured, converted, normalized and analyzed using the Dice coefficient with Bionumerics software version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). Means of the Unweighted Pair Group Method using the Arithmetic Average (UPGMA) clustering algorithm led to the formation of the species- and strain specific dendrogram. Furthermore, species identification was achieved since two to five representative strains from each different cluster (distance >90%) were selected to species identification by MALDI-TOF MS method as described by Windholtz et al. (2022).

Screening the technological properties of the isolated yeasts

Important technological characteristics such as production and sensitivity to killer toxin, acetic acid production, β-glucosidase production, resistance to SO_2 and H_2S production were tested for the yeast screening (Rodríguez et al., 2004; Comitini et al., 2011; Domizio et al., 2011; Konate et al., 2014). All assays were replicated twice. Precultures were grown in YPD broth at 28°C for 48 h.

Production and sensitivity to killer toxin

The killer character determination was performed using the plate assay described by Domizio et al. (2011), with positive activity (K+) recognized by inhibition of growth of the sensitive strain (*S. cerevisiae* SO classic, Martin Vialatte, France), seen as a clear zone surrounding the seeded strain. The *S. cerevisiae* killer strain VIN13, (Anchor, France), showing killer activity, was used as positive control. Sensitive character was observed when colonies could not grow onto agar substrate which was poured with a killer yeast strain (*S. cerevisiae* VIN13, Anchor, France); the isolate was designated as sensitive (K-, R-). The *S. cerevisiae* sensitive strain (SO classic, Martin Vialatte, France), was used as positive control. Yeasts with negative reaction to the killer character (K-) and negative sensitivity (R+) were characterized as neutral (de Ullivarri et al., 2011). The *S. cerevisiae* killer strain VIN13, (Anchor, France), showing killer activity, was used as positive control.

Acetic acid production

Acetic acid production *was* noticed by formation of clear zones around colonies of the strains which were implemented and spotted on Hestrin-Schramm $CaCO_3$ agar (Aydin, 2009). This medium was composed of [g/L: $CaCO_3$ 5.0, Yeast extract 3.0, Agar 15.0 and Dextrose 15.0] (Konate et al. 2014). Cultures were incubated at 28°C for 5 days. The ability of the colonies to form clear zones through the hydrolysis of the white salt was considered as positive reaction to this test.

β-glucosidase production

The β-glucosidase activity was evaluated as described by Rodríguez et al. (2004) on agar plates containing arbutin as substrate. Screening was carried out on agar plates with arbutin as substrate [g/L: Yeast Nitrogen Base/YNB (Condalab, Madrid Spain) 6.7, arbutin (Sigma Aldrich, USA), 5, agar, 20]. The pH was adjusted to 5.0 and after sterilization 2 mL of a sterile 1% (w/v) ferric ammonium citrate solution was added to 100 mL of melted medium. Each plate was inoculated by spot assay, incubated at 28°C and examined after 8 and 15 days. Enzymatic activity was noticed visually when brown color develops in the agar.

Resistance to SO_2

The SO_2 resistance was determined by screening on plates with synthetic substrate. Based on the protocol described in detail by Comitini et al. (2011), the isolates were inoculated onto YPD agar plates at pH 3 (with citrate–phosphate buffer), added with increasing doses of $K_2S_2O_5$ in different concentration corresponding to 0, 100, 200, 300, 400, 500 mg/L of free SO_2 and incubated at 28°C. The yeast growth was observed after 2, 5 and 8 days after the inoculation.

H_2S production

H_2S potential production was estimated by spreading the yeasts onto Biggy Agar (Condalab, Madrid, Spain) (Domizio et al., 2011). On this medium, H_2S-positive isolates create brown colonies, while H_2S negative isolates create white colonies. The H_2S effect was noticed after 2, 5 and 8 days of incubation at 28°C when the color of the colonies was recorded. The following arbitrary scale was used: 0 = white (no production); 1 = light brown; 2 = brown; 3 = dark brown.

Screening on grape must (micro-fermentations)

The fermentation potential of the yeast strains was evaluated in micro-fermentation trials. Fermentations were carried out at 18°C in 50 mL of pasteurized (72°C, 10 min) Assyrtiko must which was provided by Gaia (Sanotrini, Greece) winery (vintage 2021) under static conditions. The initial pasteurized grape must (pH = 3.2, total acidity = 5.77 g tartaric acid/L, YAN = 609 mgN_2/L, 16.6 mg SO_2, 5.1 free SO_2) contained 119.5 g/L glucose and 120.1 g/L fructose. Precultures were grown in YPD broth at 28°C for 48 h, and then used to inoculate each fermentation (10^6 cell/mL). Residual sugar (glucose and fructose) determination was performed on the wines in daily basis using Enzytec kit-liquid Glucose-Fructose (r-biopharm, Germany). All fermentations were assessed duplicated and analysis was conducted twice.

Free sorting task

In the end of the fermentation all produced wines were categorized based on their aromatic profile (odor) by means of a free sorting task test (Sáenz-Navajas et al., 2012). A total of 10 experienced panelists participated in the pilot study. Participants were provided with the produced dry wine samples (15 mL) in ISO approved wine glasses coded with different three-digit numbers and arranged in random order. Participants were asked to sort the 13 wines on the basis of similarity attending to the global sensations perceived in nose (p.e. intensity, floral, fruity, off odor characteristics). Panelists could make as many groups as they wished. Upon completion, they recorded the three-digit codes of the samples of each group on a paper sheet. All wines were served at room temperature. The sessions took place in a ventilated and air-conditioned tasting room (at around 20°C). Panelists were not informed about the nature of the samples.

Data analysis

Univariate analysis

Data obtained from the phenotypic tests, were converted into numerical from character data (+, −) as it is illustrated in Table 2. Consequently, data are further investigated by means of statistical analysis. Firstly, MANOVA was attempted but the scaled data of H_2S experiment during the MANOVA application process could not satisfy its application assumptions. MANOVA assumes multivariate

TABLE 2 Phenotype coding based on the character that resulted after the five different tests.

Digit	Test	Characterization
1st	Killer	1 = neutral, 2 = sensitive
2nd	H_2S	0 = no production, 1 = low production, 2 = high production, 3 = very high production
3rd	Acetic acid	0 = production, 1 = no production
4th	SO_2 resistance	0 = no resistance, 1 = resistance until 300 μg/L, 2 = resistance between 400–500 μg/mL
5th	β-glucosidase	0 = positive response, 1 = negative response

normality and homogeneity of variance–covariance tables between groups. These assumptions were not met, and we considered a univariate analysis using ANOVA more appropriate for each variable. Univariate method may offer simpler and more straightforward interpretations of the results. Using ANOVA for each dependent variable separately allows focusing on each variable's unique response to the independent variables. More specifically, the possibility of having statistically important differences was examined at origin and species level. Therefore we apply the model Yij = μi + εij, i = 1, 2, 3, … where with i we denote the levels of the H_2S factor and j = 1, 2, … the observations we have for each level (Koutras and Evagelaras, 2010). In the current analysis the average level of H_2S production per origin or species was considered as a dependent variable Y and time of incubation as variable X. To obtain safe statistical conclusions, (a) the assumption of equality of dispersions at the levels of factor at the level of significance of 5% and (b) the test of the normality and independence of errors at the level of significance of 5% were carried out. To check the equality of variations, Levene's test was used, where we do not reject the zero hypothesis to be checked and therefore ensure homoscedasedality. Then, to check the normality and independence of the errors, Studentized residuals were used. Utilizing the non-parametric test of Kolmogorov–Smirnov does not reject the null hypothesis that errors follow Normal Distribution. Regarding the test of independence of errors, the non-parametric, Run's test was used and the null hypothesis that errors are independent cannot be rejected. Having ensured the above conditions, the test for whether there are significant differences between the levels of the factor is of the form:

$$H_0 : \mu_1 = \mu_2 = \mu_3 vs\, H_1 : \mu i \neq \mu j \ \textit{for a combination}\,(i, j), i \neq j$$

The value of p of the test is less than 0.05 and therefore at a significance level of 5%, zero assumption that there are no significant differences in the levels of the factor is rejected.

Classification

Hiercharchical Cluster Analysis (HCA) of the different phenotypes based on the results of the five phenotypic tests was performed under R (3.6.2) software using Euclidean distance and Ward method. Among various hierarchical clustering methods such as Single Linkage Method, Weighted Average Linkage Method, Centroid Method, Flexible Strategy Method etc., the Ward's Method was chosen as it is the most effective. This method differs from others and is designed to minimize variance within groups. In particular, the method has some very good properties and usually creates groups with a similar number of observations. The development of logical rules that lead to finding the optimal number of groups of a dataset has occupied several researchers active in the field of cluster analysis, since it is obvious that this problem is of great practical interest. Thorndike (1953) proposed a graphical approach to the problem whereby an axis is first depicted on one axis of the average within-cluster distance and on a second axis of the number of groups. With each increase in the number k of groups there is a corresponding decrease in the average distances within the groups. In most cases a position appears where we have a sharp decrease in the average distances within the groups and then "leveling" the graph. In order to find the number of groups the datagram resulting from a hierarchical cumulative method was examined and from it determine the optimal number. More specifically, at that point of the dendrogram where the greatest change in the quantity recorded on the horizontal axis (distance) is observed, we can bring a parallel line to the vertical axis and see at how many points the datagram intersects. The number k for which we observe large concatenation distances relative to the previous one (k-1 groups) is a reasonable value for the optimal number of groups. For this reason, 4 groups were selected.

Based on the phenotypic test results, we encoded the positive and negative responses to the microbiological phenotypic assays. The data were organized according to oenological significance to establish an overall phenotype. Priority was given to the production of killer toxin, followed by H_2S production, acetic acid production, SO_2 resistance, and, finally, β-glycosidase activity. All parameters were considered, with particular attention to the sequence of data. This arrangement was determined with oenological requirements in mind, aiming for yeast strains that are insensitive to killer toxin, are low producers of H_2S or acetic acid, exhibit resistance to SO_2, and possess desirable β-glucosidase activity. A detailed description is provided of the development of the proposed selection method (Figure 1).

Sensory analysis

Encoding free sorting data was the key to categorize wine samples based on the results of the sensory assessment. For each group, results are encoded in an individual similarity matrix (wines × wines), in which 1 stand for two wines set in the same group and 0 for two wines put in different groups. These individual matrices are summed across subjects; the resulting co-occurrence matrix represents the global similarity matrix where larger numbers indicate higher similarity between samples. The assumption underlying this method is that samples grouped together are more similar than samples sorted into different groups. The resulting cooccurrence matrix was submitted to HCA (Ward method) in order to derive a spatial arrangement of wines with R (3.6.2) software analysis.

Results

The community structure of yeast in wine samples collected directly from wineries was determined at the end of alcoholic fermentation (AF). A total of 14 wine samples were collected, including two from Santorini, five from Pelion in central Greece, five from Nemea in southern Peloponnese, and two from Goumenissa in northern Greece (Table 1). A total of 190 yeast isolates were obtained, and their geographic origins are shown in Figure 2. The obtained RAPD-M13 PCR fingerprints were clustered using UPGMA analysis with Dice as a coefficient, and a representative number of isolates from each cluster were identified using MALDI-TOF MS. Six different species were identified, namely *Saccharomyces cerevisiae* (168 isolates), T*rigonopsis californica* (1 isolate), *Brettanomyces bruxellensis* (5 isolates), *Zygosaccharomyces bailii* (8 isolates), *Priceomyces carsonii* (1 isolate), and *Pichia manshurica* (7 isolates). Specifically, *S. cerevisiae* was the most dominant species with the isolation frequency exceeding 88.4% (data not shown). Although 7 isolates of *P. manshurica* were found, all of them were obtained from a single sample and there was no repetition across samples. Thus, it is not possible to make assumptions based on the presence of a

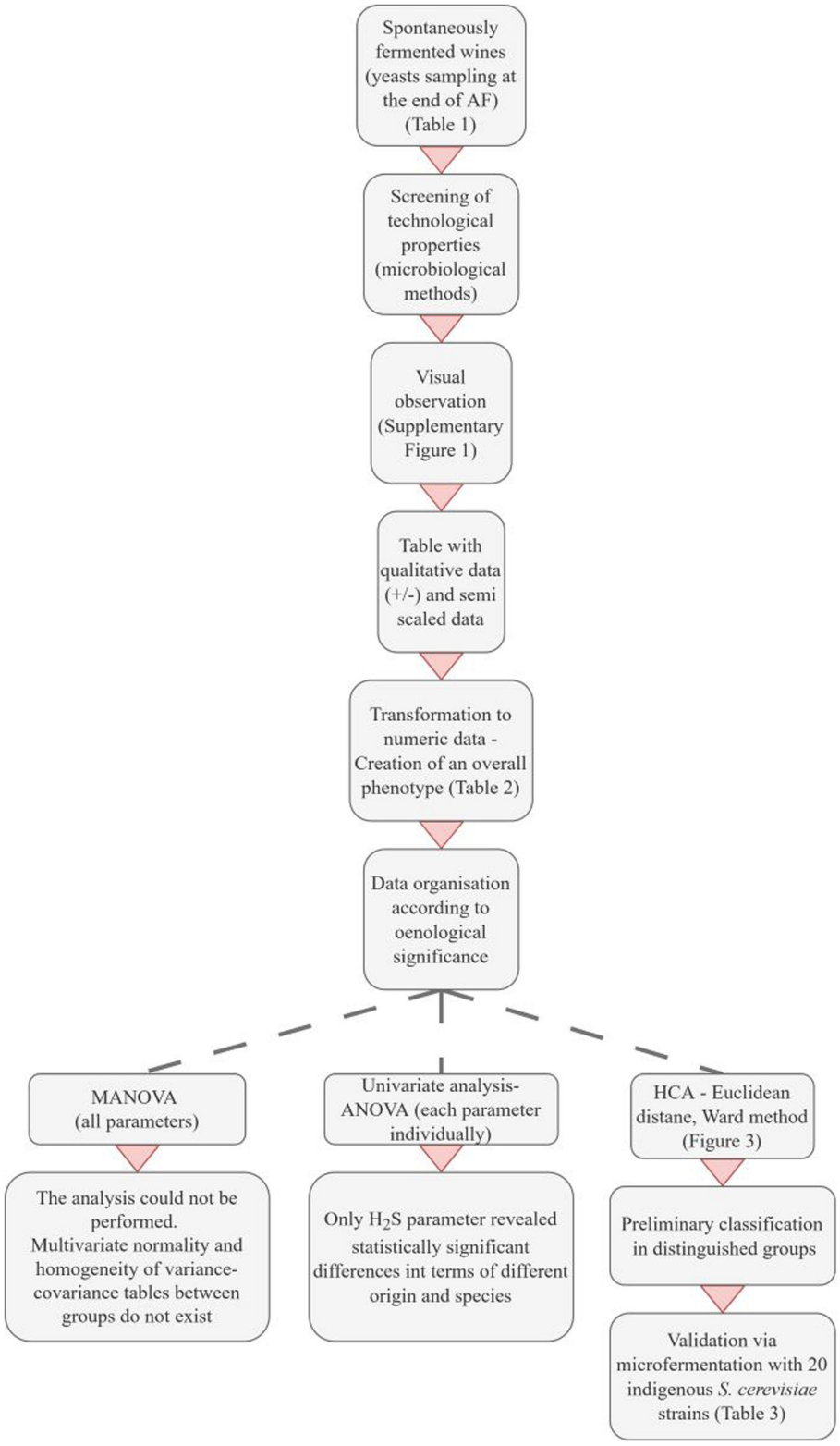

FIGURE 1
Overview of the development of the proposed phenotypic classification method. AF, Alcoholic fermentation; HCA, Hierarchical cluster analysis.

random spoilage yeast/fungus species in just one sample. Conversely, *Z. bailii* was detected in minor amounts in 5 different samples from 3 different regions (Nemea, Goumenissa, and Pelion).

Technological properties of the isolates

All 190 isolates were subjected to some phenotypic tests to monitor their technological properties. These yeasts were analyzed for

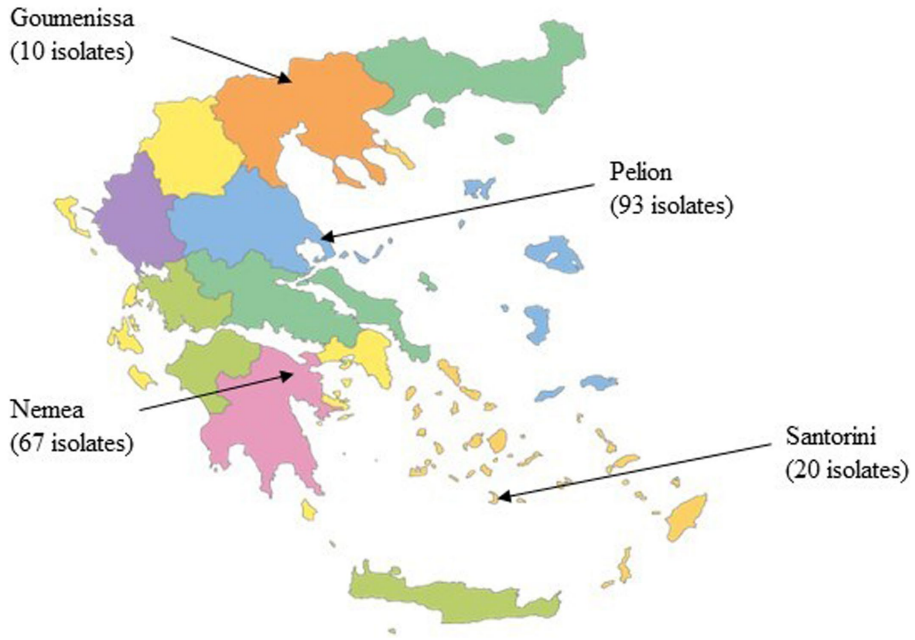

FIGURE 2
Geographical distribution of the yeast isolates.

different characteristics, such as killer activities, acetic acid production, β-glucosidase activity, hydrogen sulphide production and sulphur dioxide resistance, revealing both inter-generic and intra-generic biodiversity.

Among our observations, only 12% of the yeasts (23 isolates) were characterized as sensitive to killer toxin and the rest were noted as neutral. No killer yeast was reported. Among these 23 yeasts the only isolate of *T. californica* is sensitive to killer toxin, 20 isolates belong to *S. cerevisiae* and 2 isolates of *Z. bailii*. 137 yeasts were observed to produce clearly acetic acid and thus forming clear zones around the colony in $CaCO_3$ agar; whereas 53 did not produce acetic acid. From these isolates, *T. californica* made the only exception since the rest 52 isolates belonged to *S. cerevisiae* group. All yeasts showed low or absence of β-glucosidase activity because no color change from white to brown/dark brown was noticed. However, 12 colonies (8 *S. cerevisiae*, 1 *T. californica* and 3 *Z. bailii*) were slightly darker, compared to the others, revealing low enzymatic activity (6,3% of the total isolates). Moreover, regarding the potential H_2S production at species level *B. bruxellensis* followed by *T. californica* and *P. carsonii* perceived significantly the highest levels of H_2S. All isolates of *Z. bailii* proved to be low H_2S producers while *S. cerevisiae* isolates expressed great variability. Sulphur dioxide resistance, a very desirable oenological characteristic, was determined to be a common trait to almost all tested isolates. Only 6, 12 and 11 isolates were sensitive at the concentrations of 100 mg/L, 200 and 300 mg/L, respectively. It is noteworthy that SO_2 inhibited the growth of most *Z. bailii* isolates and 82% of the isolates were resistant to the extreme concentrations of 400 and 500 mg/L.

Hydrogen sulphide production was the only phenotypic characteristic which exhibited statistically significant differences in terms of origin and species characterization. The production of H_2S was measured at 2, 5 and 8 days and color grading indicated its quantity [0 = white (no production); 1 = light brown; 2 = brown; 3 = dark brown]. In the current analysis the average level of H_2S production (0,1,2,3) was considered as a dependent variable Y per origin and species (data not shown), and time of incubation as variable X that takes the values 1: for H_2S that was produced after 2 days of incubation, 2: for H_2S that was produced after 5 days of incubation, 3: for H_2S that was produced after 8 days of incubation. Test of homogenicity of variances, one sample Kolmogorov–Smirnov test and test off between-subjects effects proved that significant differences between the levels of factor H_2S-production exist in terms of origin and species. Isolates from the regions of Nemea and Pelion revealed lower levels of H_2S production, regardless of the species parameter. Moreover, *B. bruxellensis* followed by *T. californica* and *P. carsonii* perceived significantly the highest levels of H_2S. More specifically, *B. bruxellensis* released more H_2S at 5th and 8th day, whereas *T. californica* and *P. carsonii* emissions did not change after the 2nd day. *Z. bailii* proved to be low H_2S producers, *P. manshurica* fair producer and *S. cerevisiae* strain-dependent.

Preliminary categorization of the isolates

A novel approach that employs biostatistical tools for rapid screening and classification of large collection of indigenous wine yeasts, allowing for efficient isolate selection is introduced. Following the results of the screening tests, the positive or negative responses to the phenotypic assays were coded (Table 2) and an overall phenotype has been created. In total, 29 different phenotypes were observed. Hierarchical cluster analysis was performed and four main clusters/groups were finally obtained (Figure 3). The algorithm could discriminate the phenotypes based on the 5 tested different parameters. The first group (yellow) consists of isolates which were characterized as sensitive to killer toxin and consequently the yeasts of this group

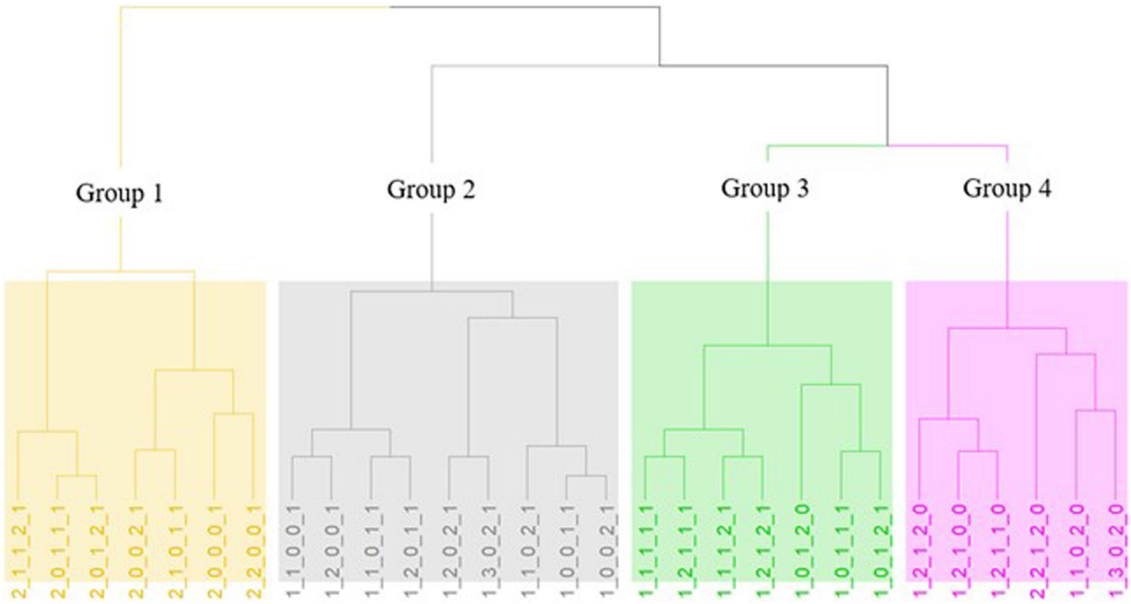

FIGURE 3
Hierarchical cluster analysis (HCA) of the different phenotypes with Eudlidean distance and Ward method. The four groups are distinct by colors: Group 1 = yellow, Group 2 = grey, Group 3 = green and Group 4 = pink.

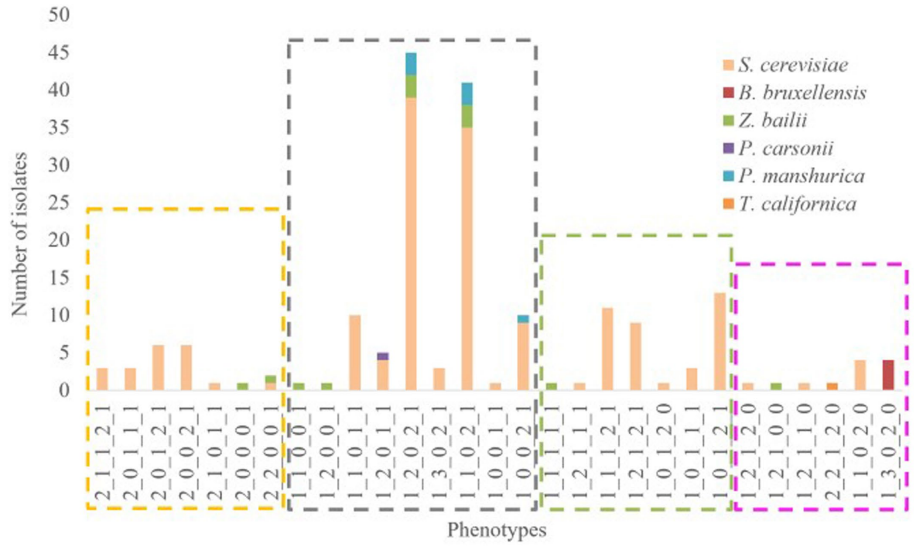

FIGURE 4
Composition plot of species among the different phenotypes. The captions distinguish the groups which were obtained from HCA (Group 1 = yellow, Group 2 = grey, Group 3 = green and Group 4 = pink).

cannot be proposed as starter cultures. Additionally, the second group (grey) is characterized by neutral, no acetic acid production, low resistance to SO_2, no β-glucosidase activity and varies regarding H_2S production. The third group (green) includes neutral yeasts, with low H_2S production, positive acetic acid productivity and absence of β-glucosidase. Finally, in the last group (pink) belong neutral to Killer yeasts (with one exception), high production of H_2S and acetic acid but resistant to SO_2 and possible β-glucosidase activity. Therefore, the most preferred groups are the 2nd – grey and the 3rd – green. Additionally, species allocation among phenotypes was also examined (Figure 4). The isolates of *S. cerevisiae* and *Z. bailli* were distributed in the four created phenotypic groups. On the contrary *P. manshurica* and *B. bruxellensis* only in the second and fourth group, respectively.

Strain identification of *Saccharomyces cerevisiae* isolates

During spontaneous fermentation process, non-Saccharomyces yeasts dominate at the beginning of AF and the conversion of sugars

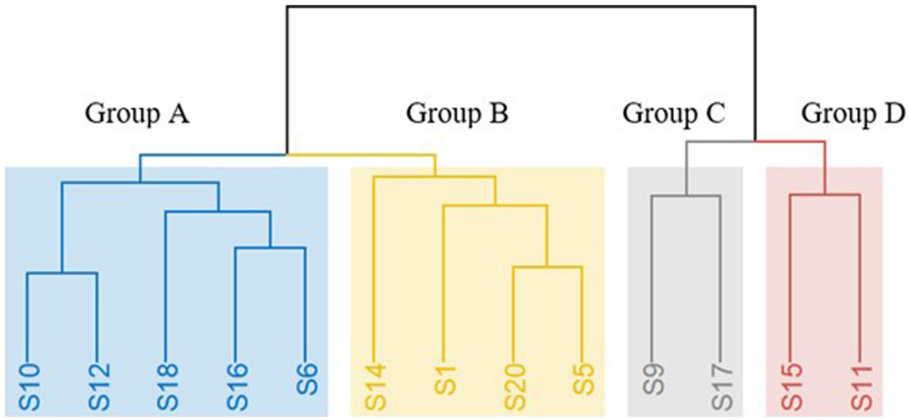

FIGURE 5
Hierarchical cluster analysis (HCA) of the different aromatic profiles of the 13 produced dry wines from different *S. cerevisiae* strains, based on the results of the free sorting task with Ward method. The four groups are distinct by colors: Group A = yellow, Group B = grey, Group C = green and Group D = pink.

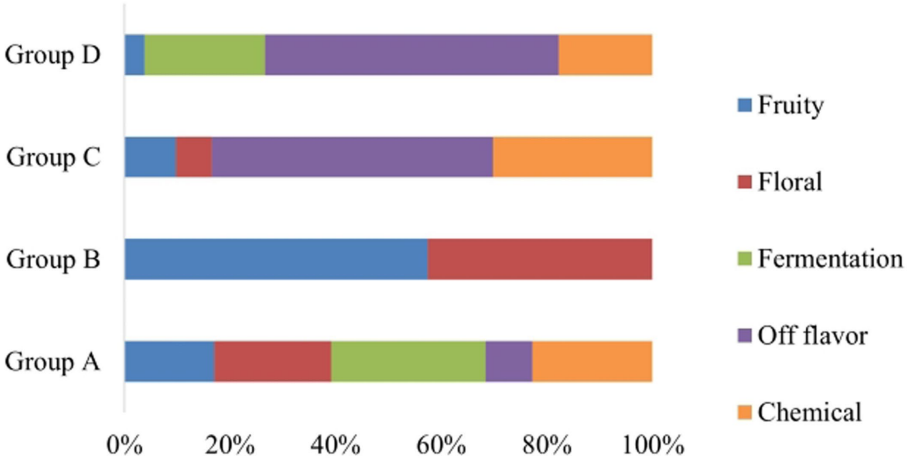

FIGURE 6
% Composition plot of the descriptors defining each cluster (Group A, Group B, Group C, and Group D) according to the panelists of the sensory assessment.

into ethanol is completed by *S. cerevisiae* yeasts (Di Maro et al., 2007; Garofalo et al., 2016). Consequently, validation of the proposed categorization and also the interest for additional insights into the geographical distribution was focused on the isolates which were identified as *S. cerevisiae*. Strain typing of *S. cerevisiae* revealed the existence of 20 distinct strains performing interdelta PCR, namely S1 to S20. To assess the performance of each *S. cerevisiae* strain under fermentative conditions, laboratory-scale fermentations were conducted, and sugar consumption was measured on a daily basis. It was observed that only 35% of the inoculated *S. cerevisiae* strains (S2, S3, S4, S7, S8, S13, S19) had a lower ability to catabolize sugars, resulting in wines with residual sugar levels of greater than 10 g/L. Therefore, those strains are not suggested to be used as starter cultures. Based on the sensory results of free sorting task, four main clusters were identified (Figure 5). The wine samples that were clustered in groups C and D based on Hierarchical Cluster Analysis (HCA) were deemed as undesirable wines (S). On the other hand, Group B exhibited floral and fruity characteristics, while Group A presented a more complex aromatic profile, which was highly preferable (Figure 6). Hence, the proposed strains are those belonging to groups A and B and it is noteworthy that S1, S10, S14, and S20 produced the most desirable wines with no problematic catabolism of sugars and different organoleptic perception.

Table 3 clarifies that the current findings are in line with the preliminary selection of yeasts, as all isolates from the same strain were also grouped in the same cluster based on their phenotypic characteristics. More specifically, from the preliminary selection, the green cluster (Group 3) and the grey cluster (Group 2) consisted mostly of strains from Group A and B, along with some strains that could not catabolize all sugars, namely S1, S7, S10, S13, S18, and S20 for the green cluster, and S5, S6, S8, S12, S14, S16, and S19 for the grey cluster. Additionally, strains that resulted in abnormal fermentations with off-odor characteristics were clustered in the less beneficial

TABLE 3 Fermentation capacity, sensory profile, preliminary group classification, and validation of the classification of the 20 different *S. cerevisiae* strains.

Strain	Fermentation capacity	Sensory profile	Preliminary group classification	Validation of the classification
S1	✓	Group B	3-Green	✓
S2	X	-	1- Yellow	✓
S3	X	-	1-Yellow	✓
S4	X	-	1-Yellow	✓
S5	✓	Group B	2-Grey	✓
S6	✓	Group A	2-Grey	✓
S7	X	-	3-Green	✓
S8	X	-	2-Grey	✓
S9	✓	Group C	1-Yellow	✓
S10	✓	Group A	3-Green	✓
S11	✓	Group D	4-Pink	✓
S12	✓	Group A	2-Grey	✓
S13	X	-	3-Green	✓
S14	✓	Group B	2-Grey	✓
S15	✓	Group D	4-Pink	✓
S16	✓	Group A	2-Grey	✓
S17	✓	Group C	1-Yellow	✓
S18	✓	Group A	3-Green	✓
S19	X	-	2-Grey	✓
S20	✓	Group B	3-Green	✓

groups: 5 strains in the yellow/Group 1 (S2, S3, S4, S9, and S17) and two in the pink/Group 4 (S11 and S15).

Finally, the geographical distribution of the isolated *S. cerevisiae* strains was examined. Three distinct types of *S. cerevisiae* communities were identified among the investigated samples. The first type consisted of wine samples (GB, A9, K16, A19) with up to two different strains, indicating the prevalence of only one or two strains. The second type consisted of samples that were observed with three to four different strains, while the third type included samples A6, K29, K32, and K33, which exhibited the most complex *S. cerevisiae* community structure. In these samples, seven to eight different strains interacted and survived until the end of the alcoholic fermentation process. Additionally, how the contribution of geographical origin, type of wine, and vintage effects to the dispersion of *S. cerevisiae* strains was investigated. Venn diagrams are illustrated in Figure 7, revealing that one unique strain (S10) was isolated from all tested regions, while three strains were found only in Santorini (S15, S17, S18), three only in Nemea (S11, S12, S13) and two Pelion region (S1, S2). Based on the current results, 10 strains (S2, S3, S4, S5, S6, S7, S8, S9, S10, S20) were found in both red and white wines, while 6 strains (S1, S11, S12, S13, S14 and S19) and 4 strains (S15, S16, S17 and S18) were isolated only from red and white wines, respectively.

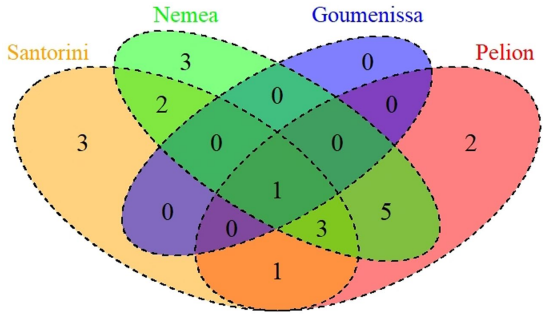

FIGURE 7
Venn diagram showing the number of unique and shared yeast species per sampling region.

Discussion

Nowadays climate change leads to even more stressful conditions for wine yeast, due to the higher concentrations of sugars on grapes during harvest. Consequently, commercial yeasts, which have been isolated in the past, may not be able to adapt to this new challenging environment (Fleet, 2008; Rossouw et al., 2012; Reiter et al., 2021). The commercialization of new selected indigenous strains to drive alcoholic fermentation is a necessity for the wine industry. Therefore, time efficient methods are crucial when isolating new strains. The process of screening and selecting wine yeasts typically involves several sequential steps (Sidari et al., 2021; Pulcini et al., 2022). However, it is necessary to initially exclude certain isolates when dealing with a vast collection of yeast isolates during the selection process. Assessing technological properties through phenotypic plating methods is a well-established approach. However, manual

selection and rejection may not always be feasible especially when no ideal combination of technological properties is discernible, as suggested in previous studies (Mestre Furlani et al., 2017; Sidari et al., 2021).

Yeast selection is a very interesting field not only in wine microbiology, but also in food microbiology. Previous research on indigenous strain selection focused on examining similar characteristics (Caridi et al., 2002; Settanni et al., 2012; Aponte and Blaiotta, 2016). Numerous researchers promote the isolation and selection of indigenous microbiota from various fermented products such as table olives and cheese in order to promote the quality of the final product (del Castillo et al., 2007; Bleve et al., 2015; Bonatsou et al., 2015). In the present study, a similar selection process for indigenous strains was followed, but additionally all the phenotypic results were coded analyzed and further categorized. To the best of our knowledge, no other relevant work managed to transform the qualitative data into numeric in order to perform HCA. Up to now phenotypic results are mostly depicted by tables with negative and positive response and the selection was resulting manually. The proposed idea is to find a way to allow the preliminary rejection of some isolates among a plethora of isolates where no perfect or worst combination is noticed. Qualitative data were transformed into numerical values, and the phenotypic characteristics were arranged based on their impact on the predominance of the selected strain and on the quality of the final product. All isolates were obtained from wines that reflect tolerance to high alcohol and sugar concentrations. Even though no perfect phenotypic combination was noticed, this classification allows the categorization of a large collection of isolates under more than one parameter in a more efficient, low-cost and rapid way.

One necessary trait for a strain, in order to be used as a starter culture, is tolerance to killer toxin (Liu et al., 2015). In the current study, the first digit reveals the killer character and the majority of the isolates were classified as neutral. Previous research has also indicated that autochthonous yeasts are predominantly sensitive or neutral (Comitini et al., 2011; Domizio et al., 2011; de Ullivarri et al., 2014; Velázquez et al., 2016). The lack of killer properties in the isolated yeasts of the current survey, justifies the strain biodiversity among wine samples at the end of AF (Puyo et al., 2023). Furthermore, a brief overview of the literature over the past few decades supports that the most preferable strains should be characterized by non or low production of H_2S in order to be used as starter cultures (Caridi et al., 2002; Settanni et al., 2012; Aponte and Blaiotta, 2016). Thus, H_2S production is evaluated as the second most important oenological characteristic of those examined. From another point of view, organic winemaking process emphasizes on the higher risks of oxidation, microbial contamination and H_2S production. Hence, new starters 'low H_2S – SO_2 – acetaldehyde producers' are desired (Comitini et al., 2017). Additionally, acetic acid screening was performed because some non-Saccharomyces yeasts produce undesirable concentrations of acetic acid and ethyl acetate from sugars, regarded as unsuitable for winemaking (Caridi et al., 2002; Rodríguez et al., 2004). Moreover, recently *S. cerevisiae* and *Z. bailii* adaptive response and tolerance to acetic acid have been investigated based on functional and comparative genomics strategies (Palma et al., 2018; Capece et al., 2022). Sulphur dioxide resistance is a very desirable oenological characteristic and most studies have been conducted in autochthonous *S. cerevisiae* strains, showing high resistance of this species to SO_2 and differentiation at strain level (Divol et al., 2012; Settanni et al., 2012). Although legacy allows up to 150–200 mg/L SO_2 addition for dry wines, while in exceptional cases it can reach up to 400 mg/L for some sweet wines, in the present study the isolates were tested in more extreme concentrations such as 500 mg/L of free SO_2 (OIV, 2023). Consequently, higher concentrations of SO_2 can delay the growth of these isolates, but we have to take into account that the experiment is designed *in vitro* and intermediate concentrations were not examined. Yeasts are the main producers of β-glucosidase which is an important enzyme for the hydrolysis of grape glycosides during winemaking. The importance of glycoside hydrolysis in aroma, flavor, color, and color stability was underlined previously (Mansfield et al., 2002; Settanni et al., 2012; Zhang et al., 2021). Modern winemaking techniques often use specific strains of Saccharomyces or non-Saccharomyces yeasts with known β-glucosidase activity to compensate the insufficient enzyme activity in grapes (Aponte and Blaiotta, 2016). Almost all of the abovementioned results are in line with previous studies, in which it is stated that β-glucosidase activity of the species *S. cerevisiae, P. carsonii* and *Z. bailii* is mostly low or even absent (Rodríguez et al., 2004; Cordero-Bueso et al., 2013; Aponte and Blaiotta, 2016; Vilela, 2020; Zhang et al., 2021). On the contrary, our results do not align with those of previous investigations regarding *P. manshurica*, where many species in the Pichia genus have been characterized for their moderate to high β-glucosidase producing ability and the enhance of beneficial volatile compounds in the final product (Zhang et al., 2021; Perpetuini et al., 2020).

The isolates' classification was validated by the species and strain dispersion. After pilot fermentations assay was conducted, the majority of *S. cerevisiae* strains completed successfully AF and led to wines with exceptional sensory characteristics. Based on recent literature, it is also mentioned that *B. bruxellensis,* in general, intensifies the off-flavor characteristic by producing high concentrations of H_2S (Avramova et al., 2018). According to the results presented in this study, *B. bruxellensis* isolates were all clustered in the group with the highest H_2S production. Notably, there is significant intra-species variability, particularly between *S. cerevisiae* and *Z. bailii* species. The validation of the proposed categorization is further validated by the fact that all isolates belonging to the same strain of *S. cerevisiae* exhibited the same phenotypes without exceptions. It is important to highlight that the strains with the most preferable sensorial characteristics were clustered in the 2nd and 3rd group and the opposite. The evaluation of the alcoholic fermentation and produced wines was achieved by monitoring the sugar consumption and the basic sensory evaluation of the final product. The aim was to be able to discriminate the wines based on their basic organoleptic characteristics, that are also examined by the clustering method [pe off odor aromas (acetic acid, H_2S), fruity/floral aromas (β- glucosidase)], in order to examine the correctness of the proposed classification. The kinetics of alcoholic fermentation reveals the possibility of having a stacked or delayed fermentation. A comparative sensory analysis was chosen in order to evaluate the final product in a more global point of view. According to previous research the free sorting task is an efficient technique for assessing the perception of a set of products by a panel of subjects (Courcoux et al., 2015). This holistic and non-verbal task is an effective tool to be used in product development. Moreover, is a technique that is widespread also in the wine science (Rossouw and Bauer, 2016; Binati et al., 2020).

Hence, the proposed coding and classification method offers the advantage of not requiring expensive molecular techniques and

provides valid results within a short timeframe of only two days. Biostatistical tools enhance the categorization of a large collection of yeasts based on their phenotype and allow a preliminary selection of the isolates. This preliminary rejection is time and cost efficient and therefore a very useful tool not only for wineries but also for yeast suppling companies. In the current study the selection some yeast strains and their direct application in the wine industry is not possible since more analysis of oenological, biochemical and aromatic point of view should be implied.

Wine samples collected at the end of AF revealed the great predominance of *S. cerevisiae*, with a high intraspecific biodiversity (Garofalo et al., 2016). Furthermore, *S. cerevisiae* strains are mostly selected as starter cultures due to their unique biotechnological characteristics, such as fermentation capacity, the production of alcohol and CO_2 and its resilience to adverse conditions of low pH and osmolality (Dimopoulou et al., 2020). The strain collection created in this research verifies the abovementioned observations. The identified different strains varied in richness and evenness among the wine samples, indicating the complex microbial interactions that occur during spontaneous fermentation. Microbial interactions play a dominant and complex role during AF. Despite the presence of multiple *S. cerevisiae* strains, some of which exhibited off-flavor characteristics during the final fermentation stage, none of the wine samples exhibited an off-flavor odors. It is well known that some indigenous strains persist in wineries for multiple years and are referred to as resident strains (Le Jeune et al., 2006). Particularly intriguing is the isolation of a single strain from samples from all regions, vintages and different varieties. This strain could be considered as a universal *S. cerevisiae* strain in Greek terroir. The genetic evaluation of this strain and its expansion all over Greece would be rather intriguing and further research is proposed. Martínez et al. (2004) propose a procedure that could be used as a tool for evaluating if a native isolate derives from the region where it was collected or if it is a strain derived from a commercial strain by microevolution. It is important to highlight that this strain showed different genomic fingerprint based on interdelta analysis among 5 different commercial strains and also it was isolated from a winery which has never used commercial strains. According to a previous study, two *S. cerevisiae* strains were isolated from the final stage of fermentation from different wineries of Beijing and possess important region-specific oenological characteristics (Sun et al., 2009). Finally, it is worth noting that 50% of the isolated strains were found in both white and red wines, suggesting that the assertion of certain strains being exclusively suitable for white or red vinification is questionable.

Conclusion

The aim of this study was to develop a novel, rapid and applicable method for preliminary yeast preliminary selection of alcoholic fermentation starters in wine. The proposed phenotypic classification method was validated by the results of fermentation kinetics and sensory evaluation of the tested *S. cerevisiae* strains. A future perspective is to focus on some of the strains that are presented in the current survey, perform bigger volume laboratory fermentations, large scale pilot fermentations and afterwards to propose new functional indigenous yeast to the wine industry. Additionally, there is no doubt that it would be quite interesting in the future to test the proposed classification test in a larger collection. Interestingly, the geographical distribution of the species revealed the presence of one ubiquitous strain with great oenological potential. Further research work could be done for the evaluation of this unique strain under large scale fermentation in order to examine the commercialization potential by the wine industry.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

AT: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. VT: Data curation, Formal analysis, Methodology, Software, Writing – original draft. IP: Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing. MD: Conceptualization, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – review & editing, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1301325/full#supplementary-material

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Bacterial communities during composting cultivation of oyster mushroom *Pleurotus floridanus* using broken eggs as the nitrogen source and study of agronomic and nutritional properties

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Introduction: Broken eggs are a byproduct of the poultry industry and a potential nitrogen source for mushroom cultivation. However, its feasibility needs to be evaluated experimentally.

Methods: In this study, a series of different addition amounts (0, 1.8, 3.6, 5.3 and 8.5%, w/w) of broken egg mixture (BEM) were applied in the composting cultivation process of oyster mushroom. The physicochemical properties and bacterial communities of composting substrate, and agronomic and nutritional properties of fruiting bodies were determined.

Results and discussion: The results showed that the BEM addition significantly ($P < 0.05$) increased the total nitrogen content in the composted substrate, and the contents of crude protein, total amino acids and essential amino acids of mushrooms. The P3 treatment (initial C/N of 26:1) showed the highest biological efficiency (BE) of 100.19% and a low contamination rate (CR) of 7.00%, while the higher dosage of BEM (P4 and P5) led to a sharp decrease in BE and a sharp increase in CR. High throughput sequencing revealed that the addition of BEM significantly ($P < 0.05$) changed the bacterial communities in the substrate at the beginning of composting. *Streptococcus* and *Lactococcus* were predominant bacterial genera in BEM treatments at the beginning stage of composting, while *Acinetobacter* became predominant at the ending stage. The co-occurrence network analysis showed that the P3 treatment demonstrated a much more complex bacterial community. The structural equation model analysis indicated that the addition of BEM affected the bacterial communities and nitrogen metabolism during composting, which further affected agronomic and nutritional properties of oyster mushrooms. An appropriate amount of BEM combined with composting processes can significantly improve the yield and quality of oyster mushroom, providing a new way for efficient utilization of BEM.

KEYWORDS

***Pleurotus floridanus*, broken eggs, composting cultivation, nutritional properties, bacterial communities**

Introduction

Poultry eggs are a high-quality protein resource and one of the most important animal protein sources for humans (Tang et al., 2022). According to the data of the National Bureau of Statistics, the annual output of poultry eggs in China reached 34.68 million tons in 2020. However, due to the fragility of eggshells, broken eggshells have brought huge economic losses to the egg industry in the production, packaging, storing, transporting, and selling processes (Ma Y. et al., 2020). Approximately 10% of the poultry egg production is lost due to breakage of eggshells, which not only leads to huge economic losses, but also brings about the problem of using broken eggs (Sah et al., 2018; Jiang et al., 2021). Moreover, the broken eggs can serve as a potential high-quality nitrogen source for mushroom cultivation, especially for oyster mushrooms.

Oyster mushroom species, belonging to the genus *Pleurotus*, are one of the most cultivated and consumed mushrooms in the world, accounting for more than 16% of global annual production of edible mushroom (Wan Mahari et al., 2020). The oyster mushrooms have become world popular foods due to their delicious taste, rich nutrition and abundant biological activities (Correa et al., 2016; Rizzo et al., 2021). They are rich in protein and dietary fiber, and low in calories. Moreover, massive researches reveal that mushroom polysaccharides and glycoproteins demonstrate antitumor, antioxidant, anti-inflammatory, immunostimulatory, antidiabetic, anti-hyperlipidemia, hepatoprotective and detoxicating properties (Correa et al., 2016; Rodrigues Barbosa et al., 2020; Jacinto-Azevedo et al., 2021; Rizzo et al., 2021; Koutrotsios et al., 2022).

Oyster mushrooms are efficient lignocellulose decomposing fungi, which can be cultivated using various agricultural and forestry waste (Bellettini et al., 2019; Wan Mahari et al., 2020; Liu et al., 2022). The composting cultivation has become very popular in China, in which the raw materials undergo a short-term composting process (about 5–10 days) before pasteurization (or not) and mushroom spawning processes (Kong et al., 2020; Guo et al., 2021). In the process, the composted substrate is directly bagged and inoculated with spawns, or undergoes a short-term steam pasteurization process (100°C, about 1–4 h) before inoculation (Liu et al., 2022; Yang et al., 2022). The non- or short-term pasteurization of compost leads to the survival of microorganisms (especially bacterial species) in the composted substrate, which play important roles in improving the mushroom yield and reducing the CR (Kong et al., 2020; Yang et al., 2022). At the same time, different raw materials and cultivation techniques also lead to differences in the nutritional properties of fruiting bodies. Previous studies revealed that the crude protein and β-glucan contents of *Pleurotus ostreatus* grown on oil palm by-product formulated substrates were significantly higher than those grown on the control (Aubrey et al., 2022, 2023).

The nitrogen source is one of the most important factors for mushroom cultivation. It can affect the enzyme activities of mycelia, as well as the yield and quality of mushrooms. Agricultural by-products such as wheat bran and soybean meal are often used as nitrogen sources for oyster mushroom cultivation, while there is relatively little research on using poultry by-products as nitrogen sources (Bellettini et al., 2019; Wan Mahari et al., 2020). In addition, a proper amount of high-quality nitrogen source can improve the yield and quality of fruiting bodies (Bellettini et al., 2019; Kumla et al., 2020). However, few studies have reported the effects and mechanisms of composting microorganisms on the nutritional properties of oyster mushroom cultivated with the composted substrate. It is necessary to analyze the microbial communities in the composted substrates and their correlations with the nutritional properties of fruiting bodies. The aim of this study was to evaluate the feasibility and optimum amounts of broken eggs as the nitrogen source during the composting cultivation of oyster mushrooms, bacterial communities in the compost and their effects on the agronomic and nutritional properties. The results will also provide a sustainable approach for the application of broken eggs.

Materials and methods

Materials

The strain of oyster mushroom used was *Pleurotus floridanus* "Heiping 6," which was collected in Laboratory of Edible and Medicinal Fungi, Beijing University of Agriculture (BUA, Beijing, China). The stock culture and grain spawns were prepared using the potato dextrose agar and wheat grain medium, respectively and cultured at 25 ± 2°C. Peach sawdust (2–20 × 2–3 mm) was crushed using naturally dried peach branches which were collected from Dahuashan Town (Beijing, China). The broken egg mixture (BEM), consisting of egg liquid, eggshells and water, was donated by a local layer farm. The corncob, wheat bran and lime were purchased from local markets. The physicochemical properties of raw materials were determined as described by Zou et al. (2020) and showed in Supplementary Table S1.

Composting design and sampling

The composting formulas were determined based on the initial C/N ratio of substrates of approximately 20:1–30:1 and listed in Table 1 (Wan Mahari et al., 2020). The BEM treatments P1–P5 were added 5.60–28.00 kg BEM as the nitrogen source. A common formula using wheat bran as the nitrogen source was treated as the blank control treatment (CK) (Yang et al., 2022). Three parallel composting experiments were performed in the Beijing Science and Technology Backyard No. 38, Dahuashan Town, Beijing, China. The raw materials were well-mixed and adjusted the initial moisture content (MC) to approximately 60–65%, and then piled into trapezoidal piles as described by Yang et al. (2022). According to the actual production of farmers and our preliminary studies, the short-term composting process lasted for 3 days, and the compost was turned at the 2nd and 3rd day (Guo et al., 2021). Samples were collected at the beginning and ending of composting (numbered as BM and CP, respectively), and stored at − 80°C or 4°C for further analyses. Nine points random sampling method was adopted for sampling, and three samples were randomly collected at the top, middle, and bottom depths of the composting pile for mixing as one sample (Guo et al., 2021).

Mushroom cultivation

After the short-term composting, the composted substrate was bagged (22 × 48 mm, approximately 2.0 kg fresh weight each), followed

TABLE 1 Composting formulas and their C/N ratio.

Formula	Peach sawdust (kg)	Corncob (kg)	Broken egg mixture (kg)	Wheat bran (kg)	C/N
CK	210	90	0	50	32:1
P1	210	90	5.60	0	36:1
P2	210	90	11.20	0	33:1
P3	210	90	16.80	0	26:1
P4	210	90	22.40	0	24:1
P5	210	90	28.00	0	22:1

by a steam pasteurization at 100°C for 4 h (Wan Mahari et al., 2020; Yang et al., 2022). Each treatment contained 300 cultivation bags, which was randomly selected and equally divided into three parallel groups (100 bags each). After cooling down to room temperature, the cultivation bags were inoculated using the spawns (2%, v/v) and incubated in the dark at 25 ± 2°C until the mushroom mycelia fully colonized the substrate. Subsequently, the matured cultivation bags were moved into a greenhouse for the fruiting management and harvest (Yang et al., 2022). The fruiting bodies were collected for three flushes.

Physicochemical analysis

The physicochemical properties of the composted substrate were determined, including the pH, electrical conductivity (EC), the content of moisture (MC), ash, organic matter (OM), total carbon (TC) and total nitrogen (TN), and the C/N ratio, followed the methods described by Guo et al. (2021). The degrading enzymes in the composted substrate were determined by the spectrophotometric methods at 50°C, including protease (Pro), filter paper cellulase (FPase), xylanase (Xyl), and laccase (Lac) (Wang et al., 2012; Zhang et al., 2019).

Agronomic and nutritional properties

After the harvest, the agronomic properties of mushroom fruiting bodies were determined, including incubation period (IP, time for mycelia fully colonized the substrate), yield of the first flush, total yield of three flushes, biological efficiency (BE), contamination rate (CR), fresh weight of single mushroom (FWSM), and length of stipe (LS), diameter of stipe (DS), thickness of pileus (TP), and diameter of pileus (DP) (Ma N. L. et al., 2020). The BE refers to the amount of fresh fruiting bodies produced from a certain amount of raw materials, and the contamination rate is the proportion of contaminated sticks to total mushroom sticks. They were determined as described by Yang et al. (2022). In detail, BE (%) = the fresh weight of fruiting bodies of each 100 bags harvested from the first three flushes/the dry weight of the relative substrate × 100. CR (%) = number of contaminated bags per 100 cultivation bags. The BE was determined based on the yield of three flushes, whereas the agronomic properties of single mushroom were determined based on the fresh fruiting bodies of the first flush.

The nutritional properties of the first-flush mushrooms were determined, including contents of crude fiber, crude fat, crude protein and amino acids. The fresh fruiting bodies were dried at 60°C in a drying box until a constant weight. The dried samples were subsequently pulverized by an electric crusher, and sifted through a 200-mesh sieve. The obtained powder was used for further assays on nutritional properties. The crude fiber content was determined according to the Chinese National Standard Method (GB/T 5009.10-2003). The crude fat content was determined using a Soxhlet apparatus (GHYK-4A, Gaohuan Youke, China) (Jacinto-Azevedo et al., 2021). The dried mushroom powder was hydrolyzed with HCl (6 mol/L) containing 5 mg/mL of phenol under vacuum at 110°C for 24 h, subsequently analyzed by an automatic amino acid analyzer (L-8800, Hitachi, Japan) (Wang et al., 2022; Guo et al., 2023). The contents of total amino acids (TAA), essential amino acids (EAA), non-essential amino acids (NEAA), sweet amino acids (SAA, Ala, Gly, Pro, Ser and Thr), bitter amino acids (BAA, Arg, His, Ile, Leu, Met and Val), flavour amino acids (FAA, Asp and Glu) and aromatic amino acids (AAA, Phe and Tyr) were further calculated (Liang et al., 2020; Guo et al., 2023).

High throughput sequencing

The composting samples at the beginning (BM) and ending (CP) stages of CK, P1, P3, and P5 treatments were further analyzed by the high throughput sequencing. The total genomic DNA of composting samples were extracted using a soil DNA kit (E.Z.N.A.®, Omega Bio-tek, United States) following the standard protocol. The concentration and quality of DNA obtained were evaluated using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, United States) and 1% agarose gel electrophoresis. Subsequently, the high throughput sequencing based on 16S rRNA gene was performed by Majorbio Bio-Pharm Technology Co. Ltd. (China) using a MiSeq PE300 platform (Illumina, United States) (Yang et al., 2022). The primer pairs used for sequencing were 338F/806R for V3–V4 region of 16S rRNA gene. The raw 16S rRNA gene sequence data was further uploaded to the NCBI Sequence Read Archive (SRA) database with the accession number of PRJNA884292.

Bioinformatics analysis

The raw sequencing data were quality-filtered and assembled into high-quality reads using fastp (v0.20.0) and FLASH (v1.2.7). The operational taxonomic units (OTUs) were picked based on 97% similarity using the high-quality reads and USEARCH (v 7.1). Taxonomy assignment of the 16S rRNA gene sequences was performed using the SILVA database (v138) with the RDP classifier (v2.2) (Yang et al., 2022). The basic analyses were illustrate using the

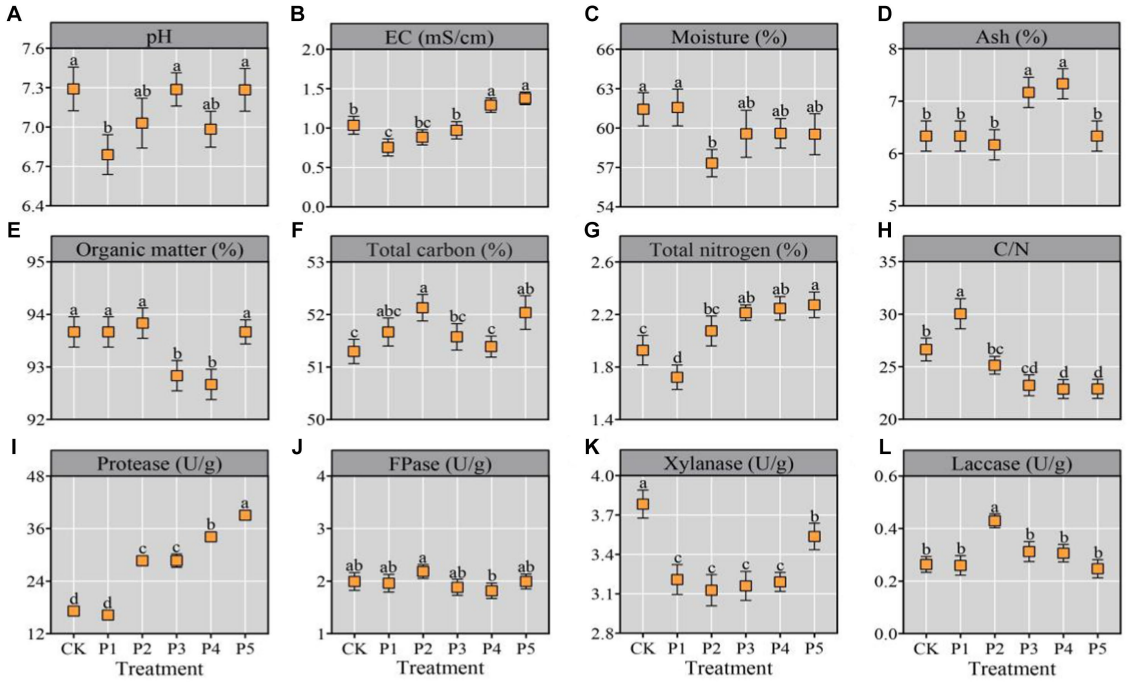

FIGURE 1
Physicochemical properties of substrate after composting. All the data were expressed as means ± SD ($n = 3$). Different lowercase letters denote significant differences ($p < 0.05$).

online Majorbio platform[1] (Guo et al., 2021). The Co-occurrence network analysis was conducted using R software (v4.1.3) with the Spearman's correlation coefficient (r) > 0.6 and statistically significant (P) < 0.05 (Barberan et al., 2012). The prediction of bacterial metabolism was performed using the PICRUSt2 tool (v2.2.0) based on the KEGG database. The correlations between physicochemical and bioinformatic parameters were conducted using the Mantel test via online R software[2] (Yang et al., 2022).

Statistical analysis

All the data were analyzed using Microsoft Excel (v2016) and Origin (v2020b), and presented as means ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA using SPSS (v.25.0). A $p < 0.05$ was considered statistically significant. The structural equation modeling (SEM) analysis was conducted using SPSS Amos Graphics (v26.0) (Wu et al., 2021).

Results and discussion

Physicochemical properties of compost

The pile temperature of each treatment sharply increased by over 50°C and maintained a thermophilic stage of 51–78°C for two days (Supplementary Figure S1). High temperature promotes the proliferation of thermophilic microorganisms and the decomposition of lignocellulose, which is a critical factor determining the success or failure of composting (Kong et al., 2020; Guo et al., 2021). The physicochemical properties of the composted substrate were shown in Figure 1. All the treatments shared a near-neutral pH range (6.79–7.29) (Figure 1A), which resembles the optimal pH range (6.5–7.0) for mycelial growth of most oyster mushrooms (Wan Mahari et al., 2020). There was a small amount of eggshells in BEM, which ash content was close to that of wheat bran (Supplementary Table S1). With the continuous increase of BEM content from P1 to P5, the EC of compost also continuously increased from 0.76 to 1.38 mS/cm (Figure 1B). Moreover, all the treatments represented relatively low EC, which would not damage the growth of mushroom mycelia (Yang et al., 2022). The P4 and P5 treatments demonstrated significantly ($p < 0.05$) high ash content and low OM content (Figures 1C,D), indicated that the two treatments consumed more OM during composting. Moreover, all the treatments shared a OM range of 92.67–93.87%, which was very close to that of compost after a short-term composting of 4–5 d (Yang et al., 2022).

The TC, TN, and C/N ratio are key factors for oyster cultivition, which directly affect the mycelial growth ratio and mushroom yield (Bellettini et al., 2019). In this study, the TC, TN, and C/N ratio were 51.57–52.13%, 1.72–2.27%, and 23/1–30/1, respectively (Figures 1E–H), which were close to the previous reports (Guo et al., 2021; Yang et al., 2022). Previous reviews on oyster mushroom cultivation indicated a optimun TN range of 1.84–2.08% and an optimum C/N range of 28/1–30/1 (Bellettini et al., 2019; Wan Mahari et al., 2020). Nitrogen participates in many important metabolic pathways in cells, including amino acid, protein and nucleic acid

1 www.i-sanger.com
2 https://www.cloudtutu.com/#/manteltest

TABLE 2 Agronomic properties of fruiting bodies in different treatments (100 bags each).

Treatment	Incubation period (days)	Contamination rate (%)	Yield of first flush (kg)	Total yield (kg)	Biological efficiency (%)
CK	17 ± 2b	13.30 ± 1.71b	24.40 ± 0.19c	53.21 ± 0.77c	73.91 ± 1.06c
P1	16 ± 2b	1.60 ± 0.27c	28.30 ± 0.46b	64.40 ± 0.49b	89.45 ± 0.68b
P2	17 ± 1b	2.00 ± 0.39c	32.49 ± 0.55a	65.41 ± 0.63b	90.84 ± 0.88b
P3	19 ± 2b	7.00 ± 0.59bc	32.66 ± 0.36a	72.14 ± 0.77a	100.19 ± 1.07a
P4	32 ± 3a	68.75 ± 10.63a	7.22 ± 0.17d	16.41 ± 0.35d	22.79 ± 0.48d
P5	34 ± 3a	69.52 ± 8.61a	6.22 ± 0.15e	14.79 ± 0.24e	20.54 ± 0.33e

All the data were expressed as means ± SD ($n = 3$). Different letters in the same column indicate significant differences ($p < 0.05$).

metabolisms. Too low or too high TN will inhibit the mycelial growth of oyster mushrooms and decrease the yield (Wan Mahari et al., 2020). Properly increasing the nitrogen content in the substrate can improve nitrogen metabolism, and thus improve the yield and nutrition properties of oyster mushroom (Rizki and Tamai, 2011; Sözbir et al., 2015). Generally, wheat bran is the main nitrogen source for mushroom cultivation (Bellettini et al., 2019). Eggs are one of the most nutritious foods and perfect protein sources. In this study, the addition of BEM in P3, P4, and P5 treatments significantly ($p < 0.05$) improved the TN in the final compost for mushroom cultivation (Figure 1G). It indicates that the addition of BEM in the composting raw materials may increase the yield and quality of oyster mushroom.

Furthermore, the degrading enzyme activities towards protein and lignocellulose in the compost were determined. With the increase of BEM addition, the protease activity in the compost gradually increased and reached the maximum in the P5 treatment (Figure 1I). It may be due to the addition of BEM activating the composting microorganisms with protease activities. All the six treatments represented a FPase range of 1.81–2.18 U/g, an Xly range of 3.13–3.78 U/g and a Lac range of 0.25–0.43 U/g (Figures 1J–L), which were similar with the degrading enzyme activities in the compost reported by Yang et al. (2022). During the short-term composting processes of substrate for oyster mushroom cultivation, the degradation ratios of cellulose and hemicellulose are usually higher than that of lignin, which was basically consistent with this study (Guo et al., 2021; Yang et al., 2022).

Agronomic properties of fruiting bodies

The agronomic properties of *P. floridanus* in different treatments were summarized in Table 2. The IP partly reflects the quality of the substrate for oyster mushroom cultivation. The CK, P1, P2, and P3 treatments shared a close IP of 16–19 days, which was approximate to that of *Pleurotus citrinopileatus* mycelia cultivated on the wheat straw substrate (17 days) (Koutrotsios et al., 2022). However, the P4 and P5 treatments represented significantly ($p < 0.05$) longer IP of 32–34 days, indicating that they were not suitable for the mycelial growth of oyster mushroom. They also demonstrated the highest CR of 68.75 and 69.52%, respectively. There may be more miscellaneous microorganisms in the substrates of the two treatments, which inhibited the normal growth of mushroom mycelia. A pasteurization at 100°C for 4 h cannot kill all microorganisms in the compost (Yang et al., 2022). The survived microorganisms during composting process are one of the keys for improving the mushroom yield and reducing the CR (Kong et al., 2020; Guo et al., 2021; Yang et al., 2022). The composting formula, composting time and pasteurization time affect the physicochemical properties and microbial communities of the final substrate for mushroom cultivation (Yang et al., 2022). The addition of high dose of BEM led to higher CR in P4 and P5 treatments. The results indicate that it is necessary to reduce the dosage of BEM or change the composting duration and pasteurization time.

Oyster mushrooms are efficient lignocellulose decomposers with high yield and BE (Bellettini et al., 2019; Wan Mahari et al., 2020). Many researchers have focused on optimizing the formulas of cultivation substrate to further improve the yield and nutritional quality (Koutrotsios et al., 2022; Wang et al., 2022). In this study, the P3 treatment represented significantly ($p < 0.05$) high yield of the first flush (32.66 kg), total yield (72.14 kg), and BE of three flushes (100.19%) compared with the CK treatment (Table 2). *Pleurotus citrinopileatus* grown on wheat straw, winery and olive mill wastes represented the BE of three flushes of 53.70, 78.52, and 26.24%, respectively (Koutrotsios et al., 2022). *Pleurotus pulmonarius* grown on four kinds of substrates showed a BE range of 61.89–85.01%, based on two flushes (Wang et al., 2022). It suggests that a proper addition of BEM can improve the yield and BE of *P. floridanus*. In addition, the low BE of P4 and P5 treatment was mainly due to the high CR during the IP, resulting in a sharp decline in the total yield. Due to the inhibition of mushroom mycelial growth by miscellaneous microorganisms in the substrate, the utilization of the substrate was reduced, resulting in a decrease in mushroom yield and BE. The agronomic properties of single mushroom were summarized in Supplementary Table S2. Compared with CK, the BEM treatments P1, P2, and P3 were similar in LS, DS, TP, and FWSM (no significant difference). It indicates that a proper addition of BEM as the nitrogen source has no significant effect on the commercial properties of the oyster mushroom.

Nutritional quality of fruiting bodies

The oyster mushrooms are rich in nutrients, which nutrient composition changes with different cultivation substrates (Suwannarach et al., 2022). Low doses of BEM (P1 and P2 treatments) can significantly ($p < 0.05$) increase the crude fiber content of fruiting bodies, whereas there was no significant ($p < 0.05$) difference between high doses (P3-5) and CK treatments (Figure 2A). It indicates that BEM as the nitrogen source will not reduce the crude fiber content of oyster mushromm fruiting bodies. Furthermore, The addition of BEM

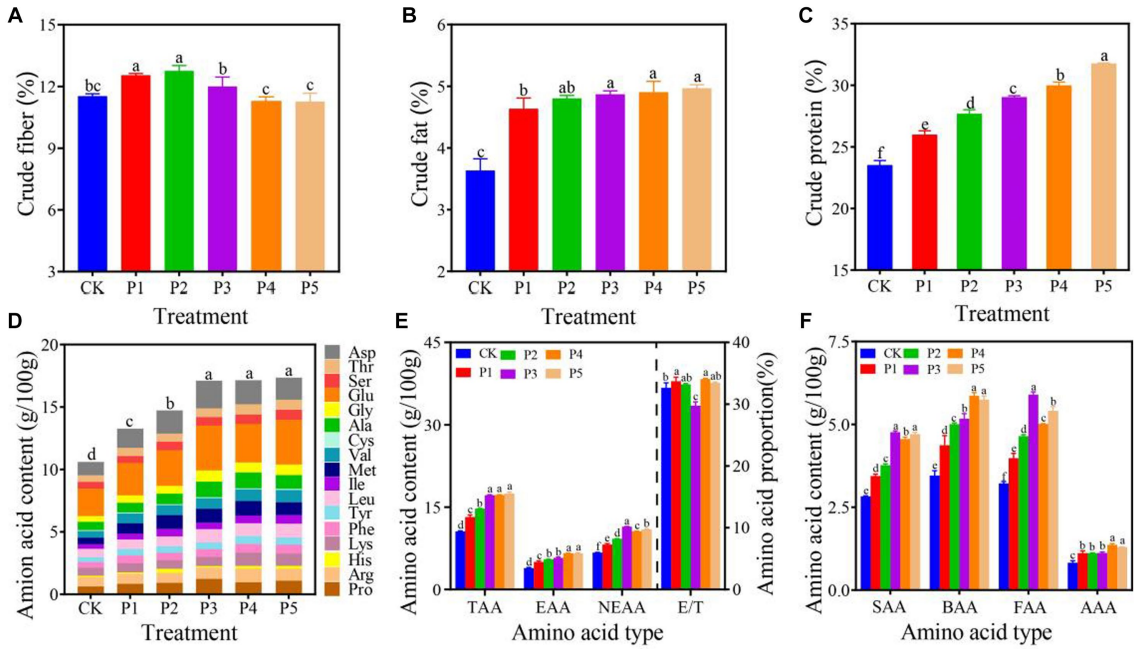

FIGURE 2
Nutritional composition of *P. floridanus* fruiting bodies grown on diffferent substrates (based on dry weight). AAA, aromatic amino acids; BAA, bitter amino acids; EAA, essential amino acids; E/T, EAA/TAA; FAA, flavour amino acids; NEAA, non-essential amino acids; SAA, sweet amino acids; TAA, total amino acids. All the data were expressed as means ± SD ($n = 3$). Different lowercase letters denote significant differences ($p < 0.05$).

significantly ($p < 0.05$) increased the crude fat content of fruiting bodies. The P1–P5 treatments represented a crude fat content range of 4.63–4.96%, which was 27.55–36.64% higher than that of CK (3.63%) (Figure 2B). The crude fat content of mushrooms varies greatly depending on their own characteristics and growth conditions such as nitrogen sources in the substrate (Asaduzzaman Khan and Tania, 2012). *Pleurotus ostreatus* and *P. cystidiosus* showed a crude fat content range of 1.32–2.78% and 2.05–3.33%, respectively, when they were grown on diffferent substrates (Hoa et al., 2015). Eggs contain certain lipids, which led to the significant ($p < 0.05$) increase in crude fat contents in BEM treatments. In addition, lipids are important energy substances and components of cells. Proper increase of the crude fat content can also improve the taste of fruiting bodies.

With the increase of BEM content, the crude protein content of fruiting bodies in BEM treatments increased significantly ($p < 0.05$) from 25.97 to 31.76%, which was 10.46–35.09% higher than that in CK (23.51%) (Figure 2C). The crude protein content of *Pleurotus* genus ranges from 11 to 42% depending on different species and cultivation conditions (Asaduzzaman Khan and Tania, 2012).The crude protein content of *P. floridanus* significantly ($p < 0.05$) increased from 23.3 to 29.8%, when it was grown on rice straw supplemented with *Leucaena leucocephala* foliage (Andrew, 2022). The crude protein content of *P. ostreatus* and *P. cystidiosus* ranged between 19.52–29.70% and 15.68–24.54%, respectively, when they were grown on different substrates (Hoa et al., 2015). Although only P3, P4, and P5 treatments had significantly ($p < 0.05$) higher TN content of substrate than the CK treatment (Figure 1G), the crude protein content of all BEM treatments was significantly ($p < 0.05$) higher than in CK. It indicates that the high content of BEM in substrate contributed to the higher protein content of fruiting bodies.

Furthermore, the amino acid content and taste characteristics of each treatment were determined. The amino acid content of BEM treatments ranged from 13.20 to 17.45 g per 100 g dry weight, which was significantly ($p < 0.05$) higher than that of CK treatment (10.59 g/100 g d.w.) (Figure 2D). The Glu and Asp were the most abundant amino acids in all the treatments, whereas the content of Ala, Arg, Asp. Glu, Gly, Ile, Leu, Met, Pro, Ser, Thr and Val in the BEM treatments were significantly ($p < 0.05$) higher than those in CK (Supplementary Table S3). The Glu and Asp are known as monosodium glutamate like amino acids, which are responsible for the characteristic umami taste of mushrooms (Bellettini et al., 2019; Koutrotsios et al., 2022). It indicates that the friuting bodies grown on BEM additive substrates taste better. Moreover, the addition of BEM also resulted in significantly ($p < 0.05$) higher contents of TAA, EAA, NEAA, SAA, BAA, FAA, and AAA in BEM treatments than in CK, which makes them have higher nutrition and better taste (Figures 2E,F). Compared with the CK treatment, the P3 treatment represented significantly ($p < 0.05$) high TAA, NEAA, SAA, and FAA of 17.17, 11.42, 4.76, and 5.90 g/100 g d.w., respectively. The TAA, SAA, and FAA contents of *P. pulmonarius* ranged between 18.90–27.97, 12.25–19.35, 0.35–1.12, and 0.69–1.16 g/100 g d.w., respectively grown on three different substrates and the first two flushes (Wang et al., 2022). In addition, the EAA/TAA (E/T) range of all treatment was 33.57–37.40%, which was close to that of *P. pulmonarius* (31.00–37.00%) and other edible wild-grown mushrooms from China (30.50–43.40%) (Wang et al., 2014, 2022). In brief, the BEM is a high-quality nitrogen source for oyster mushroom cultivation, which can improve the crude protein content, amino acid composition and flavor of the fruiting bodies.

Bacterial communities in the substrate

Thermophilic bacteria are reported to play important roles in the composting process for oyster mushroom cultivation substrates (Yang

et al., 2022). To further evaluate the impacts of composting process on the agronomic and nutritional properties of fruiting bodies, the high-throughput sequencing was performed based on the substrate of CK, P1, P3, and P5 treatments at BM and CP stages. A total of 23 phyla, 59 classes, 152 orders, 259 families, 535 genera, and 1,204 OTUs of bacteria obtained with a sequence similarity ≥97% (Supplementary Table S4). Actinobacteriota (44.77%), Firmicutes (39.42%), and Proteobacteria (13.44%) were the predominant bacterial phyla in BM_CK, whereas the predominant bacterial phyla of BEM treatments at BM stage were Firmicutes (77.61–82.90%), Actinobacteriota (12.41–13.80%) and Proteobacteria (2.34–6.71%) (Figure 3A). These phyla accounted for 97.64–99.00% of the entire representative of bacterial sequences in the samples. It indicates that almost all bacteria in substrates were detected by the high-throughput sequencing (Qiu et al., 2022). The predominant bacterial phyla of BM_CK were similar with those of samples before composting for oyster mushroom cultivation (Guo et al., 2021). The addtion of BEM strongly increased the relative abundance of Firmicutes speciese at BM stage, which could be attributed to the bacterial community of BEM itself. After the short-term composting process, Firmicutes (33.01%), Proteobacteria (28.70%), Actinobacteriota (22.77%), and Bacteroidota (11.37%) were the predominant bacterial phyla in CP_CK, whereas Proteobacteria (32.03–50.03%), Firmicutes (17.32–37.34%), and Actinobacteriota (6.67–21.18%) became the predominant bacterial phyla in BEM treatments. After composting, the predominant bacterial phyla in CK and BEM treatments tended to be similar. Firmicutes, Actinobacteriota, Proteobacteria were the most abundant bactelial phyla in the composted substrate of peach sawdust-based formulas (Guo et al., 2021; Yang et al., 2022). Proteobacteria and Firmicutes were reported to be the dominant phyla in the sugarcane straw-based substrate during a 5–15 days' composting for oyster mushroom cultivation (Vieira et al., 2019). They play important roles in the lignocellulosic decomposition and nitrogen conversion in the thermophilic stages during composting (Liu et al., 2022).

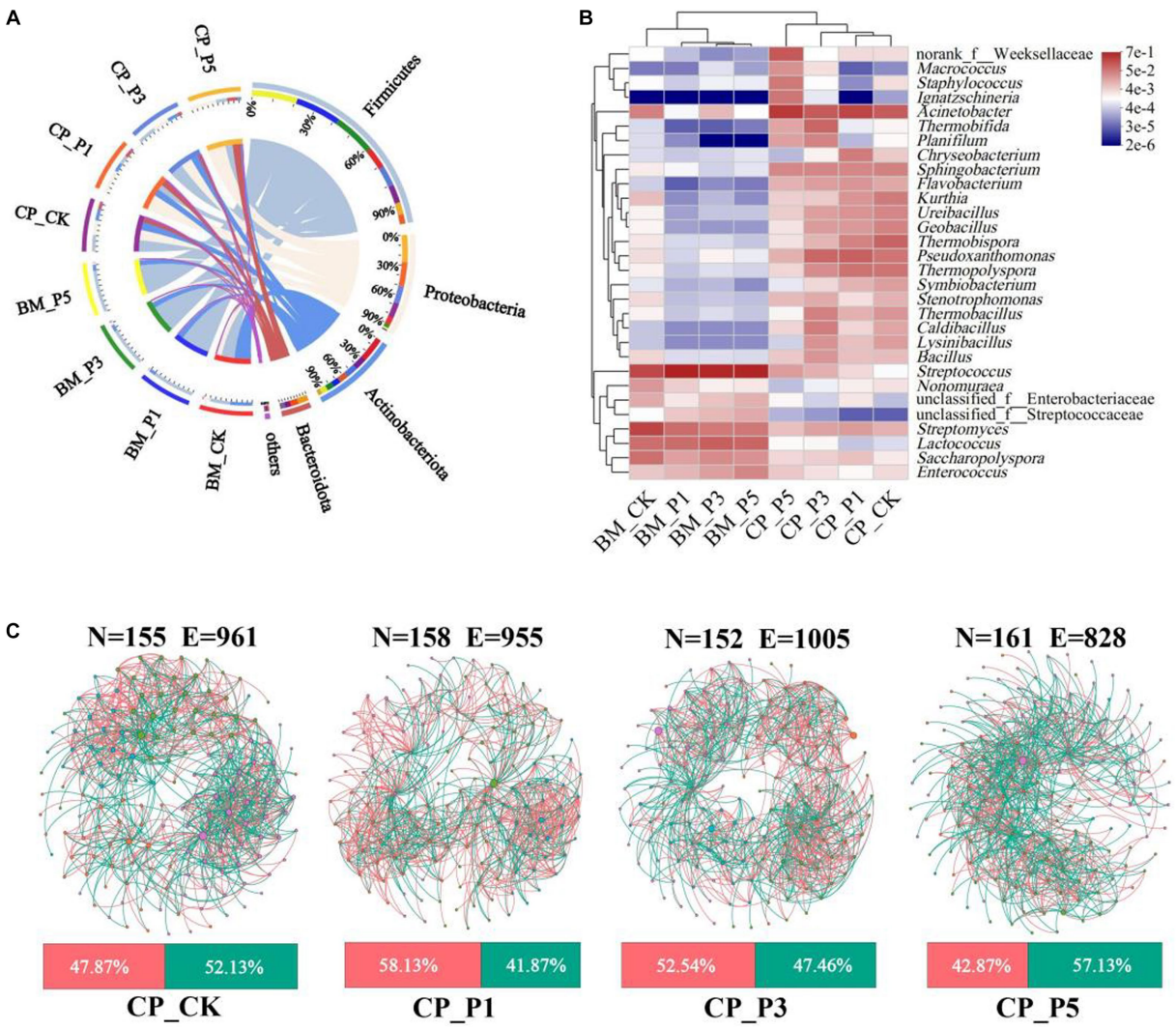

FIGURE 3
Bacterial composition and correlation in substrate samples. **(A)** Circos diagram at phylum level. Phyla with relative abundance <5% were combined together and indicated as "others." **(B)** Heatmap of log relative abundance of top 30 genera. **(C)** Co-occurrence network patterns on genus level (top 200 genera) in CP treatments ($r > 0.6$, $p < 0.05$). N, node; E, edge. The pink and green edges depict positive and negative correlations, respectively. The nodes represent individual genera, and node size corresponds to their relative abundance.

Furthermore, the heatmap analysis of top 30 genera among all samples were performed (Figure 3B). *Streptomyces* (26.32%) and *Streptococcus* (24.05%) were the most prevalent genera in BM_CK, while *Streptococcus* (59.29–70.56%) and *Lactococcus* (8.57–12.51%) were predominant genera in BEM treatments at BM stage. Guo et al. (2021) reported that *Streptomyces* was the top abundant genus (24.0%) in the substrate at BM stage, which came from the natural composting of the raw materials. The high abundance of *Streptococcus* and *Lactococcus* in the BEM treatments presumably came from the production and storage processes of BEM. After composting, the relative abundance of *Streptomyces* and *Streptococcus* in the four CP treatments decreased dramatically to 0.86–2.33% and 0.13–0.43%, respectively, while *Acinetobacter* became the predominant genus 14.20–35.79%. *Acinetobacter* was the most abundant genus in the thermophilic stage during the corncob-based and sugarcane straw-based composting, and contributed to the lignocellulosic degradation (Vieira et al., 2019). Moreover, *Thermobifida* (10.53%) and *Pseudoxanthomonas* (10.20%) became the predominant genera in CP_P3 treatment. They were thermo-tolerant and involved in carbohydrate and nitrogen metabolism (Kong et al., 2020).

Alpha and beta diversity of bacterial communities

The richness and diversity of bacterial communities can be evaluated using alpha diversity indices (Qiu et al., 2022). The coverage of each sample was ≥0.99, which indicated that the sequencing results included the majority of bacteria in the samples (Supplementary Table S5). The ACE and Chao 1 indexes of BM samples were higher than those of CP samples, suggesting that there were more OTUs in BM samples. Compared with BM treatment, the shannon index was increased and the simpson index was decreased in the CP treatments. The changes of the shannon and simpson indexes indicated that the bacterial communities increased after composting (Qiu et al., 2022).

The PCoA based on the Bray-Curtis distance can visualize the differences in bacterial community composition. The PCoA of bacterial communities in samples at OTU level was showed in Supplementary Figure S2. Three BEM treatments were clustered before composting, whereas CP_CK, CP_P1, and CP_P3 were clustered at the end of composting. However, the bacterial community of CP_P5 treatment was away from the cluster of other treatments. It indicates that CP_P5 treatment required longer composting duration, which also indirectly explained the high CR and low yield in the cultivation stage of the treatment.

Co-occurrence network analysis of bacterial communities

To further evaluate the correlations of microorganisms in the composted substrate, four co-occurrence networks were constructed with the top 200 bacterial genera (Figure 3C), and the main topological properties of the networks were summarized in Supplementary Figure S3. The power law (R^2) of 0.7885, 0.7994, 0.8201, and 0.8194 were recorded in all the networks of CP_CK, CP_P1, CP_P3, and CP_P5, respectively, indicating the non-random pattern and scale-free nature of the networks (Zhu et al., 2021). The P3 treatment demonstrated the least nodes (152), the most edges (1,005, 52.54% positive) and the highest average degree (13.22) and average clustering coefficient (0.56). This indicates that the P3 treatment enhanced the network complexity of bacterial community. With the increase of BEM dosage, the positive interaction ratio of the three BEM treatments continuously decreased, indicating that the increase of BEM dosage may promote the competition of composting microorganisms for nutrients (Bello et al., 2020; Zhu et al., 2021).

Heatmap analysis

The bacterial communities and physicochemical properties in the substrate at CP stage play vital roles in the yield and quality of oyster mushroom by the composting cultivation method (Guo et al., 2021; Yang et al., 2022). The heatmap analysis was constructed based on the top 30 bacterial genera at CP stage, physicochemical properties of substrate and agronomic and nutritional properties of fruiting bodies (Figures 4A,B). *Acinetobacter*, the predominant genus at CP stage, represented significant positive correlations with EC ($p < 0.01$) and TN ($p < 0.01$). Previous studies reveal that *Acinetobacter* is predominant in the thermophilic stage and actives in lignocellulosic degradation (Vieira et al., 2019). Moreover, *Thermobifida* showed significant positive correlations with composting temperature ($p < 0.05$), pH ($p < 0.01$), protease activity ($p < 0.001$), crude protein content ($p < 0.01$), crude fat content ($p < 0.05$), TAA ($p < 0.01$), and EAA ($p < 0.01$), whereas *Pseudoxanthomonas* demonstrated significant positive correlations with yield ($p < 0.01$) and BE ($p < 0.01$). *Thermobifida* is thermostable and can participate in cellulose degradation (Liu et al., 2022). In this study, the genus also participated in nitrogen metabolism of the substrate, and improved the nutritional properties of mushrooms. Although the relative abundance of *Streptococcus* declined sharply at CP stage, it was significantly positively correlated with compost temperature ($p < 0.01$), pH ($p < 0.001$), protease activity ($p < 0.01$), crude protein content ($p < 0.001$), crude fat content ($p < 0.001$), TAA ($p < 0.001$), and EAA ($p < 0.001$). It is worth mentioning that many genera represented significant ($p < 0.05$) correlations with compost temperature, pH, MC and protease activity of the composted substrate, and crude protein content, crude fat content, TAA, EAA, yield and BE of fruiting bodies. It indicates that the addition of BEM mainly affected the nitrogen metabolism during composting and mushroom cultivation.

Functional prediction of bacterial communities

The metabolic properties of bacterial communities in the composted substrate (CP stage) were predicted by PICRUSt based on the KEGG database and visualized in Supplementary Figure S4. The majority of predicted functional genes were assigned into metabolism (40.36–40.77%), genetic information processing (GIP, 6.78–7.24%), environmental information processing (EIP, 5.93–6.67%), and cellular processes (CP, 2.50–3.72%). Eleven pathways were observed for metabolism, three for GIP, two for EIP and two for CP on KEGG pathway level 2. It was consistent with previous reports of composting processes for oyster mushroom cultivation (Guo et al., 2021; Yang et al., 2022). The carbohydrate metabolism (9.31–10.15%), amino acid metabolism (8.54–8.74%), energy metabolism (4.58–4.78%), and metabolism of cofactors and vitamins (4.47–4.62%) were the most

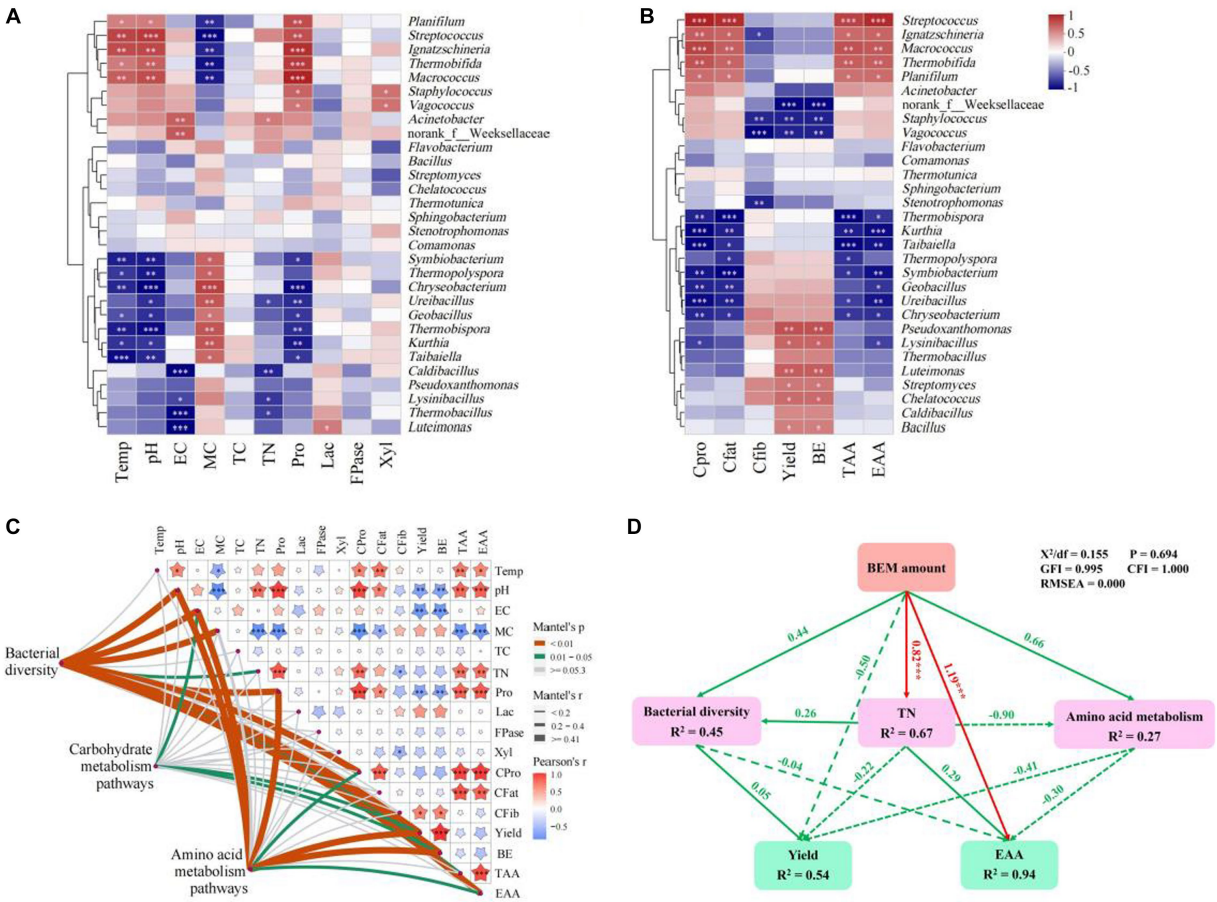

FIGURE 4
Correlation analysis of bacterial communities of substrate at CP stage with physicochemical, agronomic and nutritional properties. The correlation heatmap of the top 30 genera with physicochemical properties of substrate at CP stage **(A)** and agronomic and nutritional properties of fruiting bodies **(B)**. **(C)** Mantel test based on the Spearman's correlation coefficients. **(D)** Structural equation model. The red and green arrows represent significant and non-significant relationships, respectively. Temp, temperature; EC, electrical conductivity; MC, moisture content; TC, total carbon; TN, total nitrogen; Pro, protease; Lac, laccase; FPase, filter paper cellulase; Xyl, xylanase; Cpro, crude protein content; Cfat, crude fat content; Cfib, crude fiber content; BE, biological efficiency; TAA, total amino acids; EAA, essential amino acids. *$0.01 \leq p < 0.05$, **$0.001 \leq p < 0.01$, and ***$p < 0.001$.

predominant pathways on KEGG pathway level 2 (Supplementary Figure S4A). This indicates that the predicted genes involved in carbohydrate and amino acid metabolism were the most abundant at CP stage. A metagenomics sequencing on a corncob-based composting cultivation process revealed that carbohydrate, amino acid and energy metabolism were the top abundant pathways at the end of composting process (Liu et al., 2022). Moreover, the P5 treatment demonstrate significantly ($p < 0.05$) low abundances of carbohydrate metabolism and metabolism of other amino acids, but significantly ($p < 0.05$) high abundance of amino acid metabolism, metabolism of cofactors and vitamins, lipid and nucleotide metabolism. This may indicate that the composting process of P5 treatment was not completed at the end of composting stage, resulting in high CR and low yield of fruiting bodies.

Mantel test analysis

The correlations among bacterial communities and the matrixes of physicochemical, agronomic and nutritional properties were constructed using the Mantel test (Figure 4C). Pairwise comparisons of physicochemical, agronomic and nutritional properties revealed that temperature, pH, EC, MC, TN and protease activity were the key factors at CP stage, which would further significantly ($p < 0.05$) affect crude protein content, crude fat content, yield, BE, TAA and EAA of mushroom fruiting bodies. A composting duration study reported that pH, EC and TN were the key factors during the thermophilic stage and further affected the yield of oyster mushroom (Yang et al., 2022). The Mantel test analysis revealed that pH, EC, MC, TN and protease activity of the composted substrate significantly ($p < 0.05$) affected the bacterial communities (bacterial diversity at OTU level), while the bacterial communities in the substrate significantly ($p < 0.05$) affected the yield, BE, TAA and EAA of oyster mushrooms. The carbohydrate metabolism pathways significantly ($p < 0.05$) correlated with EC of substrate, and yield and TAA of mushrooms, whereas amino acid metabolism pathways significantly ($p < 0.05$) correlated with pH, EC and protease activity of substrate, and crude protein content, BE and EAA of mushrooms. Although the research on composting processes of agricultural wastes has been widely carried out, the research on short-term composting processes for mushroom cultivation is comparatively limited especially the impact of composting processes on the nutritional quality of mushrooms (Liu et al., 2022; Yang et al.,

2022). Guo et al. (2021) reported that TN, temperature and lignin content were the key factors for composting maturity of a short-term peach sawdust-based composting. The C/N ratio, pH, temperature and organic matters significantly affected the succession of microbiota in compost of corncob-based composting (Kong et al., 2020). In this study, the addition of BEM changed the TN, pH, EC, MC and protease activity in the substrate, which determined the bacterial communities in the compost. The bacterial communities further affected the agronomic and nutritional properties of mushroom fruiting bodies.

Structural equation model analysis

The core objective of this study was to evaluate the impacts of BEM addition on bacterial communities in the substrate, and agronomic and nutritional properties of oyster mushroom. Therefore, the SEM was constructed to further clarify the effects of BEM amount on the short-term composting and oyster mushroom cultivation (Figure 4D). The hypothetical models fit the data well with the parameters of $X^2/df = 0.155$, $p = 0.694$, GFI = 0.995, CFI = 1.000, and RMSEA = 0.000 (Wu et al., 2021). The BEM amount strongly positively affected the TN ($\lambda = 0.82$, $p < 0.001$) and EAA ($\lambda = 1.19$, $p < 0.001$). This indicates that the addition of BEM in the substrate significantly affects the TN of the substrate and nutritional quality of mushrooms. In addition, the effects of BEM amount on bacterial diversity, amino acid metabolism and yield were insignificant. This may be due to the differences of bacterial communities between P5 and other treatments. The high BEM amount in P5 treatment led to the immature substrate, which led to the lowest yield and the highest CR of mushrooms.

Conclusion

In summary, the BEM is a high-quality alternative nitrogen source for the cultivation of oyster mushrooms using the composted substrate. A proper addition of BEM can improve the physical and chemical properties of the substrate at the end of the composting stage, thus increase the yield, BE, crude fat content, crude protein content, TAA, EAA, NEAA, SAA, BAA, FAA, and AAA of the fruiting bodies. The high throughput sequencing revealed that the addition of BEM significantly changed the bacterial communities in the substrate at the beginning of composting, while those in the mature substrate tended to be similar at the end of composting. The physicochemical properties of substrate significantly affected bacterial communities, which further affected agronomic and nutritional properties of oyster mushrooms. This indicates that BEM can be appropriately added during the composting cultivation of oyster mushrooms to improve yield and quality. These findings reveal the effect of short-time composting on the nutritional quality of oyster mushrooms, and provide a new method for high-quality and efficient use of broken eggs in poultry industry.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: PRJNA884292.

Author contributions

JW: Conceptualization, Data curation, Supervision, Writing – original draft, Writing – review & editing. Y-XW: Conceptualization, Data curation, Investigation, Software, Writing – original draft. T-KG: Data curation, Investigation, Writing – original draft. Q-YW: Data curation, Investigation, Writing – original draft. JZ: Data curation, Investigation, Writing – original draft. J-YZ: Data curation, Investigation, Writing – original draft. J-LW: Data curation, Investigation, Writing – original draft. Q-JC: Methodology, Writing – original draft. G-QZ: Formal analysis, Funding acquisition, Supervision, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1274032/full#supplementary-material

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Unveiling the microbiota of sauce-flavor *Daqu* and its relationships with flavors and color during maturation

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This study investigated the microbial community in three-color sauce-flavor *Daqu* (black, yellow, and white) throughout their maturation processes, together with their physicochemical factors, culturable microbes, flavor components, and fermenting vitalities. Results from high-throughput sequencing revealed distinct microbial diversity, with more pronounced variations in bacterial community than in fungal community. Firmicutes and Ascomycota emerged as the most dominant bacterial and fungal phyla, respectively, during maturation. Genus-level analysis identified *Kroppenstedia*, *Virgibacillus*, and *Bacillus* as dominant bacteria in black *Daqu*, yellow *Daqu*, and white *Daqu*, severally, while *Thermoascus* was shared as the core dominant fungi for these *Daqu*. Physicochemical factors, particularly acidity, were found to exert a significant impact on microbial community. *Kroppenstedtia* was the key bacteria influencing the color formation of these *Daqu*. Furthermore, correlations between dominant microbes and flavor compounds highlighted their role in *Daqu* quality. Molds (*Aspergillus*, *Rhizomucor*, and *Rhizopus*), excepting *Bacillus*, played a crucial role in the formation of pyrazine compounds. Consequently, this study offers innovative insights into the microbial perspectives on color and pyrazine formation, establishing a groundwork for future mechanized *Daqu* production and quality control of sauce-flavor baijiu.

KEYWORDS

***Daqu* maturation, microbial community, *Kroppenstedtia*, color formation, pyrazines**

Introduction

Baijiu, one of the six globally recognized distilled spirits, has gained a lot of attention owing to its profound historical roots and cultural significance in China (Yan et al., 2021). Recent data from National Bureau of Statistics revealed the impressive scale of this industry, with the year 2022 witnessing a baijiu production of 6.71 billion liters, considerably contributing to national economy (National Statistics Bureau, 2023). Remarkably, sauce-flavor baijiu, is the leader among the three major categories of baijiu flavor, including light-flavor, strong-flavor, and sauce-flavor. The increasing interest among national consumers in

sauce-flavor baijiu is largely driven by its exceptional quality and pleasurable drinking experience. The intricate and traditional craftsmanship involved in brewing sauce-flavor baijiu plays a pivotal role in producing high-quality baijiu. The intricate brewing processes encompass several essential stages, consisting of *Daqu* production, solid-state fermentation, distillation, storage and aging, and blending (Jin et al., 2017; Niu et al., 2022; Yang et al., 2023). As the adage goes, "*Daqu* is the backbone of baijiu brewing." emphasizing the crucial role of *Daqu*—a fermented agent initiating the solid-state fermentation of baijiu by introducing a diverse array of microbes, enzymes, and flavor precursors, ultimately determining the final quality of base liquor (De Melo et al., 2019; Xia et al., 2022; Li H. et al., 2023; Yang et al., 2024).

In the context of sauce-flavor baijiu brewing, the *Daqu* employed is known as high-temperature *Daqu*, signifying its production under elevated temperature conditions (above 60°C) (Deng et al., 2020; Sakandar et al., 2020; Pan et al., 2023). The essential processes involved in the production of this Daqu are illustrated in Figure 1. Wheat is crushed, mixed with 34–40% water and 5–8% mother-*Daqu* powder, and then molded into *Daqu* bricks. These bricks undergo a 50-day high-temperature fermentation in a dedicated chamber, followed by a 6-month maturation period in a storage room (Niu et al., 2022). After maturation, the *Daqu* is crushed and used in the production of sauce-flavor baijiu, highlighting the critical role of fermentation and maturation in determining *Daqu* quality. Notably, the production of *Daqu* involves spontaneous processes with minimal human intervention, leading to the natural formation of three distinct *Daqu* color: black, yellow, and white (Cai et al., 2021; Shi W. et al., 2022). Previous studies have delved into the difference in microbial community of these three kinds of *Daqu* during fermentation process. Zhu's study uncovered specific insight in the microbial community succession during *Daqu* fermentation (Zhu C. et al., 2022). High temperature emerged as the most significant factor driving core functional community of *Daqu*, correlating with flavor formation throughout fermentation (Zhu Q. et al., 2022; Wu et al., 2023). Others studies compared the differences in microbial community and flavor between artificial and mechanical *Daqu* during fermentation (Huang et al., 2023). Deng's research shed light on the discrepancies in microbial composition among colored *Daqu* (Deng et al., 2020). In addition, several studies unveiled the potential factors contributing to color formation in the three types of *Daqu*, highlighting the significant roles played by amino acid metabolism and the Maillard reaction (Zhang et al., 2022; Zhu Q. et al., 2023).

Despite the big differences in the properties of the three-color *Daqu*, they are used in combination with specifical proportions to ensure the stable brewing and producing of sauce-flavor baijiu. These *Daqu* types undergo spontaneous fermentation, presenting challenges in controlling color formation and hindering the targeted production of specific *Daqu* varieties, impacting future quality control efforts. While previous researches have primarily focused on the microbiota of these *Daqu* during the fermentation process and proposed potential reasons for color formation, insufficient attention has been given to the maturation process, specifically regarding microbial composition, flavor components, and vitalities. Additionally, the microbes associated with color formation remain unclear. This study is dedicated to exploring the maturation process of these three *Daqu* types, investigating their physicochemical properties, cultivable microbes, fermenting vitalities, flavor substances, and corresponding microbial community. Hence, a systematically comparing analysis was carried out to reveal the microbiota differences among these colored *Daqu*, uncovering the core microbes related to color formation and laying a foundation for targeted production of specific *Daqu* and enhanced quality control in the future.

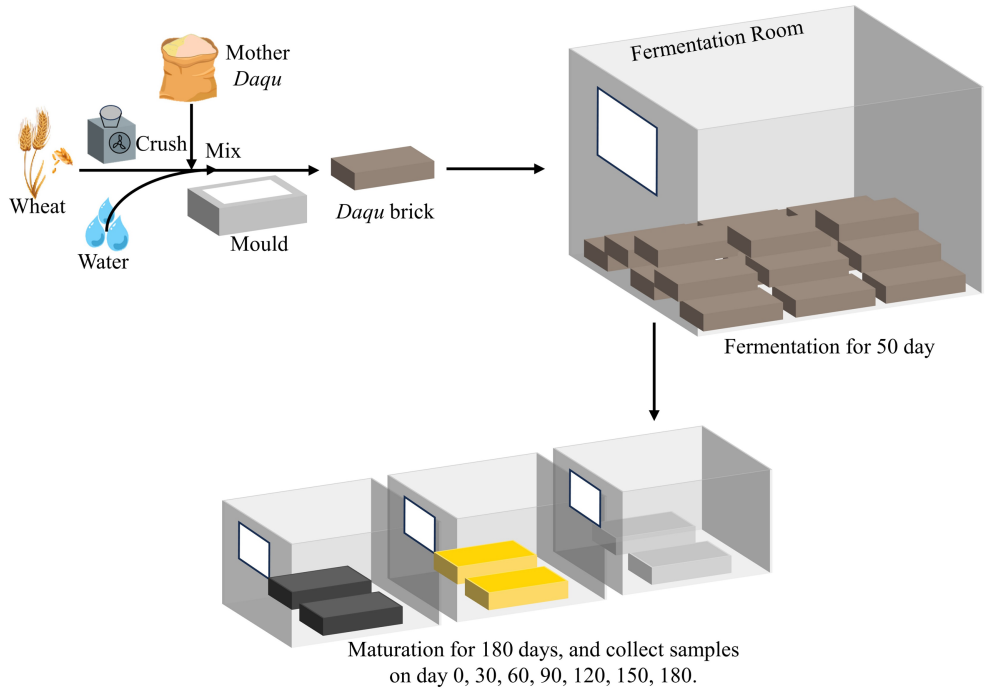

FIGURE 1
The schematic diagram of *Daqu* production.

Materials and methods

Daqu maturation and sampling

The *Daqu* bricks were fermented in the *Qu*-producing shop of Jing Brand Maotai Town Liquor Co., Ltd., Renhuai, Guizhou province, China. Following fermentation, *Daqu* was categorized by color (black, yellow, and white) and moved to the storage room for maturation with a period of 180 days. Samples were collected at intervals of 0, 30, 60, 120, 150, and 180 days from each color of *Daqu*, resulting in 54 samples (three replicates per color) for subsequent analysis. Once the samples were gathered, the brick from each kind of *Daqu* was crushed to powder for the immediate assessment of physicochemical properties, cultivable microbes, and fermenting vitalities. The powder was stored at −80°C for subsequent flavor compounds analysis and amplicon sequencing.

Physicochemical properties analysis

The analysis of moisture, acidity, and reducing sugar content in three kinds of *Daqu* were conducted based on our previous study with minor revisions (Dong et al., 2022). The amino acid nitrogen content of *Daqu* was detected following the methodology outlined in Huang's study (Huang et al., 2021). Specifically, moisture content was measured by taking 4 g of *Daqu* powder in an oven for desiccation (overnight) until a constant weight, and calculating the ratio of weight loss. Before the acidity assessing, 10 g of *Daqu* powder was added into 200 mL of ddH_2O with 30 min of static for acids extraction. Subsequently, the supernatant was used for acid–base titration to determine the acidity. The content of reducing sugar in *Daqu* was evaluated via Fehling's test in reliance on the standard reducing sugar solution. As for the amino acid nitrogen, it was evaluated based on the amphoteric behavior of amino acids. Firstly, the amino acid nitrogen was extracted form *Daqu* by mixing 20 g of samples into 60 mL of ddH_2O together with vibration at 120 rpm for 30 min, and the filtrate was collected and used for detecting. Subsequently, the pH value of filtrate was adjusted to 8.2 with 50 mM NaOH, together with 10 mL of methanal being added to fix the amino base. Finally, the titration with standard NaOH solution was performed to determine the end-point (pH 9.2) via a pH meter, and thus calculating the amino acid nitrogen content by the consuming volume of standard NaOH solution.

Cultivable microbes counting

The cultivable microbes in *Daqu* were quantified using traditional dilution and plating method, following our previous protocols with minor adjustments (Dong et al., 2022). Specifically, *Bacillus*, lactic acid bacteria (LABs), yeasts, and molds were targeted for detection. Ten grams of *Daqu* powder were combined with 90 mL of sterile H_2O and shaken at 160 rpm for 30 min. Subsequently, a gradient dilution was performed, and 100 μL of the diluted suspension was plated on respective agar plates to cultivate cultivable microbes. LB agar, YPD agar (with ampicillin), MRS agar (with nystatin), and PDA (with ampicillin) were employed to support the growth of *Bacillus*, LABs, yeasts, and molds, respectively, under appropriate conditions. Following the designated incubation period, the number of visible colonies on the respective plates was enumerated.

Fermenting vitalities analysis

The fermenting vitalities of *Daqu* was monitored by detecting neutral protease activity, α-amylase activity, glucoamylase activity, and fermentation ability. Thereinto, the neutral protease activity was determined via colorimetry at 680 nm using Folin–Ciocalteu method based on our previous study (Dong et al., 2020). The α-amylase activity was detected based on iodine-starch colorimetric assay, and the decrease in absorbance at 620 nm was indicative of α-amylase activity. Glucoamylase activity was evaluated by determining the glucose produced from starch degradation via DNS (3,5-dinitrosalicylic acid) method. Additionally, fermentation ability was assessed by quantifying the weight of CO_2 produced during sorghum juice fermentation inoculated with *Daqu* powder.

Flavor substances analysis

The flavor substances in *Daqu* were detected by a head-space solid-phase microextraction and gas chromatography–mass spectrometry (HS-SPMEGC–MS) according to our previous study (Dong et al., 2022). In this process, 2.0 g of *Daqu* powder and 3.0 g NaCl were combined in 10 mL of 10% ethanol within a headspace bottle, then internal standard substances (2-octanol and 2-ethyl hexanol) were added. After that, the flavor substances in mixture were extracted by an automatic headspace sampling system (Multipurpose Sample MPS 2XL) at 50°C for 45 min. Subsequently, the SPME fiber was introduced into the injection port, set at 250°C, for a duration of 5 min. Compounds separation was achieved using an Agilent HP-5 column (30 m × 0.25 mm; 0.25 μm film thickness) and a DB-FFAP column (60 m × 0.25 mm; 0.25 μm film thickness), followed by analysis via GC–MS analysis employing the Agilent 7890B GC system and the 5977C mass selective detector. Compounds identification was carried out by comparing mass spectral profiles with a match quality of ≥80 in the NIST14 database.

Microbial community analysis based on amplicon sequencing

The third-generation amplicon sequencing was employed to analyze the composition, structure, and succession of microbial community in three types of *Daqu* during maturation, specifically on day 0, 30, 90, and 180. Initially, total DNA was extracted from 0.2 grams of *Daqu* powder using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, United States) as per the provided instructions. After the concentration and quality assessment of total DNA were conducted, it served as the template for library construction. For the full-length 16S rDNA library of bacteria, amplification was performed using Q5 high-fidelity DNA polymerase (NEB, United States) with primers 27F (AGRGTTTGATYNTGGCTCAG) and 1492R (TASGGHTACC

TTGTTASGACTT) (Callahan et al., 2019). The amplification parameters were as follows: 98°C for 5 min, 98°C for 30 s, 55°C for 30 s, and 72°C for 45 s for 30 cycles, and final at 72°C for 5 min. Simultaneously, the full-length ITS library of fungi was constructed by Q5 high-fidelity DNA polymerase using primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCT TATTGATATGC) (Kuang et al., 2021). The amplification parameters for this library were: 98°C for 5 min, 98°C for 30 s, 53°C for 30 s, and 72°C for 20 s for 30 cycles, with a final step at 72°C for 5 min. Subsequently, the purified PCR products of these two libraries were subjected to high-throughput sequencing (HTS) using the PacBio Sequel II platform. The post-sequencing data were analyzed bioinformatically using QIIME2, with the main analytical workflow referenced from Yang's study (Yang et al., 2024).

Data availability and statistical analysis

The raw data generated from HTS has been securely archived in NCBI under the BioProject accession number PRJNA 1034761 and PRJNA 1034762. Statistical analyses in this study involved data processing with Excel (version 2019), and the determination of significance was conducted using Origin (version 9.0), with a significance threshold set at 0.05.

Results and discussions

Physicochemical properties

The physicochemical properties of the three types of *Daqu* during the maturation process were shown in Figure 2, emphasizing key parameters such as moisture content, acidity, reducing sugar, and amino acid nitrogen levels. A gradual reduction in moisture content was observed among the three types of *Daqu*, with levels decreasing from 8.09 to 7.08% for black *Daqu*, 8.43 to 7.20% for yellow *Daqu*, and 7.68 to 6.89% for white *Daqu* (Figure 2A). Notably, all three types reached a final moisture content below 9%, adhering to the standard for qualified *Daqu*, preserving enzymatic and microbial activities (Paredes-López et al., 1988; Xia et al., 2022). While the dropping trend of acidity during maturation was similar across the three colored *Daqu*, black *Daqu* exhibited the highest acidity, white *Daqu* the lowest, and yellow *Daqu* intermediate, consistent with findings in Zhang's study (Zhang et al., 2022). At the end of the maturation period, the acidity values for these three types of *Daqu* were 1.03, 0.56, and 0.35 mmol/10 g, respectively (Figure 2B), which were lower than those in previous studies (Deng et al., 2020; Zhang et al., 2022). In terms of reducing sugar, initial content varied significantly among the *Daqu* types but showcased an overall dropping trend, stabilizing at a low level of

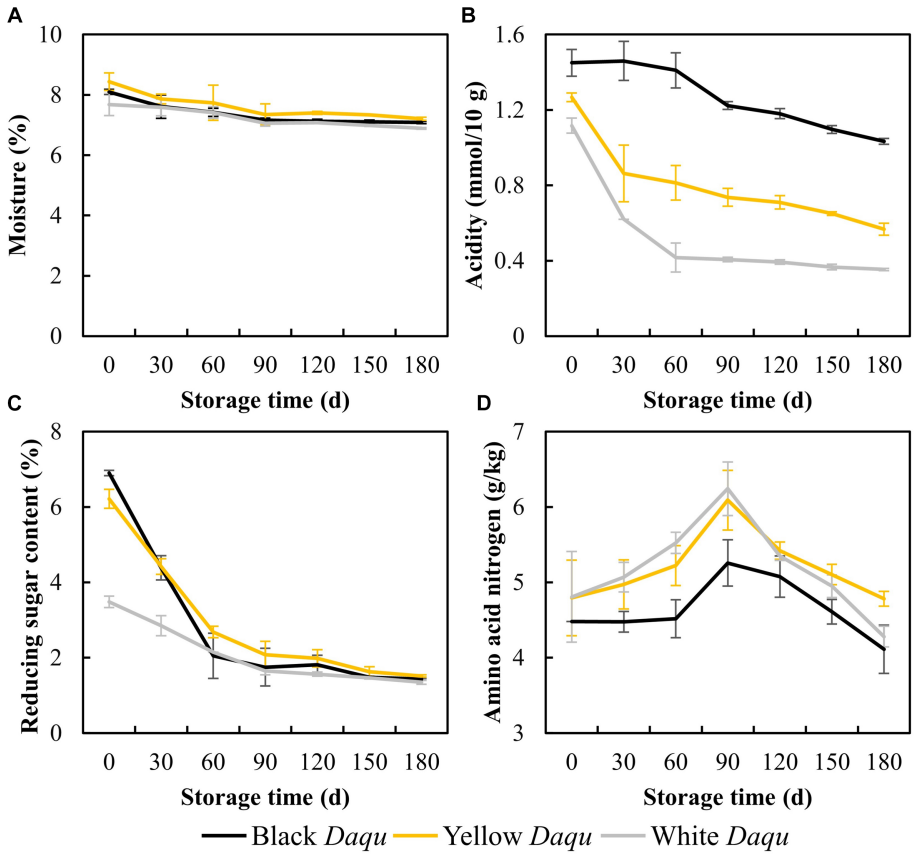

FIGURE 2
The physicochemical factors of three kinds *Daqu* during maturation, including moisture **(A)**, acidity **(B)**, reducing sugar **(C)**, and amino acid nitrogen **(D)**.

approximately 1.40% by the end of maturation (Figure 2C). This low reducing sugar content is conducive to microbial stabilization but insufficient for microbial growth in *Daqu*. Amino acid nitrogen content showed an initial increase followed by a decrease, with black *Daqu* having the lowest final content (4.11 g/kg), white *Daqu* next (4.28 g/kg), and yellow *Daqu* having the highest content (4.78 g/kg) (Figure 2D).

Cultivable microbes counting

The counts of cultivable microbes in the three-color *Daqu* manifested an overall raising trend followed by dwindling during the maturation process (Table 1), aligning with the previously noted decrease in moisture content (Figure 2A). In cases of *Bacillus*, the number ranged from 6.33×10^5 to 9.03×10^8 cfu/g, with white *Daqu* exhibiting the highest count, followed by yellow *Daqu*, and black *Daqu* (Table 1). This *Bacillus* count falls within a reasonable range reported in previous studies, where the maximal order of magnitudes for *Bacillus* is around 10^8 in *Daqu* (Sakandar et al., 2020). The prevalence of *Bacillus* in cultivable microbes may be attributed to its ability to survive in harsh conditions (Liu et al., 2019; Sakandar et al., 2020). The number of LABs were lower (6.30×10^5 to 6.21×10^7 cfu/g) than those of *Bacillus* in the three *Daqu* and exhibited an opposite trend, with black *Daqu* having the highest LAB count, followed by yellow *Daqu* and white *Daqu* (Table 1). This finding aligns with previous research indicating that LABs contribute to substrate acidity, inhibiting the growth of *Bacillus* (Zou et al., 2018). The quantity of cultivable fungi was significantly lower than that of bacteria, with yeast counts ranging from 3.62×10^2 to 3.32×10^5 cfu/g and mold counts varying from 7.66×10^2 to 3.53×10^6 cfu/g (Table 1). Among these, white *Daqu* had the highest counts of yeast and mold, while black *Daqu* had the lowest counts, suggesting a synergistic effect of yeast and mold during the maturation process.

Fermenting vitalities

The fermenting vitalities of *Daqu* play a crucial role in the production of sauce-flavor baijiu, influencing the initiation of baijiu fermentation (Wang B. et al., 2018; Li H. et al., 2023). In this study, we conducted a comprehensive analysis of neutral protease, α-amylase, glucoamylase, and fermentation ability in the three types of *Daqu* to evaluate their fermenting vitalities. Neutral protease activity displayed an initial increase followed by a decrease in all three types of *Daqu* (Figure 3A). White *Daqu* exhibited the final activity at 80.61 U/g, followed by yellow *Daqu* at 48.51 U/g, and black *Daqu* at 9.98 U/g on day 180 of maturation (Figure 3A). This result is consistent with the changes in the counts of *Bacillus* mentioned earlier (Table 1), as *Bacillus* is recognized for its superior protease production (Contesini et al., 2017). The activities of α-amylase, glucoamylase, and fermentation vitalities all exhibited similar trends, with initial increases followed by a decline and subsequent stabilization (Figures 3B–D). Overall, white *Daqu* demonstrated the highest activity, followed by yellow *Daqu*, and black *Daqu* had the lowest (Figures 3B–D). These results align with the counts of yeast and mold in cultivable microbes (Table 1), indicating that a higher count of mold and yeast promotes the production of α-amylase and glucoamylase (Wang X. D. et al., 2018), ultimately resulting in stronger fermentation activity (Yang et al., 2022). In summary, the analysis of the fermenting vitalities of the three types of *Daqu* suggests that white *Daqu* has the highest quality and provides stronger fermentation capabilities, contributing to the initiation of sauce-flavor baijiu brewing.

TABLE 1 The counts of cultivable microbes in three-color *Daqu* samples during maturation.

Maturation time (d)	*Bacillus* (cfu/g)			LABs (cfu/g)		
	Black *Daqu*	Yellow *Daqu*	White *Daqu*	Black *Daqu*	Yellow *Daqu*	White *Daqu*
0	$(3.06 \pm 0.20) \times 10^7$	$(1.49 \pm 0.20) \times 10^7$	$(1.25 \pm 0.20) \times 10^8$	$(6.21 \pm 0.10) \times 10^7$	$(4.31 \pm 0.11) \times 10^7$	$(4.54 \pm 0.24) \times 10^6$
30	$(8.69 \pm 0.40) \times 10^7$	$(8.82 \pm 0.40) \times 10^7$	$(2.43 \pm 0.20) \times 10^8$	$(4.32 \pm 0.12) \times 10^7$	$(3.94 \pm 0.40) \times 10^6$	$(2.72 \pm 0.30) \times 10^6$
60	$(2.58 \pm 0.27) \times 10^8$	$(3.66 \pm 0.24) \times 10^8$	$(4.30 \pm 0.15) \times 10^8$	$(4.05 \pm 0.27) \times 10^7$	$(3.28 \pm 0.20) \times 10^6$	$(2.52 \pm 0.20) \times 10^6$
90	$(4.93 \pm 0.52) \times 10^8$	$(5.72 \pm 0.59) \times 10^8$	$(9.03 \pm 0.05) \times 10^8$	$(3.87 \pm 0.10) \times 10^7$	$(2.87 \pm 0.20) \times 10^6$	$(2.43 \pm 0.23) \times 10^6$
120	$(1.27 \pm 0.32) \times 10^7$	$(3.01 \pm 0.21) \times 10^7$	$(5.76 \pm 0.68) \times 10^8$	$(3.28 \pm 0.10) \times 10^7$	$(2.61 \pm 0.50) \times 10^6$	$(2.07 \pm 0.40) \times 10^6$
150	$(5.50 \pm 0.31) \times 10^6$	$(2.25 \pm 0.41) \times 10^7$	$(4.71 \pm 0.46) \times 10^8$	$(2.87 \pm 0.07) \times 10^7$	$(2.07 \pm 0.40) \times 10^6$	$(1.89 \pm 0.30) \times 10^6$
180	$(6.33 \pm 0.25) \times 10^5$	$(9.02 \pm 0.85) \times 10^6$	$(3.63 \pm 0.32) \times 10^8$	$(2.07 \pm 0.10) \times 10^7$	$(1.80 \pm 0.05) \times 10^6$	$(6.30 \pm 0.05) \times 10^5$
Maturation time (d)	Yeasts (cfu/g)			Molds (cfu/g)		
	Black *Daqu*	Yellow *Daqu*	White *Daqu*	Black *Daqu*	Yellow *Daqu*	White *Daqu*
0	$(1.01 \pm 0.01) \times 10^3$	$(1.08 \pm 0.01) \times 10^3$	$(2.95 \pm 0.40) \times 10^4$	$(3.21 \pm 0.11) \times 10^6$	$(5.91 \pm 0.13) \times 10^4$	$(3.53 \pm 0.01) \times 10^6$
30	$(8.70 \pm 0.80) \times 10^3$	$(9.10 \pm 0.90) \times 10^3$	$(3.32 \pm 0.27) \times 10^5$	$(8.92 \pm 0.40) \times 10^5$	$(5.54 \pm 0.19) \times 10^4$	$(9.70 \pm 0.96) \times 10^5$
60	$(3.60 \pm 0.01) \times 10^3$	$(9.00 \pm 0.04) \times 10^3$	$(2.27 \pm 0.20) \times 10^5$	$(2.64 \pm 0.37) \times 10^4$	$(3.83 \pm 0.25) \times 10^4$	$(8.73 \pm 1.57) \times 10^5$
90	$(2.70 \pm 0.01) \times 10^3$	$(8.70 \pm 0.05) \times 10^3$	$(8.57 \pm 0.89) \times 10^4$	$(9.77 \pm 1.11) \times 10^3$	$(1.78 \pm 0.26) \times 10^4$	$(6.13 \pm 0.33) \times 10^5$
120	$(9.06 \pm 1.32) \times 10^2$	$(9.60 \pm 1.00) \times 10^2$	$(5.40 \pm 0.20) \times 10^4$	$(5.40 \pm 0.05) \times 10^3$	$(1.66 \pm 0.16) \times 10^4$	$(5.70 \pm 0.52) \times 10^5$
150	$(8.10 \pm 1.21) \times 10^2$	$(9.00 \pm 1.41) \times 10^2$	$(1.80 \pm 0.20) \times 10^4$	$(1.60 \pm 0.01) \times 10^3$	$(6.30 \pm 0.60) \times 10^3$	$(2.10 \pm 0.42) \times 10^5$
180	$(3.62 \pm 0.80) \times 10^2$	$(8.10 \pm 2.85) \times 10^2$	$(9.00 \pm 1.00) \times 10^3$	$(7.66 \pm 2.20) \times 10^2$	$(2.70 \pm 0.30) \times 10^3$	$(1.68 \pm 0.58) \times 10^5$

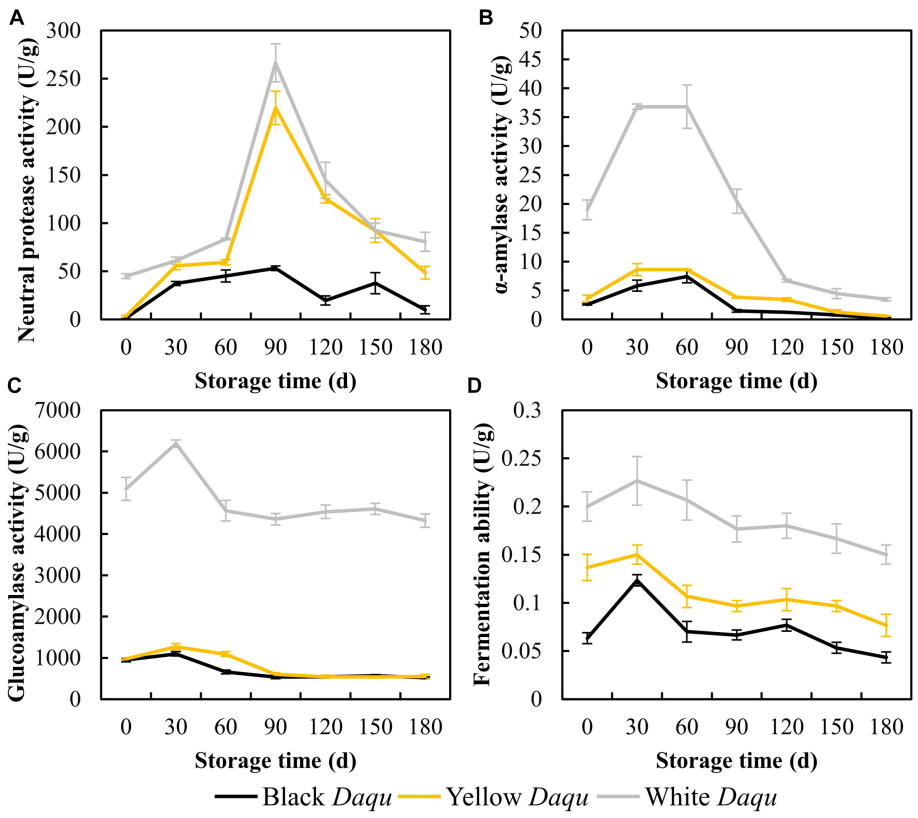

FIGURE 3
The fermenting vitalities of three kinds *Daqu* during maturation, including neutral protease **(A)**, α-amylase **(B)**, glucoamylase activity **(C)**, and fermenting ability **(D)**.

Flavor substances

The flavor substances in *Daqu* serve as the precursors to determine the final flavor and characteristics of baijiu (Li H. et al., 2023). A total of 160 different chemicals were detected, and the fluctuations in flavor substances during the maturation of the three types of Daqu are intricate. Here, we focused on analyzing alcohols, organic acids, esters, pyrazines, aldehydes and ketones (Figure 4). The content of alcohols in the colored *Daqu* all exhibited an overall decreasing trend throughout the maturation process (Figure 4A). Yellow *Daqu* initially had the highest alcohols content (79.79%) on day 0, dropping to 30.36% on day 180, while black *Daqu* started with 44.82%, decreasing to 24.33% at maturity (Figure 4A). In contrast, white *Daqu* showed the lowest initial alcohols content of 1.97%, declining to 0.68% on day 180 (Figure 4A). Organic acids content also decreased during maturation in these three types of *Daqu*, albeit with a milder decline than the variation observed in alcohols, ranging from 1.60 to 3.75% (Figure 4B). Ultimately, black *Daqu* had the highest organic acids content (1.85%), followed by yellow *Daqu* (0.73%), and white *Daqu* (0.54%) at the end of maturation (Figure 4B), in line with changes in acidity mentioned earlier (Figure 2B). Esters content varied significantly among the three types of *Daqu*, with white *Daqu* having the highest overall content, fluctuating throughout maturation, while black *Daqu* and yellow *Daqu* showed a similar upward trend (Figure 4C). Thus, the decline in alcohols and organic acids observed here can be attributed to their substantial consumption in the formation of esters. Pyrazine substances, crucial flavor compounds in sauce-flavor baijiu, contribute to pleasant aromas of roasted nuts and cocoa (Mortzfeld et al., 2020; Shi X. et al., 2022). They were considered one type of the characteristic flavor components that differentiate sauce-flavor baijiu from other types of baijiu (Zhang et al., 2013; Niu et al., 2022). The content of pyrazines in the three-color *Daqu* showed big differences (Figure 4D). White *Daqu* and black *Daqu* maintained high levels of pyrazines (>10%), with white *Daqu* reaching 19.18% and black *Daqu* at 12.37% on day 180 (Figure 4D). In contrast, the content of pyrazines in yellow *Daqu* remained at low level, reaching 4.53% on day 180 (Figure 4D). Aldehydes and ketones exhibited an increasing trend in both black *Daqu* and yellow *Daqu*, reaching 9.17 and 10.96% at the end of maturation, respectively, whereas white *Daqu* experienced a sharp increase to 13.04% during day 60–120, followed by a rapid decrease to 4.45% in the end (Figure 4E). Other flavor substances exhibited varying changes among the three types of *Daqu* (Figure 4F). In summary, the diverse changes in flavor substances during the maturation process of the three types of *Daqu* indicate each type's unique characteristics, suggesting potential combinations based on differences in flavor substances for sauce-flavor baijiu brewing.

Difference in microbial community

Through third-generation HTS, we analyzed the microbial community of the three-color *Daqu* during maturation. The results

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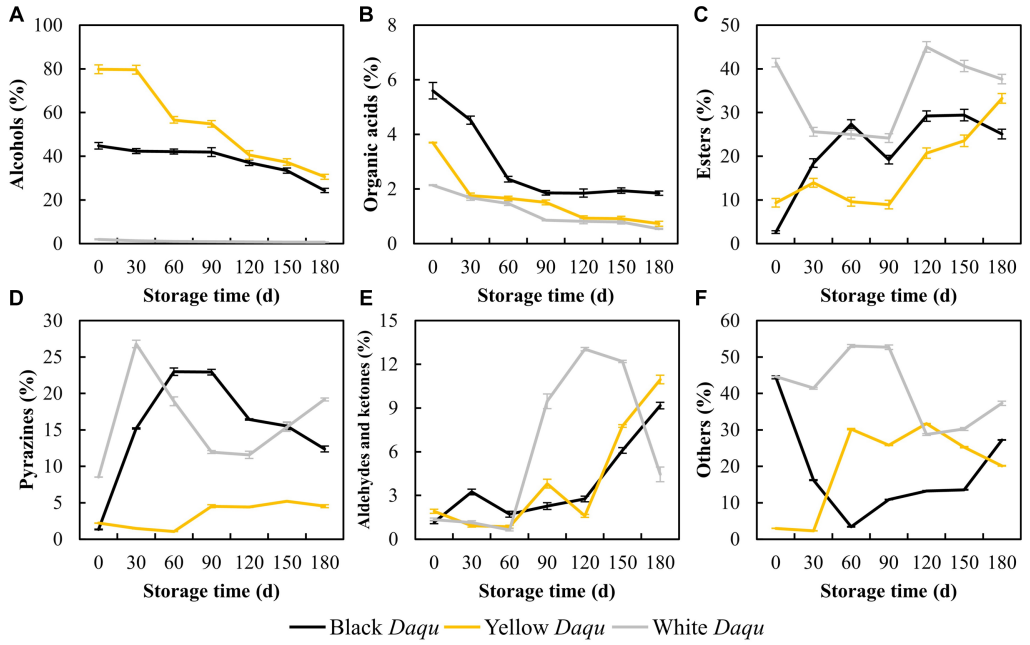

FIGURE 4
The variation of main flavor compounds in three kinds *Daqu* during maturation. Here, the alcohols **(A)**, acids **(B)**, esters **(C)**, pyrazines **(D)**, aldehydes and ketones **(E)**, and the others **(F)** were presented.

TABLE 2 The α diversity of three-color *Daqu* samples during maturation.

ID	Bacteria			Fungi		
Samples	Chao1	Shannon	Good coverage	Chao1	Shannon	Good coverage
Black *Daqu* 0d	371.84 ± 62.77	3.99 ± 0.45	0.991 ± 2.2E-03	360.46 ± 43.67	1.28 ± 0.19	0.992 ± 2.3E-03
Black *Daqu* 30d	342.08 ± 35.92	2.92 ± 0.41	0.999 ± 1.6E-03	278.06 ± 80.61	1.92 ± 0.48	0.995 ± 5.7E-03
Black *Daqu* 90d	290.96 ± 60.39	2.92 ± 0.33	0.992 ± 6.3E-03	257.13 ± 90.32	1.01 ± 0.36	0.994 ± 4.7E-03
Black *Daqu* 180d	430.04 ± 78.09	3.46 ± 0.79	0.991 ± 5.6E-04	95.61 ± 20.25	2.50 ± 0.36	0.999 ± 4.2E-04
Yellow *Daqu* 0d	150.86 ± 28.83	1.94 ± 0.44	0.997 ± 5.8E-04	466.74 ± 113.64	3.76 ± 0.86	0.991 ± 3.9E-03
Yellow *Daqu* 30d	132.89 ± 29.12	1.47 ± 0.29	0.997 ± 3.0E-03	259.80 ± 75.99	1.82 ± 0.22	0.995 ± 5.6E-03
Yellow *Daqu* 90d	106.87 ± 35.98	2.06 ± 0.42	0.998 ± 8.0E-04	190.64 ± 72.76	1.49 ± 0.42	0.995 ± 4.5E-03
Yellow *Daqu* 180d	357.77 ± 82.34	3.08 ± 0.57	0.993 ± 1.2E-03	61.12 ± 16.99	2.54 ± 0.28	0.999 ± 4.4E-04
White *Daqu* 0d	245.37 ± 61.34	3.23 ± 0.29	0.996 ± 1.1E-03	350.42 ± 20.23	2.08 ± 0.34	0.997 ± 1.7E-03
White *Daqu* 30d	243.42 ± 34.99	3.96 ± 0.33	0.995 ± 2.9E-04	310.92 ± 56.51	4.14 ± 0.65	0.995 ± 2.0E-03
White *Daqu* 90d	241.58 ± 50.66	2.98 ± 0.22	0.993 ± 2.9E-03	206.15 ± 36.84	2.21 ± 0.59	0.994 ± 5.2E-03
White *Daqu* 180d	280.74 ± 80.69	3.55 ± 0.35	0.994 ± 1.4E-03	101.48 ± 33.71	2.79 ± 0.81	0.998 ± 9.7E-04

indicated that the sequencing coverage exceeded 0.991 (Table 2), demonstrating the depth and reliability of the amplicon sequencing. Regarding bacterial α diversity, the Chao1 and Shannon indices for the three types of *Daqu* generally exhibited a trend of initial decrease followed by an increase as they mature (Table 2). Overall, the Chao1 richness was highest in black *Daqu*, followed by white *Daqu*, and lowest in yellow *Daqu*. The Shannon index was higher in black and white *Daqu* compared to yellow Daqu. For fungal α diversity, the Chao1 richness decreased for all three types of *Daqu*, indicating a decline in fungal richness during *Daqu* maturation. This decrease may be associated with the decline in moisture content during *Daqu* maturation since the lower moisture levels, below 9%, can reduce free water in microbial cells, hampering their growth and reproduction. In contrast, the fungal Shannon index remained relatively stable, with white *Daqu* exhibiting the highest value, suggesting a more even distribution of fungi during maturation. In summary, the primary diversity differences among the three types of *Daqu* are reflected in the Chao1 richness, with less variation in the Shannon index (Table 2).

To further assess the structure of microbial community during *Daqu* maturation, a Principal Component Analysis (PCA) based on Bray-Curtis distances was conducted. The β diversity results revealed that the structural differences in bacterial community among the three

types of *Daqu* were more pronounced than for fungal community. In the bacterial β diversity plot, samples representing the three types of *Daqu* were more scattered, indicating greater differences (Supplementary Figure S1A). In contrast, in the fungal β diversity plot, samples from all three *Daqu* types clustered closely and were not easily distinguishable (Supplementary Figure S1B). This implies that the differences in microbial diversity mainly exist in the bacterial community rather than the fungal community.

To investigate the composition of microbial community in *Daqu*, differences in taxa at the phylum and genus levels were analyzed during the maturation of the three-color *Daqu*. For bacterial composition, Firmicutes, Actinobacteriota, Bacteroidota, Proteobacteria, Fusobacteriota, and Verrucomicrobiota constituted the predominant phyla (Figure 5A). Among them, Firmicutes emerged as the most abundant phylum, which was congruent with Gan's study (Gan et al., 2019). However, the abundance of Firmicutes varied among three kinds of *Daqu*. In black *Daqu*, there was an initial increase followed by decrease (49.59 to 88.80 to 32.17%) (Figure 5A). Yellow *Daqu* exhibited a minor decline from 97.61 to 91.07%, while Firmicutes remained relatively stable in white Daqu, with abundances consistently above 80.52% (Figure 5A). As for fungal composition, the predominant phyla included Ascomycota, Mucoromycota, Basidiomycota, Mortierellomycota, Chytridiomycota, and Rozellomycota (Figure 5B). Ascomycota was the most dominant phylum, collaborating with previous studies (Deng et al., 2020; Zhang et al., 2022). The abundance of Ascomycota in black *Daqu* maintained above 94.29%, yellow *Daqu* exhibited fluctuations ranging from 80.51 to 99.77%, and white *Daqu* displayed an initial increase followed by a decrease (80.03 to 97.43 to 74.23%) (Figure 5B).

At the genus level, the composition of microbial community in the three-color *Daqu* showed even greater differences (Figures 5C,D). For bacterial, *Virgibacillus*, *Kroppenstedtia*, *Bacillus*, *Scopulibacillus*, *Candidatus Sulcia*, *Oceanobacillus*, *Enterobacter*, *Saccharopolyspora*, *Pediococcus*, and *Staphylococcus* were the top 10 abundant genera (Figure 5C). However, their relative abundances in the microbial community changed significantly during *Daqu* maturation. In black *Daqu*, *Kroppenstedtia* (31.58%), *Candidatus Sulcia* (17.49%), and *Virgibacillus* (10.27%) constituted the top 3 dominant bacterial genera (Figure 5C). *Kroppenstedtia*, in particular, was the most dominated genera in black *Daqu*, and its relative abundance rapidly increased from 6.48% (day 0) to 68.20% (day 30), followed

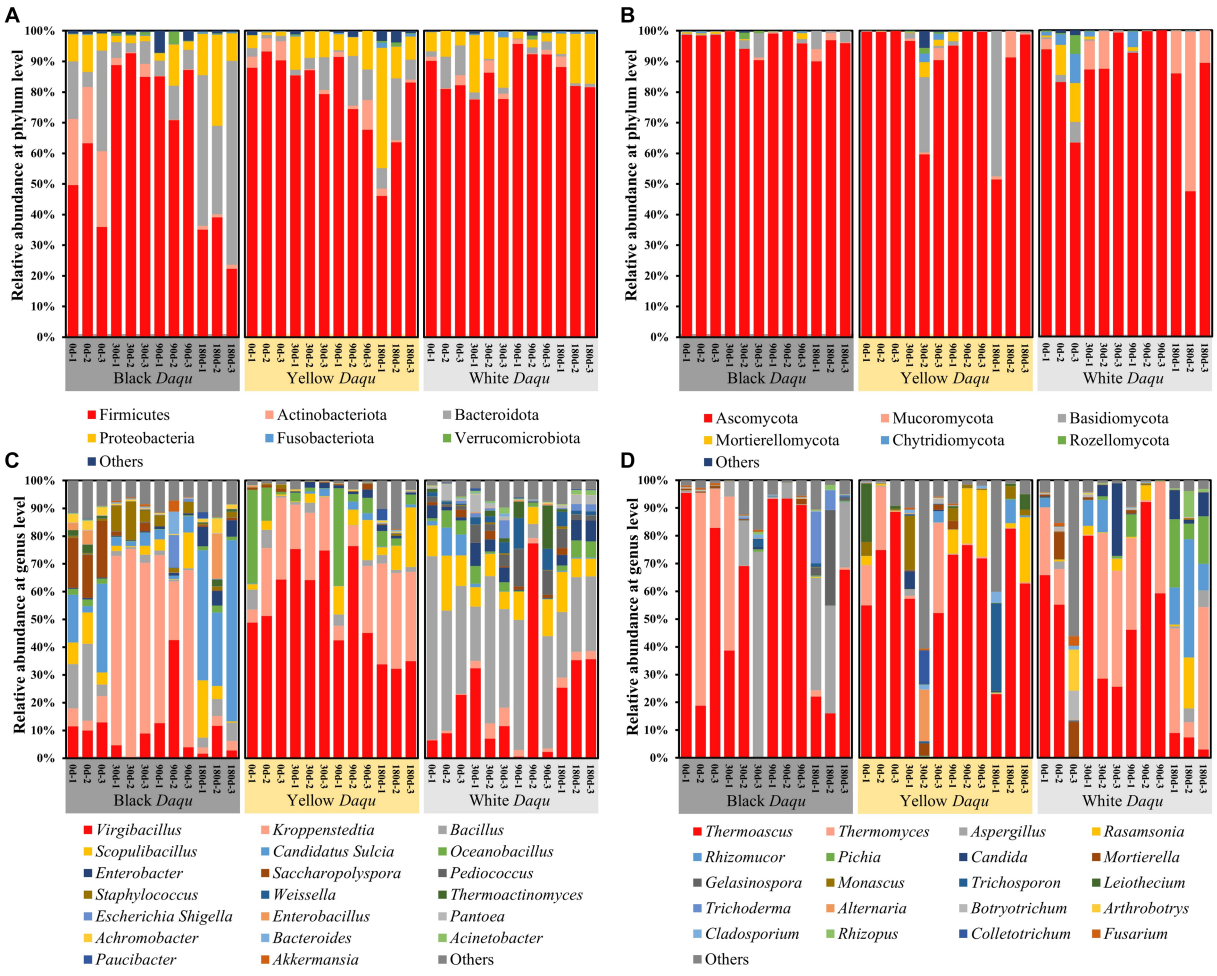

FIGURE 5
The microbial community of three kinds of *Daqu* during maturation, including bacterial phylum level **(A)**, fungal phylum level **(B)**, bacterial genus level **(C)**, and fungal genus level **(D)**.

by a continue decrease to 3.11% on day 180 (Figure 5C). Previous studies have unveiled that *Kroppenstedtia* is the core microbe in connection with the biosynthesis of organic acids (lactic acid and short chain fatty acids) (Zhu C. et al., 2023; Zhang et al., 2024), explaining the highest acidity observed in black Daqu during maturation. In yellow *Daqu*, *Virgibacillus* (54.66%), *Kroppenstedtia* (21.47%), and *Oceanobacillus* (8.57%) made up the top 3 dominant bacterial genera (Figure 5C). *Virgibacillus* was the main dominant genus in yellow *Daqu*, with its relative abundance quickly rosing from 54.83% (day 0) to 71.45% (day 30) and then gradually dropping to 33.67% (day 180) (Figure 5C). This result differs from previous studies where *Virgibacillus* was a dominant bacterium but not the most dominant in *Daqu* (Zhu C. et al., 2023; Zhu Q. et al., 2023). The roles and functions of *Virgibacillus* in *Daqu* production and baijiu brewing are not clear and needs further study. In white *Daqu*, *Bacillus* (35.53%), *Virgibacillus* (22.13%), and *Scopulibacillus* (10.01%) were the top three dominant bacterial genera (Figure 5C). *Bacillus* was the most dominant genus, and its relative abundance gradually decreased during maturation (from 49.44 to 25.86%) (Figure 5C). Despite the decreasing trend, the abundance of *Bacillus* remained above 25%, suggesting that it could still function effectively. *Bacillus* is known for secreting amylase and proteases and playing essential roles in liquefaction and saccharification (Ding et al., 2013; Wang et al., 2014, 2020), contributing to the higher fermenting vitalities observed in white *Daqu* (Figure 3). In addition, the average abundance of *Kroppenstedtia* in three kinds of *Daqu* followed the order: black *Daqu*, yellow *Daqu*, and white *Daqu*. *Kroppenstedtia* was found to be positively correlated with the production of various amino acids in *Daqu* (Zhu C. et al., 2023), which might promote the Maillard reaction with reducing sugar, leading to the formation of the yellow-brown color and contributed to the color difference in these three-color *Daqu*. Therefore, *Kroppenstedtia*, *Virgibacillus*, and *Bacillus* were identified as the three core bacteria in these colored *Daqu*, with *Kroppenstedtia* being the potential key microbe related to color formation.

For fungal genera, differences were smaller than those of bacteria among the three types of *Daqu*. The top 10 abundant fungal genera included *Thermoascus*, *Thermomyces*, *Aspergillus*, *Rasamsonia*, *Rhizomucor*, *Pichia*, *Candida*, *Mortierella*, *Gelasinospora*, and *Monascus* (Figure 5D). Among them, *Thermoascus* was the most dominant genera shared in all three types of *Daqu*, which was similar to Zhu's study (Zhu C. et al., 2022). The relative abundance of *Thermoascus* changed in the same manner, initially decreasing, then increasing, and finally decreasing again (Figure 5D). In black *Daqu*, *Thermoascus* (57.38%), *Aspergillus* (17.18%), and *Thermomyces* (12.48%) constituted the top 3 dominant fungal genera, and the relative abundance of *Thermoascus* ranged from 35.92 to 92.58% (Figure 5D). In yellow *Daqu*, the top 3 dominant fungal genera belonged to *Thermoascus* (59.84%), *Rasamsonia* (6.85%), and *Thermomyces* (6.08%), whereas the abundance of *Thermoascus* varied from 36.51 to 73.91% (Figure 5D). In white *Daqu*, *Thermoascus* (39.35%), *Thermomyces* (25.01%), and *Rhizomucor* (7.50%) comprised the top 3 fungal genera, while the abundance of *Thermoascus* fluctuated from 6.44 to 65.88% (Figure 5D). Here, *Thermoascus* is known to produce various enzymes such as catalase, endoglucanase, glucosidase, keratinase, and chitinase, which are able to degrade starch, cellulose or protein from raw materials, providing basic nutrition for other microbes' metabolism that contributed to flavor formation (Jain et al., 2014; Cai et al., 2021). Thus, *Thermoascus* was identified as the most important fungal genera during *Daqu* maturation.

CCA between microbial community and physicochemical factors

Physicochemical factors play a crucial role in shaping and influencing the composition and succession of microbial community during spontaneously fermentation processes (Guan et al., 2020). Therefore, canonical correlation analysis (CCA) was employed to examine the impact of physicochemical factors on the microbial community of the three types of *Daqu*. The results, as shown in Figure 6, indicated that acidity had the most substantial impact on microbial community, as the arrow length representing acidity was the longest among the four physicochemical factors. Regarding bacterial communities, the influence of the four physicochemical factors was as follows: acidity > reducing sugar > moisture > amino acid nitrogen (Figure 6A). Specifically, the dominant bacterium *Kroppenstedia* in black *Daqu* showed a positive correlation with acidity, reducing sugar, and moisture, while it had a negative correlation with amino acid nitrogen (Figure 6A). *Kroppenstedtia* is known to correlate with the biosynthesis of organic acids, evidencing its positive relationship with acidity (Zhang et al., 2024). Its negative correlation with amino acid nitrogen contradicted a previous study that found *Kroppenstedtia* to be positively connected to the production of various amino acids in *Daqu* (Zhu C. et al., 2023). This discrepancy might be caused by the color formation in *Daqu*, where amino acids were consumed via the Maillard reaction to form the dark color. In yellow *Daqu*, the dominant bacterium *Virgibacillus* displayed a positive correlation with reducing sugar, moisture, and amino acid nitrogen, but a negative correlation with acidity (Figure 6A). In white *Daqu*, the dominant bacterium *Bacillus* exhibited a positive correlation with amino acid nitrogen but a negative correlation with the other three physicochemical properties (Figure 6A). Here, *Bacillus* is the typical microbe for producing protease (Wang et al., 2014; Contesini et al., 2017), thus hydrolyzing protein to amino acids or small peptides, interpretating this positive correlation. When considering fungal communities, the influence of these four physicochemical factors was as follows: acidity > amino acid nitrogen > moisture > reducing sugar (Figure 6B). The dominant fungus *Thermoascus* in all three types of *Daqu* shared a positive correlation with all four physicochemical factors (Figure 6B), indicating *Thermoascus*' ability to adapt to the maturation process of *Daqu*. Therefore, acidity was the most critical factor affecting the microbial community of these three kinds of *Daqu* during maturation.

Relationships between microbial community and flavor substances and fermenting vitalities

During the maturation of *Daqu*, microbes utilize substrates such as starch, protein, and others, producing a plethora of flavor compounds and various enzymes. These, in turn, have a profound

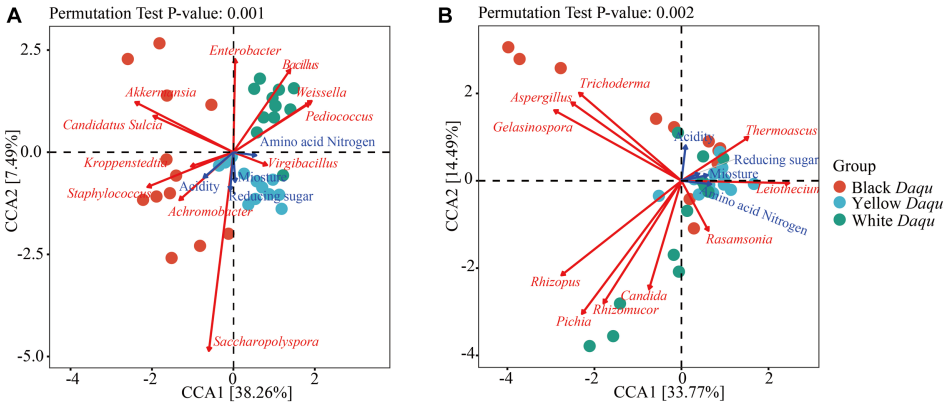

FIGURE 6
Canonical correlation analysis between microbial community and physicochemical parameters based on the top 20 dominant microbes at genus levels. The different color of dots represented different groups of samples. The blue arrows expressed physicochemical factors, and red arrows stood for core microbes. The length of arrows determines the degree of importance. The correlations of bacterial community **(A)** and fungal community **(B)** to physicochemical parameters.

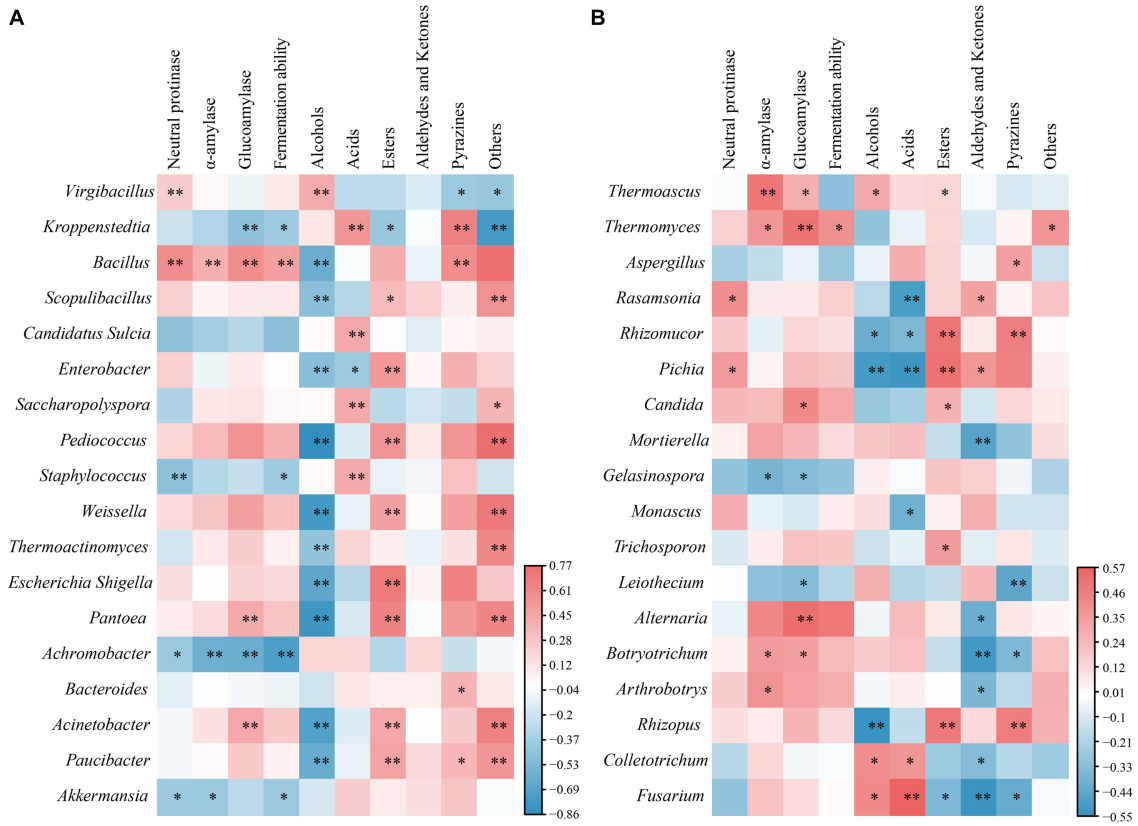

FIGURE 7
The heatmap of relationships between flavor compounds & activity and dominant microbes. Here, their relationships to bacteria were presented as in **(A)** part, and their relationships to fungi were presented as in **(B)** part. The dominant microbes at genus level (top 20) were selected to analyze the correlations to flavor compounds using Spearman correlation coefficient (*$p < 0.05$ and **$p < 0.01$). R value was set at 0.1.

impact on the subsequent production of baijiu, making microbes pivotal in the maturation of *Daqu* and significantly influencing the quality of the final product. To explore these connections, we employed Spearman correlation analysis to unravel the relationships between dominant microbes and flavor compounds, as well as fermenting vitalities. The results, presented in Figure 7, delineated the influence of dominant bacteria and fungi on flavor compounds and fermenting vitalities. In black *Daqu*, the dominant bacterium *Kroppenstedia* exhibited a substantial positive correlation with acids and pyrazines, along with a significant

negative correlation with amylase, fermenting vitality, and esters (Figure 7A). These correlations may be attributed to the unique characteristics of *Kroppenstedia*, as reported previously. *Kroppenstedtia* was identified as a core microbe related to the production of organic acids (lactic acid and short chain fatty acids) (Zhu C. et al., 2023; Zhang et al., 2024), and positively correlated with the formation of certain pyrazines such as tetramethylpyrazine and 2,3,5-trimethyl pyrazine (Zhang et al., 2021). Within yellow *Daqu*, the dominant bacterium *Virgibacillus* demonstrated a positive correlation with neutral protease and alcohols, while displaying a negative correlation with pyrazines (Figure 7A). However, the functions of *Virgibacillus* in fermented food is not clear and warrant further investigation. In white *Daqu*, the dominant bacterium *Bacillus* displayed a positive correlation with all enzymes, fermenting vitality, and pyrazines, while holding a negative correlation with alcohols (Figure 7A). *Bacillus* is known to produce various hydrolytic enzymes, including amylase, protease, and lipase, to degrade macromolecules and hence promoting the formation of flavor compounds during baijiu brewing (Jiang et al., 2021; Li Z. et al., 2023). Especially, the pyrazine compounds, providing sauce-flavor baijiu with its unique flavor characteristic, were closely linked to *Bacillus* (He et al., 2019). Considering fungi, the dominant fungus *Thermoascus* across all three types of *Daqu* showed a positive correlation with amylase, glucoamylase, alcohols, and esters (Figure 7B), hinting that *Thermoascus* could promote the formation of flavor by producing various enzymes and collaborating with others functional microbes (Jain et al., 2014; Cai et al., 2021). Notably, the formation of pyrazine substances, characteristic of sauce-flavor baijiu, exhibited a significant positive correlation with *Aspergillus*, *Rhizomucor* and *Rhizopus* (Figure 7B), implying that these fungi also played a crucial role in the formation of pyrazines. This conclusion complements the prevailing belief that *Bacillus* is the core microbe responsible for pyrazine formation, indicating the substantial contributions of molds to this process.

Conclusion

In this study, the physicochemical properties exhibited similar trends during the maturation of the three types of Daqu, except for acidity, which showed significant differences. A comprehensive analysis of the fermenting capabilities revealed that white *Daqu* attained the highest quality, followed by yellow *Daqu*, while black *Daqu* exhibited the lowest quality. Moreover, the differences in microbial community during maturation were more pronounced for bacteria than for fungi based on the third-generation HTS. *Kroppenstedia*, *Virgibacillus*, and *Bacillus* were the most dominant bacteria in black, yellow, and white *Daqu*, respectively, whereas their dominant fungi all belonged to the *Thermoascus*. Acidity acted as the most notable factor influencing the microbial community. *Kroppenstedtia* was the potential core bacterium affecting the color formation in *Daqu* via Maillard reaction. Furthermore, molds played a pivotal role in pyrazine compounds formation. Therefore, this study provides some novel explanations of color and pyrazine formation from a microbial perspective, laying a foundation for the mechanized *Daqu* production and quality control in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA 1034761 https://www.ncbi.nlm.nih.gov/, PRJNA 1034762.

Author contributions

WD: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft. XY: Data curation, Formal analysis, Writing – review & editing. LW: Methodology, Software, Writing – review & editing. MZ: Investigation, Methodology, Writing – review & editing. JM: Data curation, Software, Writing – review & editing. JL: Conceptualization, Data curation, Writing – review & editing. YF: Investigation, Resources, Writing – review & editing. SZ: Conceptualization, Resources, Writing – review & editing. QY: Resources, Supervision, Writing – review & editing. YH: Funding acquisition, Supervision, Writing – review & editing. SC: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

WD, QY, and SC were employed by Jing Brand Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1345772/full#supplementary-material

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Cultivable microbial diversity, peptide profiles, and bio-functional properties in Parmigiano Reggiano cheese

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Introduction: Lactic acid bacteria (LAB) communities shape the sensorial and functional properties of artisanal hard-cooked and long-ripened cheeses made with raw bovine milk like Parmigiano Reggiano (PR) cheese. While patterns of microbial evolution have been well studied in PR cheese, there is a lack of information about how this microbial diversity affects the metabolic and functional properties of PR cheese.

Methods: To fill this information gap, we characterized the cultivable fraction of natural whey starter (NWS) and PR cheeses at different ripening times, both at the species and strain level, and investigated the possible correlation between microbial composition and the evolution of peptide profiles over cheese ripening.

Results and discussion: The results showed that NWS was a complex community of several biotypes belonging to a few species, namely, *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* subsp. *lactis*. A new species-specific PCR assay was successful in discriminating the cheese-associated species *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, and *Lacticaseibacillus zeae*. Based on the resolved patterns of species and biotype distribution, *Lcb. paracasei* and *Lcb. zeae* were most frequently isolated after 24 and 30 months of ripening, while the number of biotypes was inversely related to the ripening time. Peptidomics analysis revealed more than 520 peptides in cheese samples. To the best of our knowledge, this is the most comprehensive survey of peptides in PR cheese. Most of them were from β-caseins, which represent the best substrate for LAB cell-envelope proteases. The abundance of peptides from β-casein 38–88 region continuously increased during ripening. Remarkably, this region contains precursors for the anti-hypertensive lactotripeptides VPP and IPP, as well as for β-casomorphins. We found that the ripening time strongly affects bioactive peptide profiles and that the occurrence of *Lcb. zeae* species is positively linked to the incidence of eight anti-hypertensive peptides. This result highlighted how the presence of specific LAB species is likely a pivotal factor in determining PR functional properties.

KEYWORDS

Parmigiano Reggiano cheese, *Lacticaseibacillus*, bioactive peptides, natural whey starter, peptidomics, starter lactic acid bacteria, non-starter lactic acid bacteria

1 Introduction

Artisanal cheeses are man-driven ecosystems inhabited by composite microbial communities that originate from various sources (Bokulich and Mills, 2013; Wolfe and Dutton, 2015; Ercolini, 2020). During the process of cheese-making and ripening, biotic, and abiotic factors affect the course of microbial community evolution (Mayo et al., 2021). As a result, differences in microbial species composition affect the organoleptic and rheological attributes of the final products. This is especially true for the Parmigiano Reggiano (PR) cheese, the most famous Italian long-ripened (at least 12 months) hard-cooked cheese produced according to the specifications of the Protected Designation of Origin (PDO).[1] PR cheese manufacturing entails the usage of raw cow milk (2.2–2.5% fat), which is a mixture of evening milk (partially skimmed by natural creaming) and morning whole milk, without adding any industrial starters or adjunct cultures (Figure 1A). The only admitted starter is the natural whey starter (NWS), produced in every dairy by incubating the whey of the previous cheese-making round at a decreasing temperature after curd cooking. The microbiota inhabiting raw milk is rapidly replaced by NWS homofermentative and thermophilic starter lactic acid bacteria (SLAB), such as *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*, and *Streptococcus thermophilus* (Rossetti et al., 2008; Bottari et al., 2010; Bertani et al., 2020; Sola et al., 2022). After curd brining, SLAB rapidly depleted in favor of non-starter lactic acid bacteria (NSLAB), mainly *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*), *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*), and *Lacticaseibacillus casei* (formerly *Lactobacillus casei*) (Coppola et al., 2000; Neviani et al., 2009; Solieri et al., 2012; Gatti et al., 2014). These mesophilic and facultatively heterofermentative species belong to the so-called *Lcb. casei* group (LCG) and are supposed to arise from raw cow milk and dairy environment (Bottari et al., 2018). LCG becomes dominant starting from 2 months of ripening (Bottari et al., 2018; Bettera et al., 2023), due to their tolerance toward pH values as low as 4.9, salt concentrations up to 6%, and a wide range of temperatures (2–53°C) (Settanni and Moschetti, 2010; Gobbetti et al., 2015).

The succession of SLAB and NSLAB during PR cheese-making and ripening assures biochemical reactions that successfully transform milk/curd into ripened cheese. SLAB present in NWS rapidly turn lactose into lactic acid, enabling gel syneresis, whey expulsion, and curd formation (Wilkinson and LaPointe, 2020). Together with rennet and milk proteases, SLAB contribute to casein hydrolysis. Accordingly, several SLAB (e.g., *L. helveticus*) are highly proteolytic species (Griffiths and Tellez, 2013). During cheese manufacturing, both abiotic (e.g., carbon starvation, curd cooking, and salt levels) and biotic conditions (e.g., prophage induction) trigger SLAB autolysis, which releases intracellular peptidases and cell-envelope proteases (CEP) into cheese matrices, further contributing to proteolysis (Gatti et al., 2008). SLAB autolysis also provides carbon skeletons and nitrogen sources to support NSLAB growth (Adamberg et al., 2005; Dea Lindner et al., 2008; Lazzi et al., 2014; Sgarbi et al., 2014; Czárán et al., 2018; Møller et al., 2020). NSLAB affect cheese flavoring compounds through three metabolic pathways: (i) lactate and citrate metabolism, (ii) free fatty acid release and their subsequent metabolism, and (iii) protein breakdown and amino acid catabolism. It is well known that several NSLAB strains possess a comprehensive proteolytic system (as reviewed by Tagliazucchi et al., 2019), which is functionally active in cheese (Bove et al., 2012; Randazzo et al., 2021; Solieri et al., 2022). NSLAB isolated from PR cheese release casein-derived bioactive peptides and oligopeptides shorter than 20 aa, which exert anti-hypertensive, antioxidant, and antidiabetic activities (Tagliazucchi et al., 2020). Therefore, the extent of proteolysis carried out by intact NSLAB cells and their released enzymes strongly contribute to the rheological, organoleptic, and functional attributes of PR cheese (Martini et al., 2020, 2021; Solieri et al., 2020; Tagliazucchi et al., 2020).

Based on the importance of SLAB and NSLAB in PR cheese-making, this study aimed to explore the cultivable microbial fraction in NWS and PR wheels at different ripening times (namely 12, 18, 24, and 30 months) and to determine the evolution of peptidomic profiles over time. Possible correlations between the observed microbial diversity, the peptidomic profiles, and the related bio-functional properties were unraveled.

2 Materials and methods

2.1 Sampling

In the present study, a total of three NWS and 12 cheese samples were collected from February 2020 to January 2021 from three different dairies located in the province of Reggio Emilia (Italy) and belonging to the Parmigiano Reggiano Cheese Consortium. For each dairy, the sampling flowchart is shown in Figure 1. Specifically, we collected three NWS as well as 12 and 24 months-ripened PR cheese samples in the first sampling. The second sampling was carried out after 6 months, collecting PR cheese samples from the same wheels after 18 and 30 months of ripening. Samples were aseptically handled and immediately brought into the laboratory under refrigerated conditions for the subsequent analysis. Each sample name consisted of a letter (corresponding to the respective dairy farm) and a number (corresponding to the ripening time).

2.2 Materials and type strains

All media and chemicals were purchased from Oxoid (Basingstoke, Hampshire, United Kingdom) and Sigma-Aldrich (St. Louis, MO, United States), respectively, except where differently indicated. Anaerobic systems and molecular biology reagents were purchased from Thermo Fisher Scientific (Waltham, MA, United States). Oligonucleotide and Sanger sequencing services were provided by Bio-Fab Research (Rome, Italy).

The type strains used in this study are listed in Table 1. All the strains were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Deutschland) and cultivated according to DSMZ culture condition specifications.

1 http://www.parmigianoreggiano.com/consortium/rules_regulation_2/default.aspx

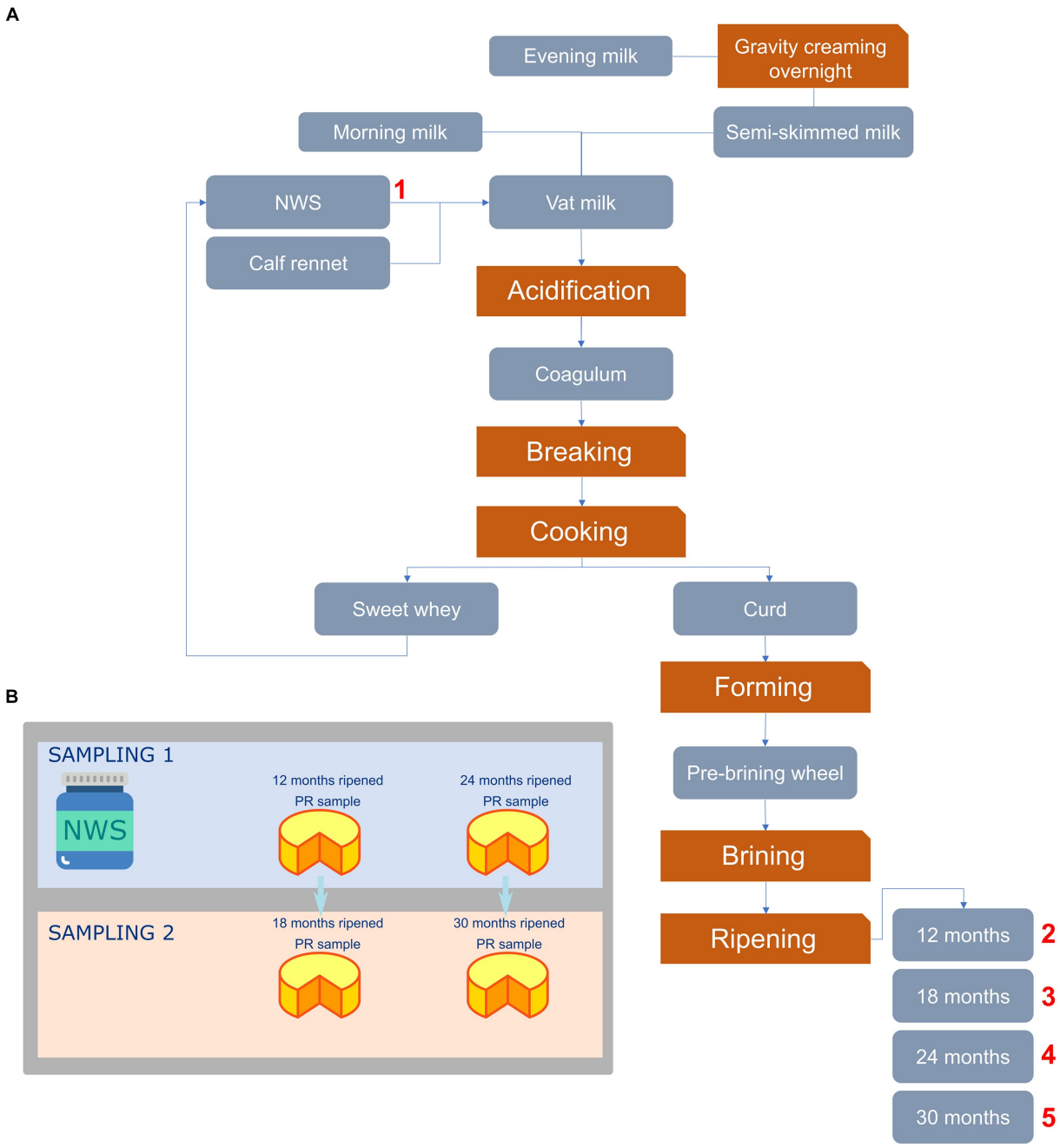

FIGURE 1
Flowchart depicting the PR cheese manufacturing process **(A)** and sampling strategy used in this study **(B)**. In panel **(A)**, numbers indicate sampling points. NWS, Natural whey starter.

2.3 Physicochemical characterization of NWS and compositional analysis of cheeses

Natural whey starter samples were analyzed for pH and titratable acidity as previously reported (Sola et al., 2022). Concerning cheese samples, after removing 15 mm of the rind, cheese cores were finely shredded and immediately analyzed for moisture, NaCl, protein, and fat contents, according to Lolli et al. (2021). NaCl/DM (dry matter), protein/DM, and fat/DM were also calculated. All analyses were performed using the NIRFlex N-500 (Büchi Labortechnik AG, Flawil, Switzerland) working with near infrared reflection (NIR) in the region 800–2,500 nm. The analyses were carried out in triplicate.

2.4 Microbiological counts and LAB isolation

To prepare the cheese samples, 5 g of each cheese sample from the medial section of the core (the length varied between 18 and

TABLE 1 Reference strains used in this study.

Strains	Species	Culture conditions
DSM20011^T	*Lacticaseibacillus casei*	MRS pH 6.2–6.5; 48 h; 37°C; anaerobiosis
DSM 5622^T	*Lacticaseibacillus paracasei* subsp. *paracasei*	MRS pH 6.2–6.5; 48 h; 37°C; anaerobiosis
DSM20021^T	*Lacticaseibacillus rhamnosus*	MRS pH 6.2–6.5; 48 h; 37°C; anaerobiosis
DSM20178^T	*Lacticaseibacillus zeae*	MRS pH 6.2–6.5; 48 h; 37°C; anaerobiosis
DSM20617^T	*Streptococcus salivarius* subsp. *thermophilus*	MRS pH 6.2–6.5; 48 h; 37°C; anaerobiosis
DSM20075^T	*Lactobacillus helveticus*	MRS pH 6.2–6.5; 48 h; 37°C; anaerobiosis
DSM 20052^T	*Limosilactobacillus fermentum*	MRS pH 6.2–6.5; 48 h; 37°C; anaerobiosis
DSM20074^T	*Lactobacillus delbrueckii* subsp. *delbrueckii*	MRS pH 6.2–6.5; 48 h; 37°C; anaerobiosis
DSM20081^T	*Lactobacillus delbrueckii* subsp. *bulgaricus*	MRS pH 6.2–6.5; 48 h; 37°C; anaerobiosis

22 cm) were aseptically transferred into a sterile stomacher bag, supplemented with 45 mL of sterile saline solution (9 g/L NaCl), and homogenized for 4 min at 220 rpm in a Stomacher LAB Blender 400 (PBI International, Milan, Italy). NWS samples were 10-fold diluted with sterile saline solution (9 g/L NaCl) before plating. Enumerations of LAB populations were carried out for NWS and cheese samples using de Man, Rogosa, and Sharpe (MRS) agar medium at a pH of 5.4, incubated at 37°C for 72 h under anaerobic conditions, and M17 medium, incubated at 42°C for 72 h under anaerobiosis. The NWS M17 medium supplemented with 7% (v/v) of sterile skimmed milk (SSW) (Morga AG, Ebnat-Kappel, Switzerland) was prepared and incubated at 42°C for 72 h under aerobiosis, as described by Fornasari et al. (2006). All media were added with the antibiotic cycloheximide (100 mg/L) to inhibit yeasts. Viable cell counts were recorded as a number of colony-forming units (CFU)/mL recovered from plates with CFU/mL ranging from 20 to 200 and expressed as Log_{10} CFU/mL means of at least three replicates.

Individual bacterial colonies were randomly selected, sub-cultured on the same isolation medium, screened for catalase reaction and Gram staining, and microscopically checked before storing at −80°C in liquid culture using 25% (v/v) of glycerol solution.

2.5 LAB identification

Genomic DNA was obtained through mechanical lysis of bacterial cells in the late exponential phase and organic solvent extraction as previously described (Tagliazucchi et al., 2020). The purity and quantity of DNA were estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). The DNA quality was also measured by electrophoresis on 0.8% (w/v) agarose gel containing ethidium bromide (0.5 mg/mL) in 0.5× TBE buffer (45 mmol/L Tris–HCl, 45 mmol/L boric acid, and 1 mmol/L EDTA, with a pH of 8.0) at a constant voltage of 90 V for 1 h at room temperature. Gel pictures were taken under UV light using a BioDoc analysis system (Biometra, Göttingen, Germany). Finally, the DNA samples were diluted to 50 ng/μL in ddH_2O and were stored at −20°C for subsequent analyses.

Natural whey starter isolates were identified by pentaplex PCR as previously reported (Cremonesi et al., 2011), except for primer concentrations reduced to 1 μmol/L for each primer. Cheese isolates were identified by *mutL* gene multiplex PCR targeting the species *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, and *Lacticaseibacillus rhamnosus* (Bottari et al., 2017), with the following improvement. A fourth target species, *Lacticaseibacillus zeae*, was implemented in the multiplex PCR assay using a primer pair designed on gene KRK10099.1 [locus_tag = "FD51_GL001918" on *Lcb. zeae* DSM20168^T genome (AZCT01000025.1:29282.30181), namely, Zeae_F1 (5′-TTTGACCGGTTAGATGACCAGCAT-3′) and Zeae_R1 (5′-CGCGACATGTTGGTAAGGTGCG-3′)]. A comprehensive list of primers and PCR cycling conditions used in this study is reported in Supplementary Table S1. All PCRs were carried out using a thermal cycler T100 (Bio-Rad, Hercules, CA, United States) in a final volume of 20 μL containing DreamTaq Green Buffer 1 × 2 mmol [L of $MgCl_2$, 200 μmol/L of each dNTP, 50 ng of DNA template, 0.5 U DreamTaq Green DNA polymerase (5 U/μL), and 0.5 μmol/L of each primer in multiplex PCR and 1 μmol/L of each primer in pentaplex PCR, respectively]. When required, 16S rRNA gene PCR amplification and ARDRA analysis with diagnostic endonucleases, such as *Mse*I, *Eco*RI, and *Hha*I, were carried out for NWS and cheese isolates, respectively (Sola et al., 2022).

2.6 Phylogenetic analysis

PCR amplicons of the 16S rRNA gene were purified using the DNA Clean & Concentrator™-5 Kit (Zymo Research, Orange, CA, United States) and were sequenced on both strands using 27f and 1,490 primers through a DNA Sanger dideoxy sequencing process performed by Bio-Fab Research (Rome, Italy). When required, internal primer WLAB2 (5′-TCGAATTAAACCACATGCTCCA-3′) (Lopez et al., 2003) was also used for sequencing. Contig sequences were merged using the program SeqMan (DNASTAR, Madison, WI, United States), and the poor-quality ends were edited manually to remove primers. The resulting contig sequences were used as queries in a Blastn search against the NCBI RefSeq database (O'Leary et al., 2016) (14 September 2023). A cutoff of 98.7% 16S rRNA gene similarity was used for species attribution (Stackebrandt, 2006). The related sequences, i.e., four outgroup species (*Weizmannia coagulans*, *Bacillus subtilis*, *Bacillus vallismortis*, and *Enterococcus faecalis*) (Ventura et al., 2009), were aligned with the Muscle program (Edgar, 2004) in Mega X software (Kumar et al., 2018), and the resulting alignment was subjected to a DNA substitution model analysis to select the best-fitting model. Phylogenetic relationships were inferred using the maximum likelihood method. Among sites, rate variation was modeled by a

gamma distribution (+G). Bootstrap support values were obtained from 1,000 random resamplings. Trees were visualized using the Interactive Tree of Life (ITOL) (Letunic and Bork, 2019) and were rooted at outgroup reference species. The sequences obtained in this study were deposited in the GenBank NCBI database with the accession numbers ON936796–ON936814 and OM091849–OM091851.

2.7 rep-PCR and fingerprinting analysis

Genotyping of LAB isolates was performed by the repetitive sequence-based polymerase chain reaction (rep-PCR) using marker $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′), as previously described (Tagliazucchi et al., 2020). Fingerprinting patterns were analyzed using BioNumerics software v8.10 (Applied Maths, Sint-Martens-Latem, Belgium). A unique dataset was used to analyze 65 SLAB. For 189 NSLAB submitted to UGMA analysis, we created four datasets based on the ripening time, namely, 12_m (12 months of ripening), 18_m (18 months of ripening), 24_m (24 months of ripening), and 30_m (30 months of ripening). Particularly, the Pearson correlation coefficient was used to calculate similarity matrices from densitometric curves. To define appropriate parameters (optimization and curve smoothing), we used the software's optimization tools, which build dendrograms to identify distinct isolates and conduct bootstrap replications to test a range of possible values and find the most parsimonious branching solution. Clustering analysis of similarity matrices was performed using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm with 1,000 bootstrapping replicates to evaluate the consistency of the group. The resulting trees were visualized using ITOL, as reported above. LAB isolates displaying a similarity greater than 91% were considered to have the same biotype.

2.8 Extraction of low-molecular-weight water-soluble peptides from PR cheese samples

Water-soluble peptides were first extracted from PR cheeses by following the protocol reported in Martini et al. (2021). Briefly, 5 g of cheese were mixed with 45 mL of 0.1 mol/L HCl, and the mixture was homogenized using an Ultra-Turrax homogenizer. Three cycles of homogenization lasting 1 min were carried out, alternated with 1 min in an ice bath. Subsequently, the homogenates were centrifuged (40 min; 4°C; 4,000 *g*) and then filtered using Whatman filter paper 4. The clear water-soluble peptide extracts were then subjected to ultrafiltration to get the low-molecular-weight peptide fractions for peptidomics analysis. Ultrafiltration was carried out with a membrane of 3 kDa cutoff, as previously described (Tagliazucchi et al., 2017).

2.9 Peptides identification and semi-quantitative analysis

Low-molecular-weight peptide fractions were analyzed via the peptidomics technique for outlining PR cheese peptide profiles from a qualitative and semi-quantitative point of view. The samples were injected into a UHPLC system (UHPLC UltiMate 3000 separation module, Thermo Scientific, San Jose, CA, United States) coupled with a high-resolution mass spectrometry (Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer, Thermo Scientific, San Jose, CA, United States). The complete details about the chromatographic conditions, mass spectrometry, and tandem mass spectrometry parameters were reported in Martini et al. (2021). Qualitative and semi-quantitative analyses were carried out using Mascot and Skyline software, respectively, as previously described (MacLean et al., 2010; Dallas and Nielsen, 2018; Martini et al., 2021; Helal et al., 2023).

Bioactive peptide identification was performed using the Milk Bioactive Peptide Database, considering only the peptides with 100% sequence homology with previously characterized bioactive peptides (Nielsen et al., 2017).

2.10 Statistical analysis

Significant differences among samples were evaluated by one-way ANOVA with Tukey post-test. Data were considered significantly different when $p < 0.05$. Three analytical replicates for each sample were used for all analyses.

The semi-quantitative bioactive peptide data of the cheese samples were utilized for chemometric analysis by using online software MetaboAnalyst 5.0 (Xia et al., 2015) (REF). Data were normalized by median and Pareto scaling before principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). The analysis was validated by multiple correlation coefficients (R2) and cross-validation (Q2). The significance of the biomarkers was ranked using the projection variable importance score (VIP score > 1) of the PLS-DA.

The correlation among the variables (semi-quantitative bioactive peptide data, microbiological data, and compositional data) was assessed by Spearman rank analysis ($p < 0.05$) with MetaboAnalyst 5.0.

3 Results

3.1 NWS physicochemical characterization and microbial counts

In this study, three NWS samples from three manufacturers in the Italian province of Reggio Emilia, referred to as R, C, and L, were considered. The samples NWS_R, NWS_C, and NWS_L showed pH values of 3.46 ± 0.01, 3.46 ± 0.05, and 3.49 ± 0.04, respectively. Titratable acidity ranged from 27.29 ± 0.05 to 29.90 ± 0.04, while lactic acid concentrations were from 12.29 ± 0.21 to 13.48 ± 0.28 (Supplementary Table S2).

To detect the broadest spectrum of NWS cultivable fraction as possible, we used four different growth conditions. Microbial counts are reported in Figure 2. The SLAB counts ranged from 8.17 ± 0.05 (NWS_R, MRS at 42°C) to 5.72 ± 0.02 (NWS_L, MRS at 42°C) Log_{10} CFU/mL. NWS_R generally had higher counts of SLAB than NWS_C and NWS_L in all growth conditions tested ($p < 0.05$). The only exception was SLAB counts in M17-SSW medium from dairy L, which were similar to those scored in R (MRS 42°C; M17-SSW 42°C).

3.2 SLAB diversity and species distribution

A total of 65 SLAB isolates were submitted to species attribution, which entailed pentaplex PCR and 16S ARDRA analysis with endonucleases *Mse*I and, in case of *L. delbrueckii*, 16S ARDRA with *Eco*RI. Details on molecular identification results are reported in Supplementary Table S3. Both approaches were consistent in attributing 53.85% of isolates to *S. thermophilus* species, followed by 27.69% to *L. helveticus* and 16.92% to *L. delbrueckii* subsp. *lactis* (16.92%). The remaining 1.54% of isolates did not give any results with pentaplex PCR, while 16S ARDRA analysis resulted in a restriction pattern A that did not match those exhibited by the type strains considered in this study. At least one strain for each sample and for each 16S ARDRA profile was submitted to 16S rRNA gene partial sequencing. Two phylogenetic trees were constructed based on 16S rRNA gene sequences with their closest phylogenetic neighbors for rod-shaped and cocci isolates, respectively (Figures 3A,B, respectively). Strains CBB09 and CBA12 formed a monophyletic group with *L. delbrueckii* subsp. *lactis*, while strains RBB02, RBB03, LBB02, and LBB04 shared higher than 99% homology with *L. helveticus* NBRC1519 (Figure 3A). The analysis grouped LBC06, CBC05, RBC06, RBC20, and RBN16 with *S. thermophilus* ATCC19258^T, while strain RBC05 was grouped separately with *Staphylococcus capitis* JCM2420 (Figure 3B).

Genotyping with microsatellite primer $(GTG)_5$ resulted in 65 reliable banding patterns with band size ranging from 580 to 4420 bp. The number of amplicons ranged from 7 to 18 for each isolate. UPGMA analysis of fingerprinting data using 91% as a cutoff of reproducibility discriminated 37 biotypes, namely, 12 subclusters (S) and 25 singletons, with a discriminatory power of

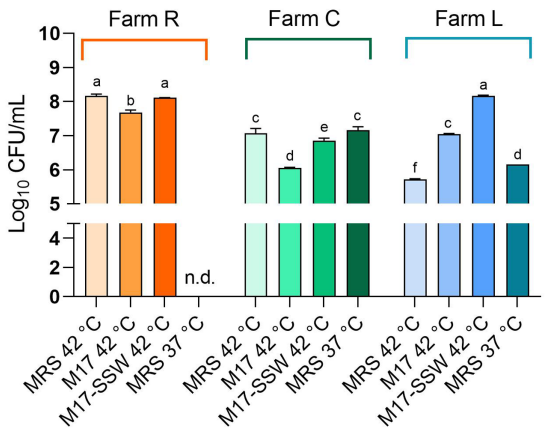

FIGURE 2
Starter lactic acid bacteria (SLAB) plate counts from NWS samples. Data from dairies R (orange), C (green), and L (blue) are mean ($n = 3$) of Log_{10} CFU/mL values, while error bars represent standard deviations. Different letters indicate significant differences among samples and conditions ($p < 0.05$). nd—, Not determined.

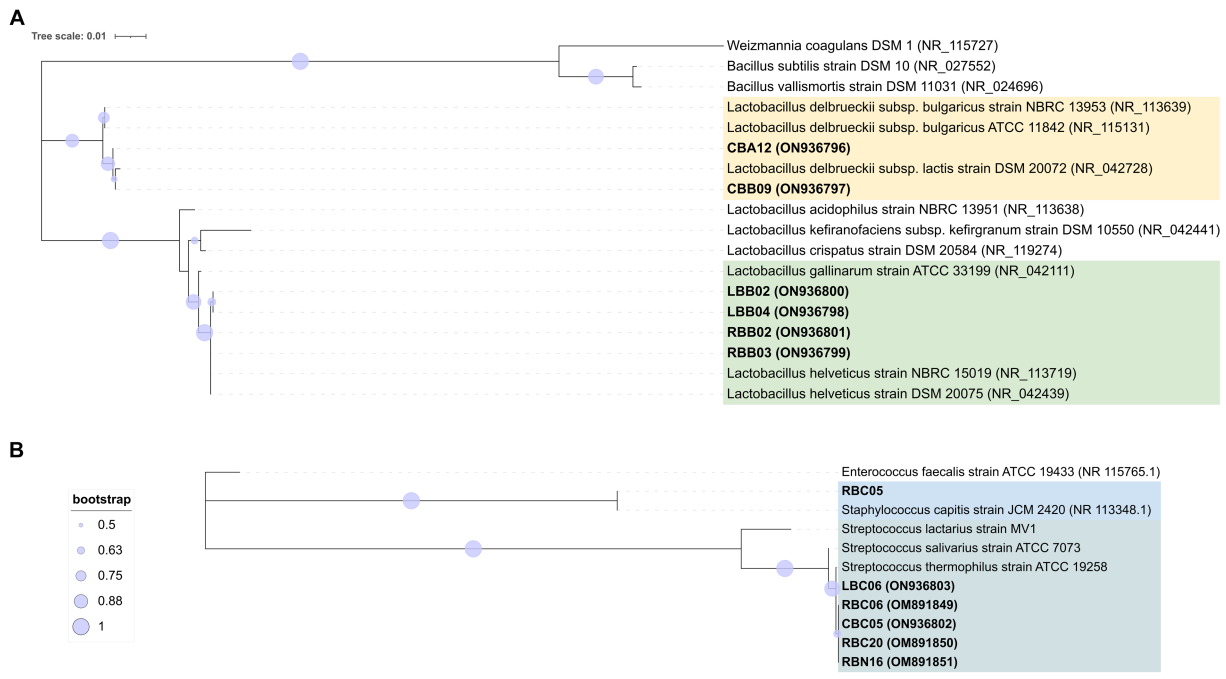

FIGURE 3
Phylogenetic trees based on 16S rRNA gene sequences showing the relationship among rod-shape **(A)** and cocci **(B)** SLAB strains isolated from three NWS samples and the related neighbor's species. The trees were inferred using the maximum likelihood method and the Kimura's two-parameter model (Kimura, 1980) with Mega X software (Kumar et al., 2018). Representative isolates for each sample are shown with the sequence accession numbers indicated in brackets, while the sequence data of reference strains were from the NCBI RefSeq database. A discrete Gamma distribution was used to model evolutionary rate differences among sites. Bootstrap values are indicated at branch points based on 1,000 replications. Bootstrap values below 50% are not shown. Bar: 0.01 substitutions per nucleotide position. The trees are drawn in scale, with branch length measured in the number of substitutions per site. The trees were rooted using the branch leading to three outgroup species: *W. coagulans*, *B. subtilis*, and *B. vallismortis* for the panel **(A)** and *E. faecalis* for the panel **(B)**.

0.970, as calculated by Simpson's index of diversity (Figure 4). These 37 biotypes were grouped into four major clusters (named from A to D). Major clusters A and D grouped *S. thermophilus* strains, while B and C were promiscuous and grouped isolates belonging to at least two different species. Within the largest major cluster B, 11 *L. delbrueckii* strains were grouped congruently with their taxonomic positions and were divided into five biotypes. Additionally, 21 and 8 subclusters were found to group 35 *S. thermophilus* and 18 *L. helveticus* isolates, respectively. In a few cases (subclusters S10 and S12), *L. helveticus* and *S. thermophilus* isolates did not cluster according to their taxonomic attribution. Generally, the genetic relatedness was congruent with the sampling site, with a few exceptions (subclusters S10 and S12). According to Figure 4, dairies R, C, and L had 13, 14, and 14 biotypes, respectively.

Species distribution per sampling site is shown in Figure 5. *Streptococcus thermophilus* was the dominant species in dairies R and L, followed by *L. heleveticus*. In sampling site C, only *L. delbruecki* and *S. thermophilus* were detected.

3.3 Chemical composition and microbial counts in PR cheese

In the present study, a total of 12 PR samples were collected from the same dairy, which provided NWS samples. The ripening periods varied from 12 to 30 months. Salt, fat, protein, and moisture contents along with other parameters are reported in Supplementary Table S4. Generally, the moisture content exhibited an inverse trend compared with the ripening time.

Three different cultivation conditions were considered to recover NSLAB cultivable fractions from PR cheese samples. As shown in Figure 6, we did not find any significant differences in Log_{10} CFU/gr values among growth conditions within the same samples ($p > 0.05$), with a few exceptions. In sample C30, MRS medium at 37°C was the only condition suitable to sustain bacterial growth, while in L18 and L30, M17-SSW medium at 42°C resulted in lower Log_{10} CFU/gr compared to the other conditions. Generally, NSLAB counts ranged from 7.19 ± 0.01 to 0.36 ± 0.10 Log_{10} CFU/gr in R12 incubated in MRS at 42°C and L30 incubated in M17-SSW at 42°C, respectively

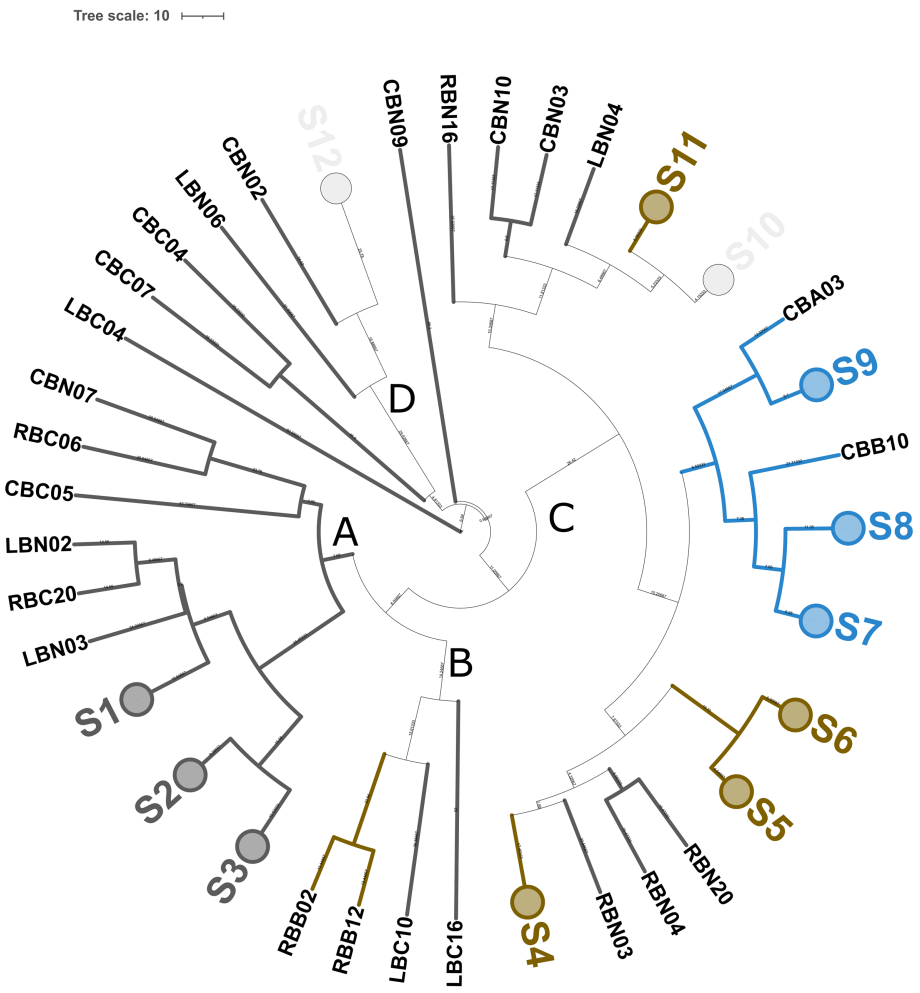

FIGURE 4
Genotyping of 65 SLAB isolates from NWS samples. Dendrogram of $(GTG)_5$-PCR fingerprints was built according to the Pearson correlation coefficient and UPGMA. Collapsed clades (S1–S12) represent 12 clusters with more than 90% similarity coefficient. Upper case letters from A to D indicate major clusters. Each knot shows the length of the arms. Colors were according to the species, as follows: blue, *L. delbrueckii* subsp. *lactis*; orange, *L. helveticus*; dark gray, *St. thermophilus*; and light gray, mixed species. The tree was visualized with ITOL (Letunic and Bork, 2019).

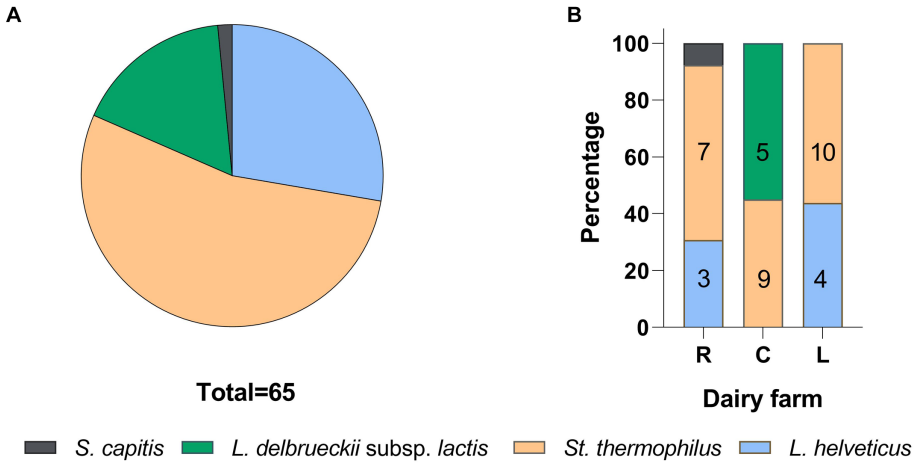

FIGURE 5
SLAB species frequencies **(A)** and distribution **(B)** in PR NSW samples. The numbers in the columns represent biotypes scored by the UPGMA analysis of $(GTG)_5$ rep-PCR fingerprinting data.

(Figure 6). A general decrease in the NSLAB cultivable fraction was scored over time in all the samples ($p < 0.05$). Variations in microbial loads among wheels of the same dairy were observed (Figure 6). For instance, the C24 sample had a NSLAB population slightly higher than the C18 sample ($p < 0.05$). This could be due to the homemade nature of PR cheese, manufactured with raw cow milk and NWS. Comparing dairies with each other, the lowest Log_{10} CFU/gr values were recovered from the samples collected in dairy L in all tested conditions, whereas the highest ones were collected in all the samples from dairy R ($p < 0.05$), except for the 18-month ripening time (Figure 6). These results could mean that viable cells with an integrous cellular wall vary among samples, resulting in putative differences in releasing intracellular peptidases and in the extent of proteolysis.

3.4 Validation of improved multiplex PCR for NSLAB identification

Recently, Liu and Gu (2020) reestablished *Lcb. zeae* as a separate species within the LCG. Notable 16S ARDRA with *Hha*I was unable to discriminate *Lcb. zeae* DSM 20178^T from *Lcb. paracasei*, while conventional *mutL* multiplex PCR did not distinguish *Lcb. zeae* DSM 20178^T from *Lcb. casei* DSM 20011^T (Supplementary Figure S1A). On the other hand, Laref and Belkheir (2022) reported that seven endonucleases were required to separate LCG species. To overcome this caveat, we developed a fast PCR assay targeting a *Lcb. zeae* species-specific gene. The locus FD51_GL001918 encoding glycosyl transferase family 8 has been previously demonstrated to be present in the *Lcb. zeae* genome only (Kim et al., 2020). Therefore, it was selected as a target for designing species-specific primers. The *mutL* gene multiplex PCR assay was implemented with a new *Lcb. zeae* species-specific primer pair, resulting in an improved multiplex PCR assay suitable to discriminate *Lcb. zeae*, *Lcb. paracasei*, *Lcb. rhamnosus*, and *Lcb. casei* in a single round of PCR reaction (Supplementary Figures S1B,C).

3.5 NSLAB diversity and species distribution

Two hundred and fifteen Gram-positive and catalase-negative isolates were obtained from PR cheeses at 12, 18, 24, and 30 months, respectively. An overview of isolates and details on molecular species attribution carried out with 16S ARDRA and improved multiplex PCR assay are reported in Supplementary Table S5. Finally, sequencing of the 16S rRNA gene and phylogenetic analysis were used to confirm species attribution based on 16S ARDRA and improved multiplex PCR assay (Figure 7). Based on the adopted polyphasic approach, 105 isolates were ascribed to *Lcb. paracasei*, 67 to *Lcb. zeae*, 42 to *Lcb. rhamnosus*, and 1 isolate to *Lcb. casei*.

Analysis of species frequencies showed that species patterns were strictly dairy-dependent (Figure 8A). NSLAB populations inhabiting ripened PR wheels reflect the quality and microbiological variability of raw cow milk, resulting in a strong dairy-to-dairy variation in species composition. While *Lcb. paracasei* was ubiquitous, *Lcb. zeae* and *Lcb. rhamnosus* were differently recovered depending on the sampling site. Dairy R was positive for all four species belonging to the LCG clade; dairy C was positive for *Lcb. zeae*, *Lcb. rhamnosus*, and *Lcb. paracasei*. Finally, *Lcb. paracasei* was the only species recovered in dairy L (Figure 8A). We observed that the recovery of *Lcb. rhamnosus* decreased with increasing ripening time. *Lcb. paracasei* dominated dairy R samples, regardless of the ripening time (Figure 8B), while in sampling site C, *Lcb. zeae* became dominant starting after 24 months of ripening (Figure 8B).

Out of 215 NSLAB identified at the species level, 189 resulted in reliable banding patterns by rep-PCR with microsatellite primer $(GTG)_5$. The number of amplicons ranged from 7 to 18 for each pattern. The UPGMA analysis of fingerprinting data was carried out using four datasets; each of them contained isolates belonging to the same ripening time (12, 18, 24, and 30_m, respectively). Figure 9 shows the resulting UPGMA clustering trees. When 91% reproducibility cutoff was used, 68 NSLAB isolates from 12-month ripened PR cheeses were grouped into 30 biotypes (9 subclusters and 21 singletons) (Figure 9A); 48 isolates from the 18_m dataset were divided into 20 biotypes (10 subclusters and 10 singletons) (Figure 9B); 59 isolates from 24_m into

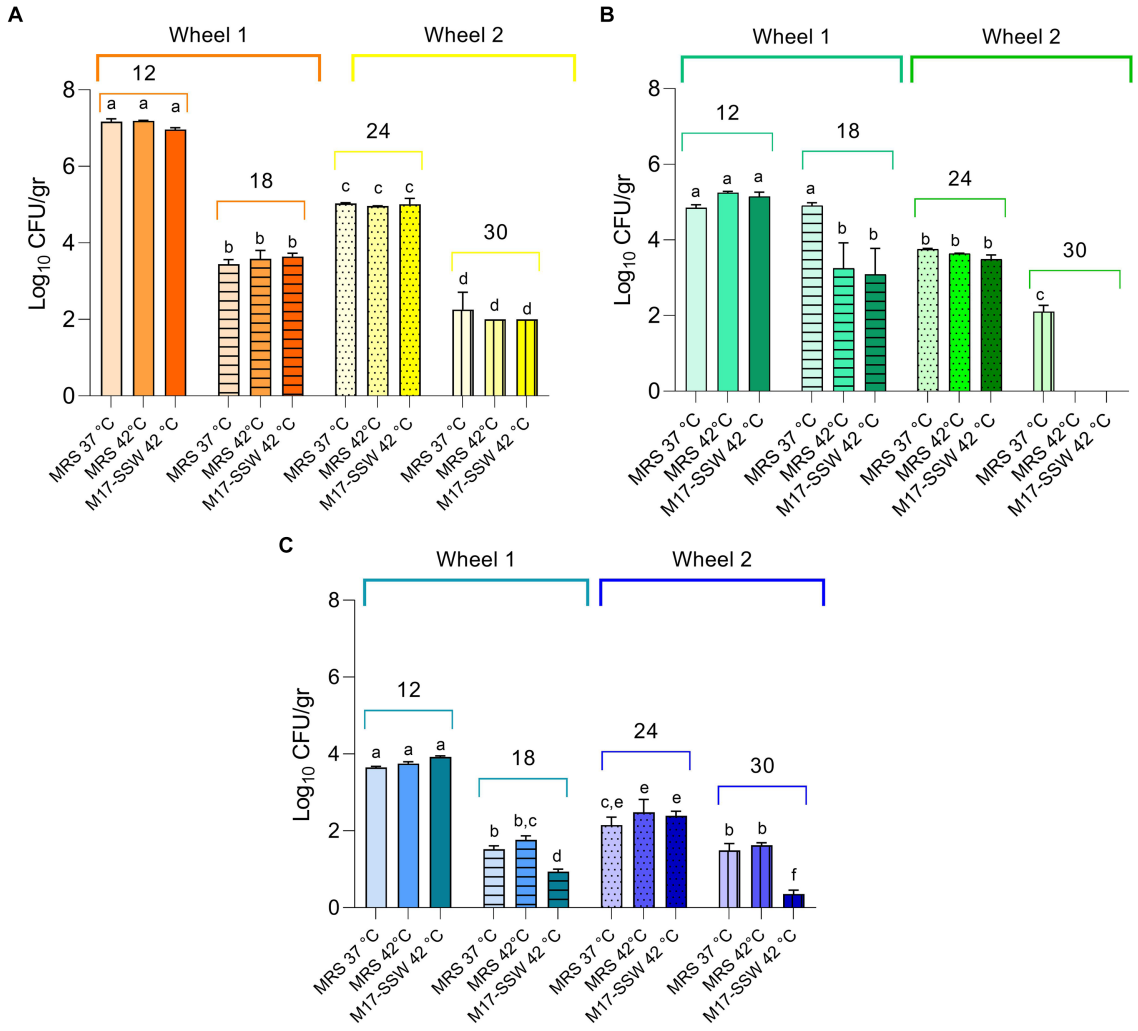

FIGURE 6
Non-starter lactic acid bacteria (NSLAB) plate counts from cheese samples. Data from dairies R (orange) **(A)**, C (green) **(B)**, and L (blue) **(C)** are mean ($n = 3$) of Log_{10} CFU/mL values, while error bars represent standard deviations. Different letters indicate significant differences among samples and conditions ($p < 0.05$).

18 biotypes (10 subclusters and 8 singletons) (Figure 9C); and 18 isolates from 30_m into 10 biotypes (3 subclusters and 7 singletons) (Figure 9D). The Simpson's indices of diversity were 0.921, 0.924, 0.915, and 0.895 for 12, 18, 24, and 30_m, respectively, suggesting that biodiversity slightly decreased with increase in the ripening time and selective pressure. In most cases, biotypes are grouped congruently with species attribution and sampling site. The only exceptions were the subclusters S2, S3, S4, and S8 in the 24_m dataset, where *Lcb. zeae* and *Lcb. rhamnosus* formed mixed subclusters (Figure 9C), as well as subcluster S7 in the 18_m dataset where *Lcb. rhamnosus* and *Lcb. paracasei* isolates were intermixed (Figure 9B). According to Figure 8, dairies R, C, and L showed a high number of biotypes.

3.6 Peptidomics analysis of PR cheeses and bioactive peptides identification

The ultra-high performance liquid chromatography high-resolution mass spectrometry (UHPLC/HR-MS) performed on the low-molecular-weight water-soluble peptide extracts (<3 kDa) of 12 different cheese samples revealed a total of 603 unique peptides in PR cheeses from dairy R, 609 in PR cheeses from dairy C, and 527 in PR cheeses from dairy L, respectively. The complete list of the identified peptides, together with semi-quantitative and MS data, is reported in Supplementary Table S6. All the identified peptides are derived from the proteolysis of the principal milk caseins, i.e., β-casein, αS1-casein, αS2-casein, and κ-casein. In all samples, the best source of peptides was β-casein, followed by αS1-casein, αS2-casein, and finally κ-casein (Figures 10A,C). C samples showed the highest number of peptides at 12 and 30 months of ripening (Figure 10A), whereas R samples showed the highest number at 18 and 24 months of ripening (Figure 10C). PR samples from dairy L displayed the lowest number of peptides at any ripening time (Figure 10B). The trend of the peptide number over time differed depending on the dairy, while no substantial differences in the number of peptides as a function of the ripening time were found, with the only exception of C30 (Figure 10D). Anyway, the average number of peptides determined during

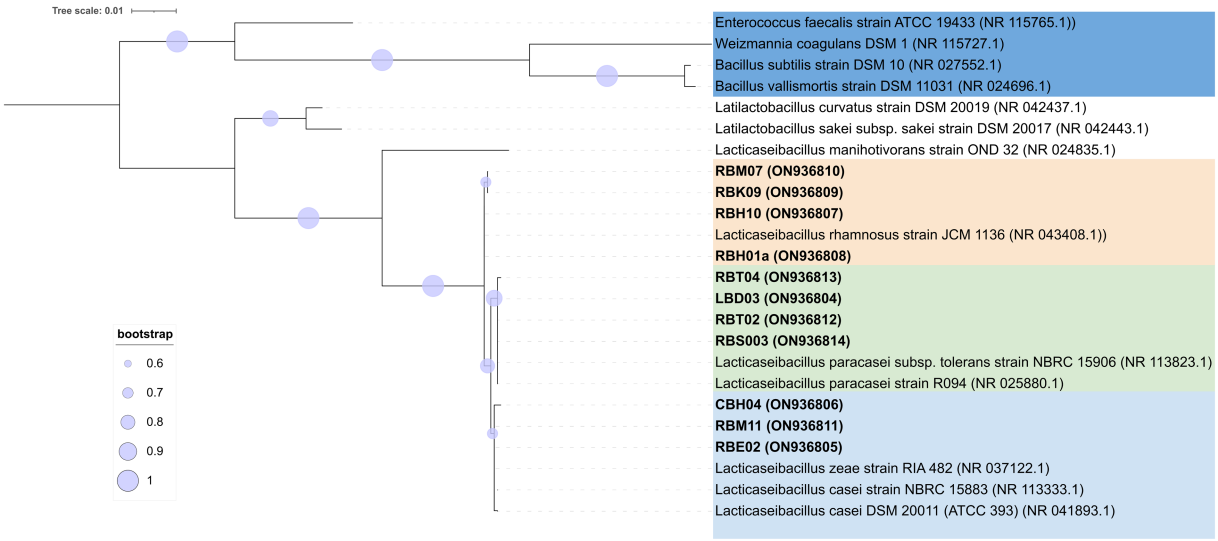

FIGURE 7
Phylogenetic tree based on 16S rRNA gene sequences showing the relationships among non-starter lactic acid bacteria (NSLAB) strains isolated from PR cheeses at 12, 18, 24, and 30 months of ripening. The tree was constructed using the maximum likelihood method and the Kimura's two-parameter model (Kimura, 1980) in Mega X software. Representative isolates for each sample are shown in bold, with the sequence accession numbers indicated in brackets. Sequence data for reference strains were from the NCBI RefSeq database. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The final dataset involved 24 nucleotide sequences for a total of 1,372 positions. Bootstrap values (>60%) are indicated at branch points based on 1,000 replications. The tree is rooted using the branch leading to four outgroup species (*W. coagulans*, *B. subtilis*, *B. vallismortis*, and *E. faecalis*). The tree is drawn in scale with branch length measured in the number of substitutions per site: bar, 0.01 substitutions per nucleotide position. The outgroup species are highlighted in the blue background, *Lcb. rhamnosus* in orange background, *Lcb. casei*/*Lcb. zeae* in green background, and *Lcb. paracasei* in light blue background, respectively. The tree was visualized with ITOL (Letunic and Bork, 2019).

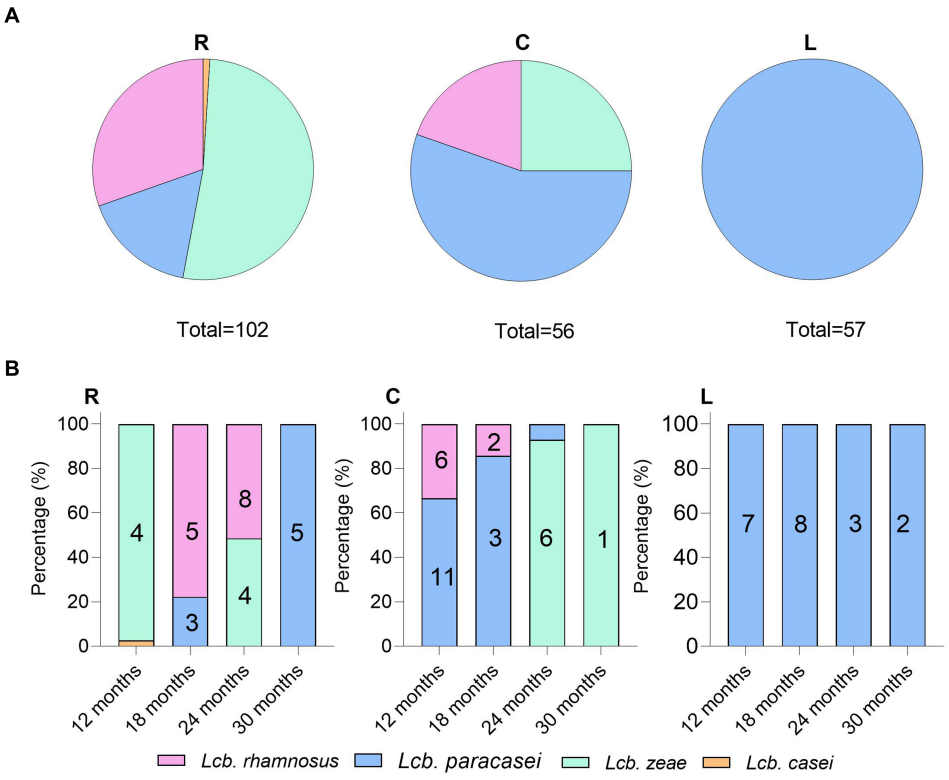

FIGURE 8
Non-starter lactic acid bacteria (NSLAB) species frequencies **(A)** and distribution **(B)** in PR cheese samples. The numbers in the columns represent biotypes scored by UPGMA analysis of $(GTG)_5$ rep-PCR fingerprinting data.

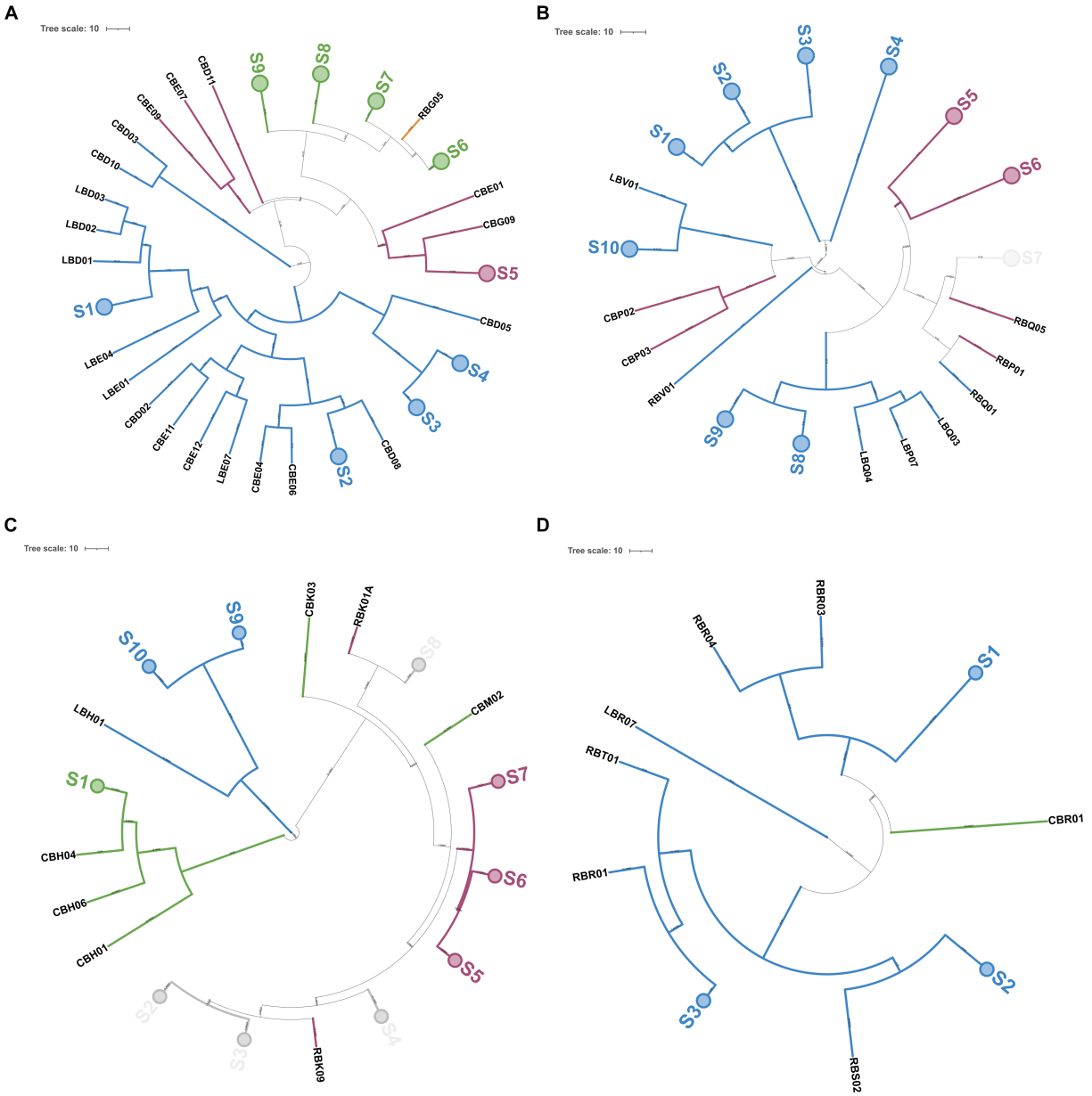

FIGURE 9
Genotyping of 189 NSLAB isolates from PR cheese samples. Trees **(A–D)** represent NSLAB isolates from 12, 18, 24, and 30 months of ripening, respectively. Dendrograms of $(GTG)_5$-PCR fingerprints were built according to the Pearson correlation coefficient and UPGMA. In each tree, collapsed clades (S) represent clusters with more than 91% similarity coefficient. Each knot shows the length of the arms. Colors were according to the species, as follows: blue, *Lcb. paracasei*; pink, *Lcb. rhamnosus*; green, *Lcb. zeae*; and light gray, mixed group. The tree was visualized with ITOL (Letunic and Bork, 2019).

ripening, considering the three dairies, did not vary significantly from each other (Figure 10D).

As shown in Supplementary Figure S2, for each dairy, samples from different ripening times shared most of the identified peptides, suggesting that there is a consistent pool of stable peptides released during the early stages of ripening that are conserved over time. Moreover, different PR samples at the same ripening time shared most of the identified peptides, indicating that the differences in NSLAB cultivable microbiota as well as in cheese manufacturing practices among distinct dairies apparently did not result in any evident peptide variability (Supplementary Figure S3).

To further investigate the effect of ripening time and dairy on peptide profiles, we carried out a semi-quantitative analysis. Data revealed significant differences among the sum of the intensity (peptide abundance measured as the area under the peak for each specific peptide) of the identified peptides in the PR cheese samples. Based on this analysis, significant differences were observed both among dairies and over time (Figures 11A–D). C12 displayed a higher total peptide intensity with respect to R12 and L12 samples ($p < 0.05$). In dairy C, the total peptide abundance strongly decreased by approximately three times from 12 to 18 months of ripening, surging with the highest total peptide intensity at

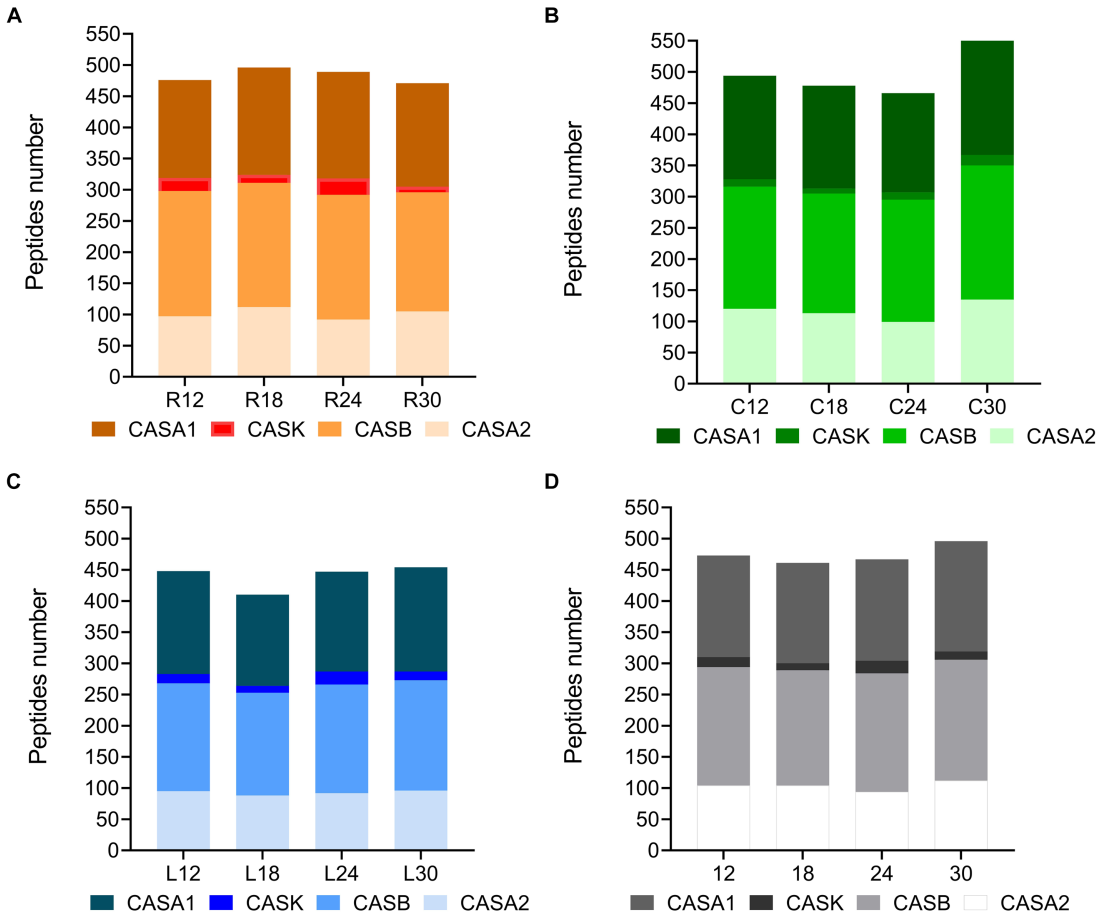

FIGURE 10
Number of peptides per protein in PR cheeses at different ripening times. Analysis was carried out on low-molecular-weight peptide fractions obtained by ultrafiltration at 3 kDa from the water-soluble peptide fractions extracted from the different PR cheeses. **(A)** Number of peptides per protein identified in PR cheeses from dairy C (orange) at 12, 18, 24, and 30 months of ripening. **(B)** Number of peptides per protein identified in PR cheeses from dairy L (green) at 12, 18, 24, and 30 months of ripening. **(C)** Number of peptides per protein identified in PR cheeses from dairy R (blue) at 12, 18, 24, and 30 months of ripening. **(D)** Number of peptides per protein averaged according to the ripening time. The complete list of identified peptides can be found in Supplementary Table S6.

30 months of ripening (Figure 11A). On the contrary, in dairies L and R, an increase in total peptide intensity from 12 to 18 months of ripening was recorded. Then, in L samples, the total peptide abundance gradually decreased to reach its lowest value in 30-month ripened PR cheese (Figure 11B). Differently, in R samples, the total peptide intensity decreased from 18 to 24 months of ripening, and finally, it reached a plateau (Figure 11C). These differences in total peptide abundance highlighted the role of the different LAB species that colonize the cheeses during ripening in the different dairies on casein hydrolysis.

Considering the total peptide intensity by protein (Figure 11), β-casein and αS1-casein displayed the highest peptide intensity in any sample, with β-casein overcoming αS1-casein at each ripening time. The trend of the total peptide intensity in β-casein and αS1-casein as a function of the ripening time overlapped that of the total peptide intensity for each dairy. Ripening also affected the percentage of incidence of peptide intensity per protein with respect to the total peptide abundance (Figures 12A–D). In fact, the percentage of incidence of the peptide intensity from β-casein continuously decreased during ripening, whereas the percentage of incidence of peptide intensity for αS1-casein increased according to the ripening time in all the dairies (Figures 12A–D). A similar trend was also observed for the percentage of incidence of peptide intensity for αS2-casein in dairies C and L (Figures 12B,C).

The intensity of individual peptides identified in the different dairies was averaged according to the ripening time (Supplementary Tables S7, S8). Some clusters of specific peptides coming from the hydrolysis of the different caseins displayed characteristic behavior. For example, poly-phosphorylated peptides released from the N-terminal region 1–28 of β-casein peaked and showed the highest intensity in the 12- or 18-month ripened PR cheeses, and then, for most of them, the intensity decreased as the ripening process continued. On the contrary, peptides from the region 38–88 of β-casein showed an increasing trend during ripening, reaching the highest intensity after 30 months of ripening. Finally, peptides from the C-terminal of β-casein (from residue 164 to residue 209) were continuously released during ripening, peaking at 24 months.

Concerning αS1-casein, most peptides from the N-terminal region 1–38 peaked after 18 or 24 months of ripening, whereas

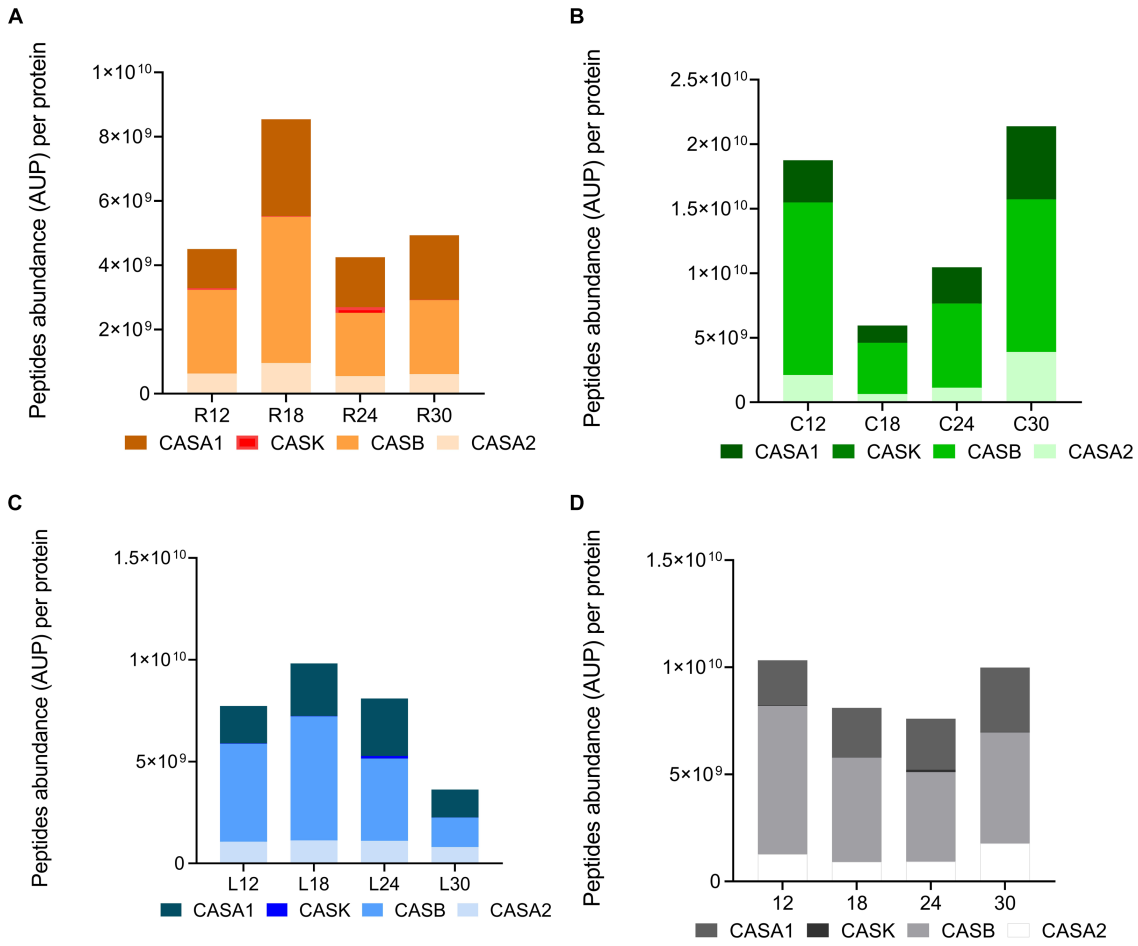

FIGURE 11
Peptide abundance per protein in PR cheeses at different ripening times. Analysis was carried out on low-molecular-weight peptide obtained by ultrafiltration at 3 kDa from the water-soluble peptide fractions extracted from the different PR cheeses. **(A)** Total peptide abundance per protein in PR cheeses from dairy C at 12, 18, 24, and 30 months of ripening. **(B)** Total peptide abundance per protein in PR cheeses from dairy L at 12, 18, 24, and 30 months of ripening. **(C)** Total peptide abundance per protein in PR cheeses from dairy R at 12, 18, 24, and 30 months of ripening. **(D)** Total peptide abundance per protein averaged according to the ripening time. Data are reported as the sum of the intensity of each identified peptide measured as the area under the peak (AUP) by Skyline analysis. The complete list of identified peptides and the semi-quantitative data can be found in Supplementary Table S6.

peptides in the region between the residues 38 and 80 of αS1-casein, rich in phosphorylation sites, constantly increased in intensity during ripening and reached the highest amounts after 30 months. The same considerations can be made for the C-terminal region of αS1-casein (from residue 80 to residue 199), whose peptides were present in a low amount or absent at the beginning of ripening, peaking after 24 or 30 months.

The presence of bioactive peptides in PR cheese samples was ascertained using the Milk Bioactive Peptides Database and considering only peptides with 100% sequence homology with previously identified bioactive peptides. A total of 49 bioactive peptides were detected, considering all the PR cheese samples (Supplementary Table S9). Most of the identified bioactive peptides came from the hydrolysis of β-casein (30 peptides), followed by αS1-casein (12 peptides) and αS2-casein (six peptides), whereas only one bioactive peptide was identified from κ-casein. Only 25 bioactive peptides out of 49 were detected in all the 12 PR cheese samples.

Most of the identified bioactive peptides presented ACE-inhibitory activity (27 peptides), followed by anti-microbial activity (17 peptides). Other identified peptides have been characterized as antioxidant (seven peptides), immunomodulatory (six peptides), anti-inflammatory (three peptides), and DPP-IV-inhibitory (three peptides) compounds. Finally, two peptides were able to inhibit cholesterol solubility; one was anti-cancer, and one poly-phosphorylated peptide was found to promote calcium uptake. Thirteen identified bioactive peptides possessed more than one biological activity.

As reported above, ACE-inhibitory peptides were the most common bioactive peptides identified in PR cheese. Most of them (15 out of 27 ACE-inhibitory peptides) have been found in all the PR cheese samples. A total of five ACE-inhibitory peptides were not identified in any PR cheese sample from the dairy L. Sample L18 also contained the lowest number of ACE-inhibitory peptides (19 peptides), whereas samples C30 and R12 displayed the highest number of ACE-inhibitory peptides (24 peptides).

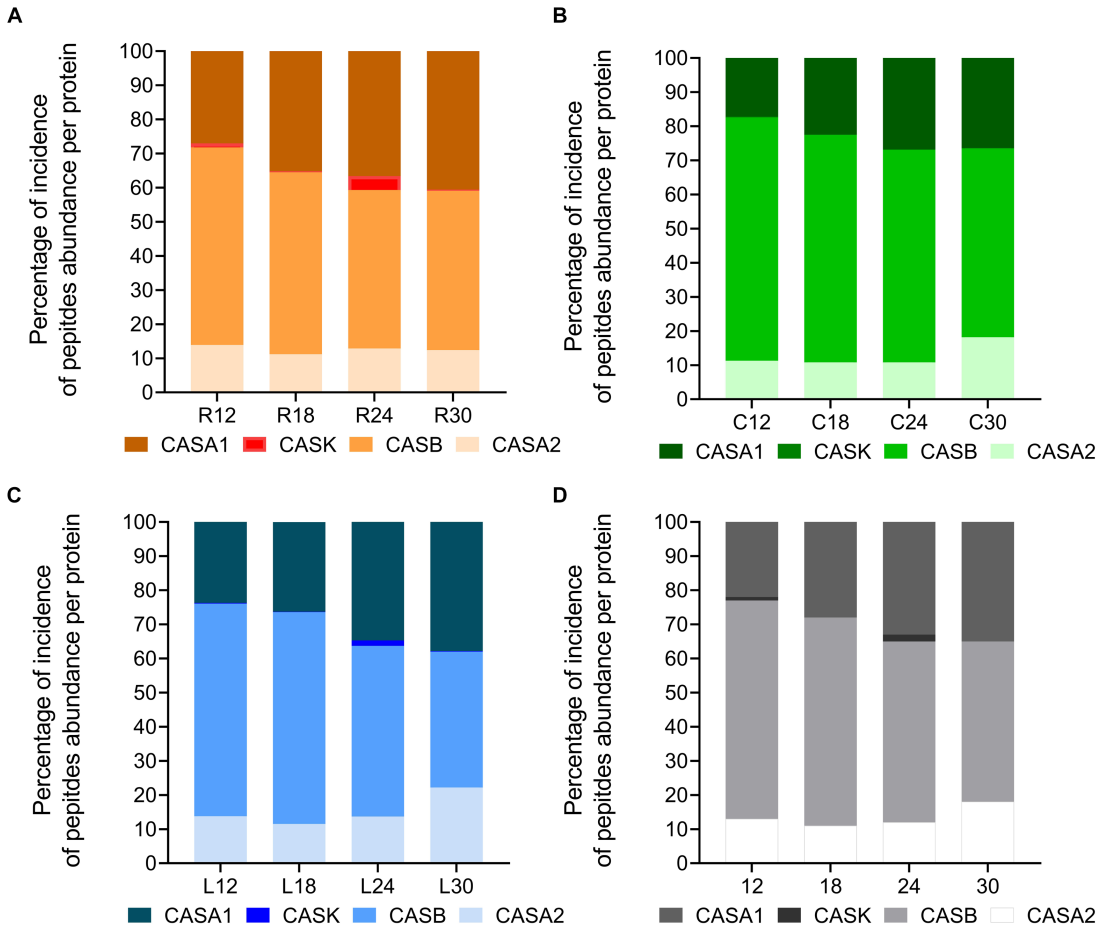

FIGURE 12
Percentage of incidence of peptide abundance per protein in PR cheeses at different ripening times. **(A)** Percentage of incidence of total peptide abundance per protein in PR cheeses from dairy C at 12, 18, 24, and 30 months of ripening. **(B)** Percentage of incidence of total peptide abundance per protein in PR cheeses from dairy L at 12, 18, 24, and 30 months of ripening. **(C)** Percentage of incidence of total peptide abundance per protein in PR cheeses from dairy R at 12, 18, 24, and 30 months of ripening. **(D)** Percentage of incidence of total peptide abundance per protein averaged according to the ripening time.

The evolution of the total ACE-inhibitory peptide abundance during ripening was found to be strongly dairy-dependent, highlighting the role of the different lactic acid bacteria species present in cheese samples (Figure 13). In dairy L, the total ACE-inhibitory peptide intensity increased slightly but significantly from the sample at 12 months of ripening to the sample at 18 months of ripening. Then, it remained almost constant until 24 months of ripening to experience a further decline at 30 months of ripening. Total ACE-inhibitory peptide intensity in sample from dairy R was not significantly different from that of PR cheeses from dairy L at 12 and 18 months of ripening. However, in PR cheese from dairy R at 24 months of ripening, a 2.7 time increase in total ACE-inhibitory peptide abundance was observed. Next, a drastic reduction of approximately three times was recorded in PR sample at 30 months of ripening. PR cheese samples from dairy C behaved differently with respect to the other two dairies. At the beginning of ripening, the total ACE-inhibitory peptide abundance declined, passing from the sample at 12 months of ripening to the sample at 18 months of ripening. As the ripening proceeded, the total ACE-inhibitory peptide abundance started to increase, reaching its highest value at 30 months of ripening.

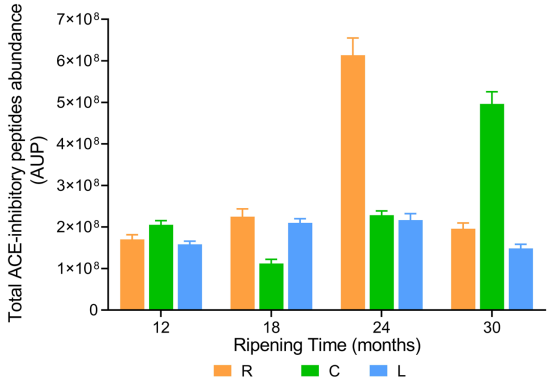

FIGURE 13
ACE-inhibitory peptide abundance in PR cheeses at different ripening times. Analysis was carried out on low-molecular-weight peptide obtained by ultrafiltration at 3 kDa from the water-soluble peptide fractions extracted from the different PR cheeses (dairy R, orange; dairy C, green; and dairy L, blue). Data are reported as the sum of the intensity of each identified ACE-inhibitory peptide measured as the area under the peak (AUP) by Skyline analysis. The complete list of identified ACE-inhibitory peptides and the semi-quantitative data can be found in Supplementary Table S9.

3.7 Effect of ripening time on bioactive peptide patterns

To investigate the effect of ripening time on patterns of bioactive peptides, we first attempted PCA analysis. PCA identified principal components (PC) 1 and 2 as suitable to explain more than 64% of data variability. However, no distinct clusters of samples were found in the score plot (Supplementary Figure S4A). The peptides with the highest loading values were YQEPVLGPVRGP with a positive load for both components 1 and 2, and RPKHPIKHQGLPQEVL with a positive loading for component 1 and a negative loading for component 2 (Supplementary Figure S4B).

Subsequently, we used the PLS-DA method to classify the samples according to the ripening time. PLS-DA analysis revealed that PR cheese samples can be divided into three clusters based on the first two PCs, explaining 34.7 and 16.4% of the total variance, respectively (Figures 14A,B). PC1 (horizontal plane) was found to have a major weight, resulting in the greatest differentiation of the samples along this plane. Two clusters clearly grouped samples with 18 months and 30 months of ripening, respectively, while the 12-month ripened samples were slightly further apart, especially when looking at PC1. The 24-month group was the most widely dispersed, especially along PC2. This can be explained by the high inter-dairy variability of PR samples, which resulted from a homemade cheese-making process.

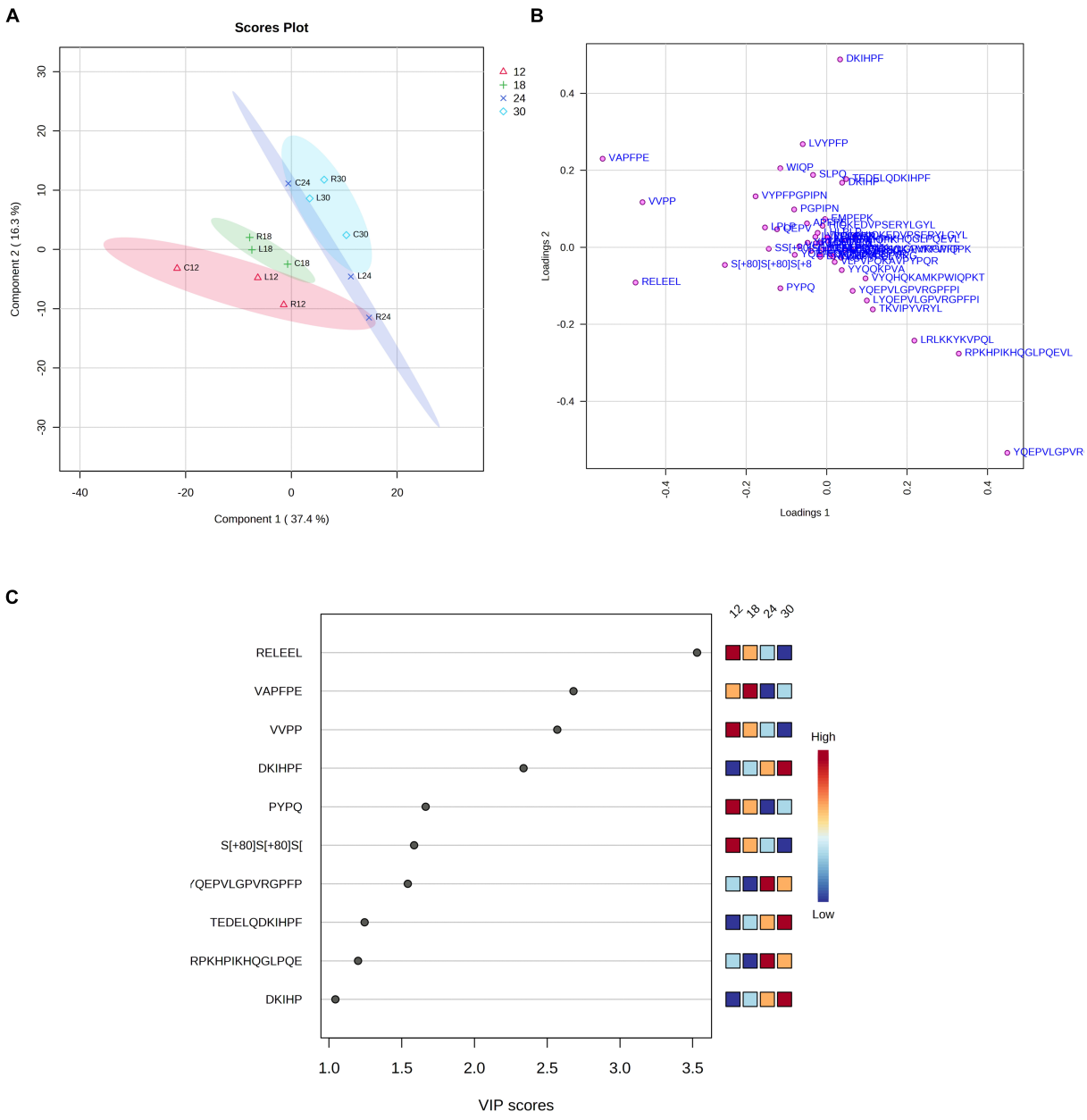

FIGURE 14
Partial least squares discriminant analysis (PLS-DA) of the bioactive peptide profile from dairies C, L, and R. Scores **(A)** and loadings **(B)** were analyzed at different ripening times (12, 18, 24, and 30 months). **(C)** Bioactive peptides with a VIP score > 1 were found throughout cheese ripening by PLS-DA analysis.

The VIP score graph generated by the PLS-DA analysis shows the 10 peptides with a VIP score > 1.0, which mostly contributed to differentiate samples into clusters and were highly present in these samples (Figure 14C). Among these 10 bioactive peptides, we found that the peptides RELEEL (antioxidant peptide), VAPFPE (cholesterol-lowering peptide), VVPP (ACE-inhibitory peptide), DKIHPF (ACE-inhibitory peptide), and PYPQ (antioxidant peptide) displayed the highest VIP values. RELEEL, VVPP, and PYPQ were present in large quantities at the beginning of the ripening process and decreased over time, while DKIHPF, TEDELQSKIHPF (anti-microbial peptide), and DKIHP (ACE-inhibitory peptide) exhibited an opposite trend, with the highest concentration in samples at 30 months of ripening.

3.8 Data correlation

The composition, microbiological data, and intensity of the 49 bioactive peptides identified in cheese samples were analyzed by Spearman rank correlations (Supplementary Figure S5). A positive significant correlation ($p < 0.05$) was found between *Lcb. zeae* and eight bioactive peptides, suggesting that the presence of *L. zeae* is pivotal for the release of these bioactive peptides. Most of these peptides were anti-microbial, whereas two had ACE-inhibitory activity. On the contrary, *L. paracasei* was negatively correlated with six out of eight bioactive peptides and was positively correlated with *L. zeae*.

Furthermore, each of the species, *Lcb. Rhamnosus*, *Lcb. Casei*, and *Lcb. Paracasei*, was positively correlated with one specific bioactive peptide (*L. rhamnosus* with the ACE-inhibitory peptide ENLLRF; *Lcb. casei* with the ACE-inhibitory peptide NLHLPLPLL; and *Lcb. paracasei* with the ACE-inhibitory peptide LPLP).

4 Discussion

Molecular ecological surveys of the LAB communities have revealed a remarkable degree of bacterial diversity in dairy products. This is especially true for artisanal raw cow milk cheeses, such as PR cheese. Recently, Fontana et al. (2023) found a huge genomic variability in the cheese microbiota, supporting the genetic uniqueness of the strains used in producing different types of PDO cheeses. Local variables on a microgeographical scale, such as temperature and humidity levels, as well as changes in technological parameters and variations in milk quality, ultimately cause fluctuations in the final organoleptic features of the dairy product. Currently, quantitative estimation of these effects is still lacking, and there is a dearth of information on how culturable microbial diversity contributes to biofunctionalities *in vivo*.

This study investigated culturable LAB microbiota in PR cheeses at different ripening times with the aim of establishing a link between cultivable species composition and peptidomic patterns, with a special regard for bioactive peptides. According to PDO guidelines, PR cheese-making entails the practice of back-slopping, where a small portion of the previous batch of sweet whey is fermented to develop the new NWS required for the next fermentation step of raw milk without adding commercial bacterial starters. NWS microbial composition is of great importance in PR cheese-making, as it determines the acidification rate and significantly impacts the overall quality of the final cheese. Three dairies included in this study exhibited remarkable diversity in NWS microbial composition, which significantly affected the subsequent cheese-making steps. Different from the previous studies that reported *S. thermophilus* as a minor species in NWS (Rossetti et al., 2008; Rossi et al., 2012; Santarelli et al., 2013; Morandi et al., 2019), lactococci thermophilic counts were comparable with lactobacilli counts in R and C and even higher than lactobacilli counts in L dairy. Furthermore, lactobacilli counts were generally lower than those found by Gatti et al. (2006) in NWS for Grana Padano cheese under the same culture conditions but comparable with those found by Morandi et al. (2022). Remarkably, *S. thermophilus* was the only species recovered in all three samples. While *L. helveticus* and *S. thermophilus* co-dominate in R and L samples, *L. helveticus* was replaced by *L. delbrueckii* subsp. *lactis* in C. A drastic reduction of *L. delbrueckii* subsp. *lactis* has been recently described in Grana Padano and Trentin Grana NWS (Morandi et al., 2019; Mancini et al., 2021). This species was described as more sensitive than *S. thermophilus* and *L. helveticus* to anti-microbial agents used for equipment cleaning (Morandi et al., 2022). Therefore, dairy-to-dairy differences in cleaning procedures can explain the variable presence of *L. delbrueckii* subsp. *lactis* in NWS. Other reasons for the huge diversity in NWS microbial composition can be related to technological parameters that have changed over space and time, such as temperature-decreasing curves and milk quality. We also consider that NWS microbiota is hard to be cultivated out of the whey environment (Fornasari et al., 2006; Sola et al., 2022). One of the reasons could be the presence of peptides and growth factors in whey, which are lacking in microbiological media. To improve the spectrum of the culturable NWS fraction, we used four different growth conditions. Accordingly, M17 supplemented with SSW mimics a better NWS environment than M17 medium and significantly improves the recovery of streptococci. Another reason for the low cultivability could be the cross-feeding interactions existing in the NWS community, which hamper the microbial growth of axenic cultures (Sola et al., 2022). We cannot exclude that the low cultivability of NWS microbiota could distort the species abundance detected by a culturable approach.

Dissection of the culturable fraction at the strain level confirmed the previous observation that NWS is a complex community composed of several biotypes belonging to a few species (Sola et al., 2022). It is puzzling how this microbial diversity is maintained through several rounds of back-slopping. Recently, phage predation has been recognized as a key evolutionary force driving microbial diversity in natural starter cultures (Mancini et al., 2019; Somerville et al., 2022). The occurrence of several biotypes could make NWS more resilient to phage attacks than dairy starter cultures composed of a few strains or mono-strain dairy starters (as reviewed by Zotta et al., 2022). According to the "kill the winner" model, virulent phages predominantly prey on fast-growing bacteria, thereby suppressing the competitive exclusion of slower-growing bacteria in natural communities (Somerville et al., 2022). This model could explain how NWS diversity remains high over time.

Based on our results, the NWS population was completely replaced by the NSLAB fraction in PR cheese. This confirmed previous studies that reported the replacement of NSLAB over SLAB in the second month of ripening (Solieri et al., 2012; Gatti et al., 2014). Recently, metabarcoding analysis revealed the presence of SLAB in hard-cooked cheeses such as Grana Padano, suggesting that

thermophilic NWS microbiota could enter a viable but not cultivable state (VBNC) when pH decreased and lactose was almost completely depleted (Zago et al., 2021). Even if SLAB disappeared by a culture-dependent approach, studies on mesophilic dairy starter suggested that VBNC lactococci are metabolically active in cheese, and their contribution to the cheese flavor increases in a non-growing state, for example, by expressing genes involved in the production of the flavor compounds such as diacetyl and dimethyl disulfide (Decadt and De Vuyst, 2023). While it is well known that lysate SLAB contribute to flavor and support NSLAB growth (Gatti et al., 2014), the presence of viable SLAB in PR cheese as well as the contribution of VBNC SLAB to PR quality have not been investigated yet.

It is well known that SLAB are more sensitive to low a_w values than NSLAB. Therefore, low values of moisture and high salt content found in PR samples were expected to inhibit SLAB growth in favor of NSLAB (Beresford et al., 2001). Microbial counts collected from PR cheeses were consistent with previous studies on PR cheese at the same time of ripening (Bottari et al., 2020). As expected, after 30 months of ripening, low a_w, high salt concentration, and nutrient depletion induce NSLAB cells to die and undergo spontaneous autolysis (Gatti et al., 2014). It has been well established that the main NSLAB present in hard-cooked, long-ripened Grana-type cheeses are *Lacticaseibacillus* spp., followed by *Lactiplantibacillus plantarum* (Gobbetti et al., 2015). In case of raw milk-based cheese, the raw milk microbiota acts as a primary inoculation source, resulting in a higher diversity of NSLAB compared with pasteurized milk cheeses (Vann Hoorde et al., 2010). Other sources of NSLAB are animal rennet and facility equipment, which provide the so-called "in-house microbiota" (Bokulich and Mills, 2013). LCG taxonomy is quite complicated by the high relatedness of species conventionally attributed to this cluster. Recently, the establishment of novel species within the genus *Lacticaseibacillus* further enhanced this complexity (Zheng et al., 2020), and consequently, metabarcoding studies often failed to resolve LCG at the species level (Fontana et al., 2023). Among the 17 species currently attributed to the *Lacticaseibacillus* genus, the most related to LGC is *Lcb. zeae* followed by *Lacticaseibacillus chiayiensis*, a species mainly isolated from meat. Here we developed an improved multiplex PCR assay to fast and accurately discriminate the dairy species *Lcb. casei*, *Lcb. paracasei*, *Lcb. rhamnosus*, and *Lcb. zeae*. Due to this assay, we observed that *Lcb. paracasei* and *Lcb. zeae* were dominant at a long ripening time. To the best of our knowledge, this is the first time that *Lcb. zeae* has been isolated from PR cheese. Even if more samples are required to corroborate this observation, the results suggested that *Lcb. paracasei*, followed by *Lcb. zeae*, could be the most adapted species to live in long-ripened PR wheels. Accordingly, *Lcb. paracasei* has been described as a species well adapted to survive under multiple stresses, i.e., no lactose, low pH, low a_w values, and high NaCl concentrations (Neviani et al., 2013). The low presence of *Lcb. rhamnosus* detected in this study partially disagreed with Tagliazucchi et al. (2020) who found *Lcb. rhamnosus* as a dominating species in PR wheels at 12 months of ripening. The high abundance of *Lcb. paracasei* and *Lcb. zeae* species at the highest ripening time could have a great impact on the technological and organoleptic traits of PR cheese, as *Lcb. paracasei* is known to produce several sensorially active compounds (Bancalari et al., 2017; Stefanovic et al., 2018), while *Lcb. zeae* is characterized by high proteolytic aptitude (Vukotić et al., 2016). Proteolysis could contribute to adaptive response to multiple cheese-related stresses, and, accordingly, peptidase activity increases under acidic conditions and carbohydrate depletion (Piuri et al., 2003; Papadimitriou et al., 2016). Similar to *L. helveticus* and *Lactococcus* spp., most dairy *Lacticaseibacillus* strains have CEPs highly efficient in releasing peptides and free amino acids (Solieri et al., 2022). *Lacticaseibacillus* CEPs are less inhibited by the low pH and high salt concentrations compared with lactococcal proteases (Minervini and Calasso, 2022). Among LCG, *L. zeae* strain LMG17315 was reported to possess three copies of CEP-encoding *prt* genes (Vukotić et al., 2016). Based on the previously observed reduction of viable counts over ripening time, we can speculate that CEPs and the intracellular aminopeptidase were released from NSLAB cells into the surrounding cheese matrix, contributing to the peptidomic profile of cheese.

The peptidomic profile of cheese strongly impacts the sensorial features, texture, and biofunctionalities of PR and is strongly related to the composition of SLAB and NSLAB populations. Concerning the general peptide profiles, more than 600 unique peptides were found in cheese samples from dairy C and R and more than 520 unique peptides from dairy L. This study unveiled a higher number of peptides identified in ripened PR cheeses than previously reported. Previous studies identified less than 300 peptides in ripened PR cheeses (Bottari et al., 2020; Martini et al., 2021). Therefore, the current study presents the most detailed evaluation of the PR peptidome to date.

The semi-quantitative analysis of the total peptide intensity (Figures 11, 12) pointed out that β-casein is the preferred hydrolyzed protein by the LAB proteases at the beginning of ripening by promoting the release of peptides at high concentrations, as already suggested by Bottari et al. (2020). Furthermore, as the ripening proceeds, β-casein-derived peptides are further hydrolyzed by LAB proteases into small peptides and/or amino acids or transported inside the cells. The increasing percentages of incidence of the peptide intensity for αS1-casein and αS2-casein during ripening suggest that these proteins are cleaved slowly and with less efficacy by LAB proteases, and their hydrolysis requires a longer ripening time compared with β-casein.

The semi-quantitative analysis of the individual peptide intensity revealed that peptides from the N-terminal region 1–28 of β-casein were easily released at the beginning of the ripening time. Previous studies found that these poly-phosphorylated peptides started to be produced during the first month of ripening of PR cheese and reached their maximum amount after 12 months of ripening (Bottari et al., 2020). These peptides are derived from the hydrolysis operated by LAB CEPs at the initial phases of PR manufacturing and ripening. Most identified poly-phosphorylated peptides had K_{28}, N_{27}, or R_{25} as C-terminal amino acids. Accordingly, CEPs isolated from several LAB, such as *Lcb. casei*, *Lcb rhamnosus*, *L. helveticus*, and *S. thermophilus*, recognize peptidic bonds between K_{28}-K_{29}, N_{27}-K_{28}, and R_{25}-I_{26} as common cleavage sites (Solieri et al., 2018; Ji et al., 2021). Next, intracellular LAB aminopeptidases may be responsible for the shortening of these peptides at the N-terminus. Some of the smallest identified poly-phosphorylated peptides presented a biphasic behavior. Their intensity was highest at 12 months of ripening, then decreased until 24 months of ripening, and finally increased again in the 30-month ripened PR cheeses. This is probably due to the massive LAB death observed after 30 months of ripening, which resulted in the release of a high number of active aminopeptidases in the cheese.

Differently, the intensity of peptides from the region 38–88 of β-casein continuously increased during ripening. Most of these

peptides were precursors for the anti-hypertensive lactotripeptides VPP and IPP, as well as for β-casomorphins. This region contains numerous cleavage site characteristics of CEPs, and therefore, their release is probably a consequence of the action of bacterial CEPs. Long peptides released during the first phases of ripening are further shortened by the action of the CEPs themselves or by intracellular endopeptidases/aminopeptidases (Ji et al., 2021). Similar behavior was observed for peptides from the C-terminal of β-casein. It has been previously reported that most of the characterized LAB CEPs displayed a marked preference for hydrolyzing the C-terminal region of β-casein and can be responsible for the continuous release of these peptides during ripening (Monnet et al., 1992; Lozo et al., 2011; Ji et al., 2021; Solieri et al., 2022).

Regarding the peptides released from the N-terminal portion of αS1-casein, they can be easily liberated during curding and in the first hours after curding, thanks to the action of chymosin, as well as during ripening by lactic acid bacteria CEPs due to the presence of numerous cleavage sites for CEPs (Sforza et al., 2012; Ji et al., 2021; Solieri et al., 2022; Helal et al., 2023). Most of these peptides reached their highest intensity after 18 or 24 months of ripening. Differently, the region between residues 38 and 80 of αS1-casein, rich in phosphorylation sites, has been suggested to be less susceptible to the hydrolysis by CEPs, and these peptides reached the highest amounts after 30 months of ripening (Sforza et al., 2012; Ji et al., 2021; Solieri et al., 2022; Helal et al., 2023). A similar behavior was observed for peptides released from the C-terminal region of αS1-casein, as previously suggested (Sforza et al., 2012; Ji et al., 2021; Solieri et al., 2022; Helal et al., 2023).

Among the identified peptides, 49 had previously demonstrated biological activities, and most of them were ACE inhibitors. Five ACE-inhibitory peptides identified in PR cheese samples were proven to exert *in vivo* anti-hypertensive effects in spontaneously hypertensive rats (SHR). In detail, the αS1-casein-derived peptide AYFYPEL and the β-casein-derived peptides YPFPGPIPN, LHLPLP, LPLP, and KVLPVPQ were able to decrease blood pressure in SHR to values between 7 and 31.5 mmHg (Tagliazucchi et al., 2019). Moreover, the peptides AYFYPEL and YPFPGPIPN were detected in the bloodstream of human healthy volunteers after consumption of pasteurized milk, suggesting their possible effect also in human subjects (Caira et al., 2022).

Chemometric analysis allowed us to define bioactive peptide biomarkers mostly associated with every ripening time and to establish a list of 10 peptides significantly affected by ripening. Correlation analysis also supported the positive relationship between the recovery of *Lcb. zeae* and the occurrence of eight bioactive peptides with anti-microbial and anti-hypertensive activity. This agrees with the above-mentioned proteolytic aptitude of this species. Interestingly, two β-casein-derived peptides, positively associated with *Lcb. zeae*, such as LLYQEPVLGPVRGPFPIIV and YQEPVLGPVRGPFPIIV, displayed low IC_{50} values against ACE (24 and 101 μmol/L, respectively) and have been demonstrated to decrease blood pressure in SHR (Yamamoto et al., 1994). Significantly, these peptides were found in human plasma after the intake of milk or PR cheese (Caira et al., 2016, 2022).

In conclusion, we depicted the interplay between the microbial cultivable fraction, the peptide profile, and the associated biofunctionalities in PR cheese. We demonstrated that the NSLAB cultivable fraction significantly contributes to the release of bioactive peptides in PR. The positive correlation between *Lcb. zeae* and the presence of bioactive peptides with anti-microbial and anti-hypertensive activities suggests that different species patterns can affect the biofunctionalities of PR cheese. In future, the knowledge of parameters affecting a given species pattern can assist in the improvement of bioactive peptide content in cheese. The wide portfolio of SLAB and NSLAB strains isolated from NWS and PR cheese, respectively, could be useful to select anti-hypertensive adjunct cultures for functional fermented food. Finally, the strain collection established here could be used in future studies with synthetic communities in controlled environments to understand how biotic and abiotic parameters affect the observed patterns of microbial and peptidomics diversity.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

SM: Writing – review & editing, Methodology, Investigation. LaS: Writing – review & editing, Methodology, Investigation, Data curation. AC: Writing – review & editing, Software, Data curation. MC: Writing – review & editing, Investigation. VP: Writing – review & editing, Investigation, Conceptualization. DT: Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Conceptualization. LiS: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Conflict of interest

VP was employed by Consorzio del Formaggio Parmigiano Reggiano.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1342180/full#supplementary-material

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