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Microbial diversity analysis and isolation of thermoresistant lactic acid bacteria in pasteurized milk

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Pasteurization is a common method for dairy products, typically heating at 72 °C for 15 s or 63 °C for 30 min. The 17 samples of commercial pasteurized milk were divided into three groups according to the shelf life: group A (1–5 days), group B (6–10 days) and group C (11–15 days), and the diversity composition of microbial communities in the samples was analyzed. Among all groups, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* were the dominant bacterial phyla. The lactic acid bacteria (LAB) were mostly *Streptococcus*, *Weissella* and *Lactobacillus*, and there were high proportions of *Streptococcus thermophilus* in group A, *Weissella paramesenteroides* in group B, and *Lactobacillus plantarum* in group C. Furthermore, a strain of *Enterococcus faecium* SFM2 was isolated from the A2 sample, which showed better temperature tolerance compared to the *E. faecium* SFM1 of oral origin. After treatment at 50 °C for 2 h, the survival rates of *E. faecium* SFM1 and SFM2 were 28.20 ± 0.04% and 82.58 ± 0.01%, respectively. This study investigated the diversity of microorganisms in pasteurized milk, providing effective information for analyzing the potential microbiota of commercial pasteurized milk. Meanwhile, it provided new ideas for expanding the resource pool of thermoresistant LAB.

Keywords Microbial diversity, Pasteurized milk, Thermoresistant, Lactic acid bacteria

As one of the most widely consumed beverages, milk contains various essential nutrients like proteins and vitamins. Meanwhile, the high nutrient content provides an ideal environment for the growth of many microorganisms, including lactic acid bacteria (LAB). The analysis of the biodiversity in the milk environment and the isolation and identification of microorganisms from milk are of great scientific significance, and there are many studies have analyzed the microbial populations in different raw milk. For instance, Yuan et al.[1] analyzed the variation in raw milk microbiota from three farms by 16S rRNA amplicon sequencing, providing new information on the ecology of raw milk microbiota at the farm level and contributing to the understanding of the variation in raw milk microbiota in China. Luoyizha et al.[2] sequenced the bacterial populations in donkey milk from different regions and revealed possible harmful pathogens and probiotics, which also provided a new way for potential future use of microbial resources.

In order to reduce the number of live microorganisms in raw milk, liquid dairy products are usually sterilized by ultra-high temperature sterilization (UHT) treatments and pasteurization. A typical UHT treatment is 135–150 °C for 1–10 s, which inactivates almost all microorganisms, and the treated product could be stored at room temperature for 6–9 months[3]. The low temperature long time (LTLT, 63 °C, 30 min) treatment and high temperature short time (HTST, 72 °C, 15 s) treatment are two common methods of pasteurization[4]. Pasteurization could effectively eliminate the vast majority of microorganisms in dairy products, but some heat-resistant microorganisms still survive after treatment. Khalil et al.[5] reported that after pasteurization, the content of *Enterobacteriaceae* bacteria in goat milk decreased significantly, but there were still some LAB in the samples, such as *Lactobacillus kefiranofaciens* and *Lactobacillus kefiranofaciens*. Rashtchi et al.[6] found that the number of *Escherichia coli* in cheese prepared with pasteurized milk as raw material was significantly lower than that in cheese fermented with raw milk, while the number of LAB decreased significantly in the early fermentation period and remained stable after that. Müller et al.[7] conducted 16S rRNA sequencing on pasteurized and UHT milk, which showed that there was still a higher level of LAB in milk after pasteurization, while the content of LAB in milk after UHT was very low. Because there are still some heat-resistant microorganisms in pasteurized samples, the enrichment of thermoresistant LAB can be realized after the treatment of this method[7,8].

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LAB are generally accepted as a group of Gram-positive, nonmotile, non-spore-forming bacteria that can ferment hexose carbohydrates under microaerophilic to strictly anaerobic conditions to produce mainly lactic acid[9]. LAB are widely distributed in milk, vegetables, and meat products. Some strains of LAB have potential probiotic effects, including regulating blood pressure, improving lactose intolerance, and lowering serum cholesterol[10]. LAB are usually prepared into bacterial powder by freeze-drying or spray-drying for applications in food, medicine and other fields. The productivity of freeze-drying technology is one-fifth that of spray-drying[11], and its high cost and long drying time limit its application[12]. In contrast, the cost of spray drying is more than 10 times lower than that of freeze drying, and it has the advantages of rapid, efficient and continuous production[13]. However, the high temperature during spray drying will reduce the survival rate of LAB[14]. In addition, the high-temperature environment in which *Lactobacillus plantarum* and *Lactobacillus fermentum* et al. participate in various fermentation processes also has a certain impact on the activity of LAB, thus affecting the quality of fermented food. Therefore, it is necessary to obtain thermoresistant LAB. Ramírez-Chavarín et al.[15] and Pérez-Chabela et al.[16] reported that a variety of natural thermoresistant LAB are applied to the preservation and fermentation of meat products as biological protective agents, which can improve the safety of cooked meat products. Chen et al.[17] suggested that the improved thermostable *Lactobacillus plantarum* can also be used as a silage additive to promote lignocellulose degradation and improve fermentation quality.

The main means to improve the heat resistance of LAB are mutagenesis treatment or adding protective agents. Although the success rate of ultraviolet mutagenesis or other mutagenesis techniques is high, the experiment period is long and the operation is complicated. The cost of various types of protective agents is slightly higher, and their safety is still to be verified. Using pasteurized milk as a sample to isolate thermoresistant LAB, this method does not require a long domestication process or gene editing, thus it can be used in the food industry. In this study, 17 kinds of commercial pasteurized milk were divided into three groups according to the shelf life: A (1–5 d), B (6–10 d), and C (11–15 d). The sequence information of the 16S rRNA V3–V4 region was amplified by molecular biological methods to analyze the relationship between bacterial diversity and shelf life. Furthermore, microbial isolation and culture technology were used to screen potential heat-resistant LAB in pasteurized milk and determine their thermoresistant properties. The objective of this study was to enrich the biodiversity information of pasteurized milks, thus providing a new way and idea for enriching the resource base of heat-tolerant LAB.

Materials and methods

Sample collection and preparation

Pasteurized cow milk was purchased from local supermarkets in Jinan, Shandong, China. The products originate from Beijing, Tianjin, Shanghai, Inner Mongolia Autonomous Region, Jiangsu Province, Zhejiang Province, Guangdong Province, Sichuan Province, Hubei Province, Shandong Province, Anhui Province, Henan Province, and Heilongjiang Province. The samples were placed into sterile tubes in the laboratory for metagenomics DNA extraction as follows. Pasteurized cow milk of 2 mL was mixed thoroughly and centrifuged at 10,000×g for 10 min. The supernatant was discarded, and genomic DNA was extracted from the remaining pellet using the Easy-DNA-Extraction Kit (Invitrogen, California, USA). The extracted DNA was quantified using a Qubit 2.0 spectrometer (Invitrogen, California, USA). PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 250 bp sequencing was performed using the Illlumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

Sequence analysis

Microbiome bioinformatics were performed with QIIME2 2019.4[18] with slight modification according to the official tutorials (https://docs.qiime2.org/2019.4/tutorials/). Briefly, raw sequence data were demultiplexed using the demux plugin following by primers cutting with cutadapt plugin[19]. Sequences were then quality filtered, denoised, merged and chimera removed using the DADA2 plugin[20]. Non-singleton amplicon sequence variants (ASVs) were aligned with mafft[21].

Isolation and identification of microorganisms in pasteurized milk

The pasteurized milk samples were inoculated (20%, v/v) into LB and MRS liquid medium (Haibo, Qingdao, China), respectively. After cultivation for 24 h, 1 mL of the sample was mixed with 99 mL of sterile distilled water. Appropriate dilutions of 10^{-1} to 10^{-6} were carried out in the sterilized saline water. The LAB and other bacteria were isolated on the MRS and LB agar medium, respectively. After incubation at 37 °C, the predominant, morphologically distinct, and well-isolated colonies were transferred to the corresponding medium for further enrichment.

The strains were cultured in the MRS and LB medium at 37 °C for 24 h, after which the cells of the strains were harvested by centrifugation and the genomic DNA of the strains was extracted using a genome DNA isolation kit (Vazyme, Nanjing, China). Further, the 16S rRNA gene was amplified using the universal primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR was performed by applying the 2× SanTaq PCR Mix (Yeasen, Shanghai, China). For the amplification reaction, 1 mixture was prepared in a 200-μL tube that contained 9.5 μL of sterilized ddH$_2$O, 1 μL of primer 27F, 1 μL of 1492R, 1 μL of genomic DNA, and 12.5 μL of 2× SanTaq PCR Mix. The PCR amplification program was performed as follows: denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min). Amplified 16S rRNA genes were sequenced by Sangon

Biotech Co., Ltd. (Shanghai, China), and the 16S rRNA gene sequence was identified using the BLAST program developed by the NCBI.

The optimum temperature and temperature tolerance of *Enterococcus faecium*

After overnight incubation, *E. faecium* SF1 and *E. faecium* SF2 were inoculated (2%, v/v) into MRS liquid medium, and statically cultured at 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C, respectively. After cultivation for 24 h, the OD_{600} values of *E. faecium* were measured by ultraviolet spectrophotometer. To determine the temperature tolerance of *E. faecium, E. faecium* SFM1 and *E. faecium* SFM2 were collected by centrifugation after 12 h of cultivation. The bacterial cells were suspended in an equal volume of PBS buffer solution (NaCl, 8 g/L; KCl, 0.2 g/L; Na_2HPO_4, 1.44 g/L; KH_2PO_4, 0.24 g/L, pH 7.4). After incubation at 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C for 2 h, the number of viable cell counts was counted to obtain the survival rates of the strain.

Safety evaluation

Hemolytic activity of *E. faecium* SFM1 and *E. faecium* SFM2 was assessed by streaking each isolate on Columbia CNA blood agar plates (Haibo, Qingdao, China). Plates were incubated at 37 °C for 24 h. Isolates surrounded by a transparent halo were classified as β-hemolytic, and those presenting no halo were classified as γ-hemolytic. *Staphylococcus aureus* ATCC6538 was used as a positive control (β-hemolytic).

The antimicrobial resistance profile of *E. faecium* SFM1 and *E. faecium* SFM2 was determined using the disc diffusion method. Resistance to penicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), vancomycin (30 µg), rifampicin (5 µg), ciprofloxacin (5 µg), and tetracycline (30 µg) was tested using discs from Oxoid, UK. The antibiotic tablets were placed on MRS plates containing 4.5×10^7 CFU/mL *E. faecium* and incubated at 37 °C for 24 h. The mean (M) and standard deviation (SD) of the inhibition halo diameter (x) of *E. faecium* SFM1 and *E. faecium* SFM2 were calculated for each antimicrobial, and isolates were classified as follows: $x \leq 15$ mm—resistant; 16 mm $< x <$ 20 mm—intermediate; $x \geq 21$ mm—susceptible.

Characterization of probiotic potential

The simulated gastric fluid and intestinal fluid were prepared using the method of Son et al.[22] with modification, and all digestive fluids were filtered through a 0.22-µm filter for sterilization. After overnight incubation, *E. faecium* SFM1 and *E. faecium* SFM2 were inoculated (2%, v/v) into MRS liquid medium and cultured statically at 37 °C, respectively. After cultivation for 12 h, 1 mL suspension of *E. faecium* SFM1 and *E. faecium* SFM2 was mixed with 9 mL of artificial gastric fluid (0.2% NaCl, 0.3% pepsin, pH 2.5), and incubated at 37 °C, respectively. After incubation for 1 h and 2 h, the cells were collected by centrifugation at 2000×*g* for 10 min, washed three times with PBS buffer, and then re-suspended in an equal volume of PBS buffer. 100 µL of the re-suspension was applied to MRS solid medium, the viable bacteria numbers were counted, and the survival rates of the strains in gastric fluid were calculated. Furthermore, 1 mL of the above resuspended cell solution was mixed with 9 mL of artificial intestinal fluid (0.1% trypsin, 0.6% bile salt, pH 8), and incubated at 37 °C for 8 h. The survival rates in artificial intestinal fluid were determined by the same method.

Determination of carbon sources of *Enterococcus faecium*

To detect the growth of *E. faecium* SFM1 (isolated from human oral) and *E. faecium* SFM2 (isolated from pasteurized milk) with different carbon sources, the glucose in MRS medium was replaced with 2% (w/v) fructose, sucrose, lactose, galactose, galacto-oligosaccharides (GOS), and stevioside to prepare MRS-fructose, MRS-sucrose, MRS-lactose, MRS-galactose, MRS-GOS, and MRS-stevioside medium. *E. faecium* SFM1 and SFM2 were inoculated (2%, v/v) into different kinds of MRS with different carbon sources cultured statically, and the sugar-free MRS was used as the control group. After cultivation for 24 h, the OD_{600} value was determined by a UV-5500 Ultraviolet–Visible spectrophotometer (Shanghai, China).

Statistical analysis

Each experiment was repeated at least three times. The results were statistically compared and analyzed with SPSS 22.0 software. This included calculating the mean and standard deviation of different parallel experiments. The p value was calculated by using the Student's t testing method.

Results

Sequence quality control

A total of 2,329,929, including 1,908,493 high-quality 16S rRNA gene sequences were obtained using 16S rRNA sequencing (Table S1). A total of 33,783,415 ASVs (amplicon sequence variants) were finally obtained using a 99% sequence similarity threshold. The Good's coverage was 99% on average, further indicating that it was sufficient for pasteurized milk microbiota analysis at the current sequencing depth.

Alpha and Beta diversities of milk microbiota based on shelf life

17 samples were divided into three groups according to the shelf-life: group A (1–5 days), group B (6–10 days), and group C (11–15 days). The α-diversity analysis of the three groups with different shelf life was shown in Fig. 1. Overall, bacterial community richness and diversity were generally higher in group A.

Changes in the microbial composition of pasteurized milk in different shelf life were compared by β-diversity analysis (Fig. 2A,B). The results showed that the microbial composition of group A differed significantly from that of group B and group C. PCoA at the genus level showed significant differences in microbial community composition between the three groups. Hierarchical cluster analysis revealed the similarities and differences among the three groups at the genus level (Fig. 3). It suggested that the community composition of group B and group C was similar, which was consistent with the analysis results of PCoA and NMDS. Hierarchical cluster

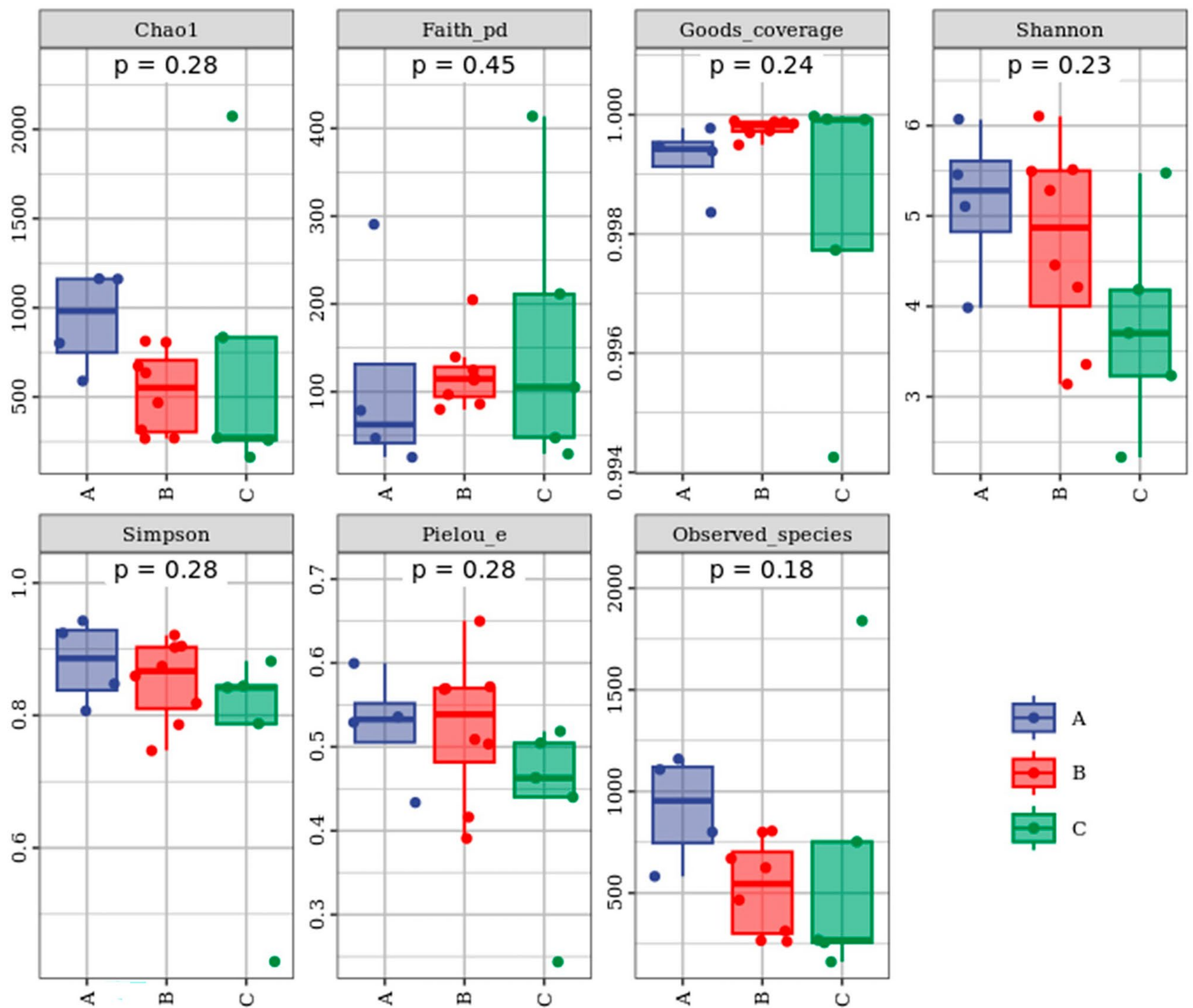

Fig. 1. Alpha analysis metrics of pasteurized milk according to different shelf lives. Richness of microbiota in pasteurized milk was evaluated according to Chao1, Observed species, bacterial evenness and community coverage were evaluated by Pielou's evenness, Shannon, and Simpson index. The evolutionary diversity and the degree of coverage were evaluated by Faith's PD and Good's coverage.

analysis represented the top 10 genera in the sample in terms of abundance, which revealed that *Pseudomonas*, *Flavobacterium*, and *Acinetobacter* in group A had a high abundance, while *Lactococcus* microbe was present in some samples. The species composition of group B and group C was similar, which mainly included *Ralstonia*, *Caulobacter*, *Bradyrhizobium*, *Sphingomonas* and other microorganisms at the genus level. PCoA, NMDS and hierarchical cluster maps were drawn to evaluate beta diversity. The results showed that the species composition of group A was significantly different from that of the other two groups, while the species composition of group B and group C was similar.

Differences in microbial composition of pasteurized milk between different shelf-life groups

As shown in Fig. 4A, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* were the dominant phyla, with relative abundance accounting for $94.54 \pm 4.22\%$ of the total bacteria. The most common classes in pasteurized milk were *Alphaproteobacteria*, *Gammaproteobacteria*, and *Flavobacteria* (Fig. 4B). At the family level, *Acetobacteraceae*, *Ralstonia*, and *Burkholderiaceae* were the dominant families (Fig. 4C). The most common genus in pasteurized milk were *Pseudomonas*, *Ralstonia*, and *Flavobacterium* (Fig. 4D). The relative abundance of microbial communities at the class, family, and genus levels varied significantly among samples with different shelf lives. The distribution of *Gammaproteobacteria* in group A samples was higher than that in group B and group C. The samples of group A contained little or no *Acetobacteraceae* and *Burkholderiaceae*, while the samples of group B and group C contained a higher number. The dominant bacteria in group A were *Pseudomonas* and *Flavobacterium*, while *Ralstonia* was the dominant bacteria in group B and group C. *Lactococcus* had the highest proportion in group A, and *Lactobacillus* had the highest number in group B and group C.

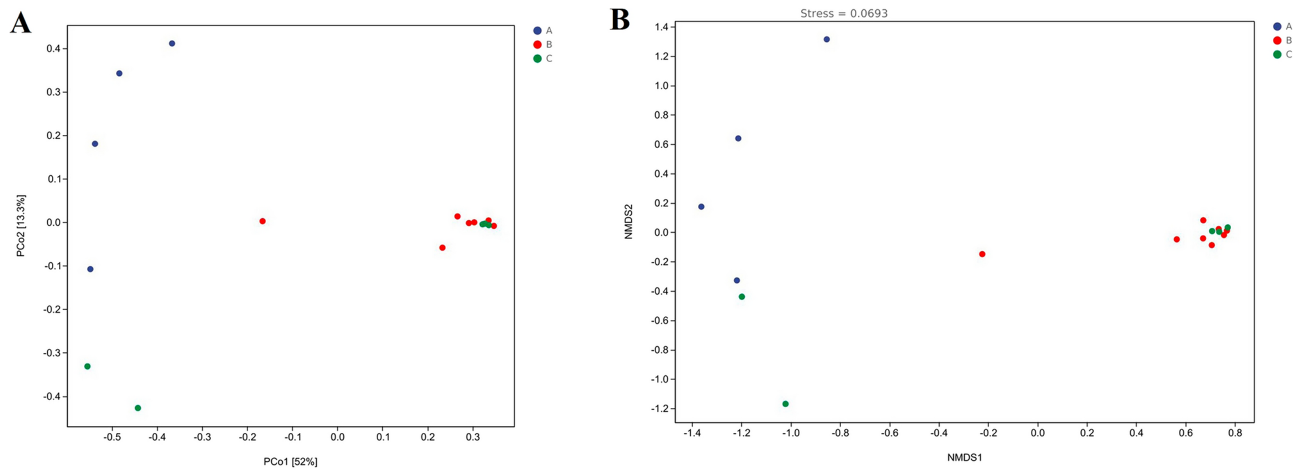

Fig. 2. Beta diversity was visualized with principal components analysis (PCoA) (**A**) and NMDS analysis (**B**). Dots with different colors represent the samples of different shelf lives. The horizontal and vertical axes represent the first and second principal coordinates, respectively, and the percentages on the horizontal and vertical axes are the contribution of that principal coordinate to the difference of the sample matrix data. The closer the projection distance between two points on the coordinate axis, the more similar the community composition.

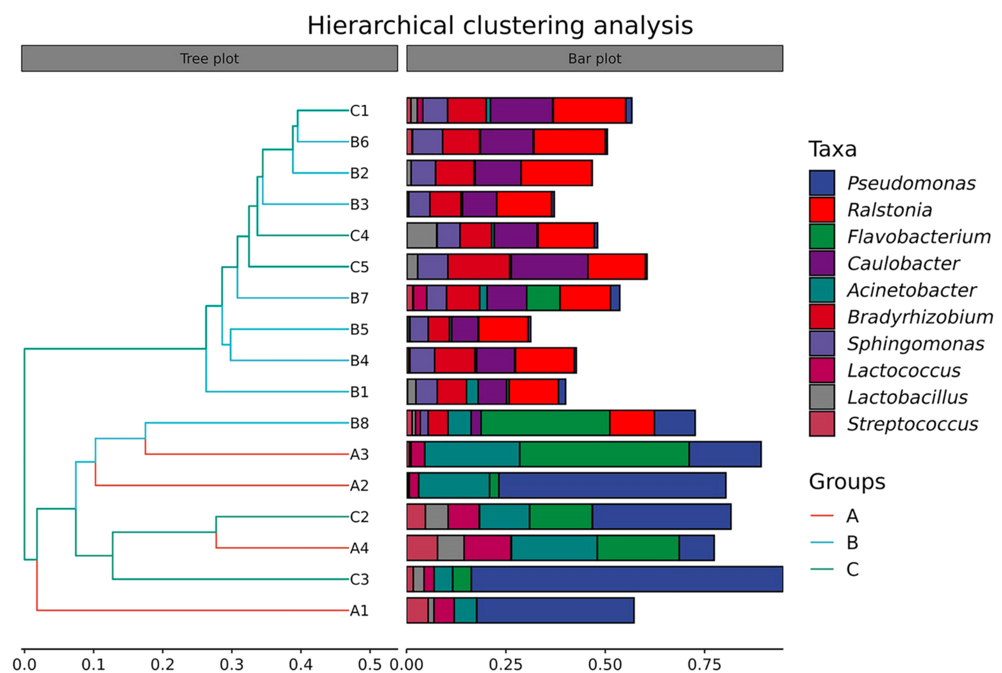

Fig. 3. Hierarchical clustering analysis. The similarity between samples was shown in the form of a hierarchical tree. The closer the projection distance between two branches, the higher similarity of the community composition between the samples.

The Krona species composition map showed the species distribution of LAB in the three groups of samples with different shelf lives. The microorganisms with the highest abundance in the three groups all included the genus of *Streptococcus*, and there was a high proportion of *Lactococcus lactis* and *Streptococcus thermophilus* present in group A samples (Fig. 5A). In group B, the microorganisms with a higher proportion were *Lactobacillus plantarum* and *Weissella paramesenteroides* (Fig. 5B). The *Lactobacillus plantarum* and *Weissella paramesenteroides* in group C samples accounted for a relatively high proportion (Fig. 5C).

Isolation and identification of bacteria in pasteurized milk

The 16S rRNA genes of isolated strains were amplified and sequenced for further genetic identification. The obtained 16S rRNA gene fragments were aligned via the BLAST program in NCBI, and the results indicated that

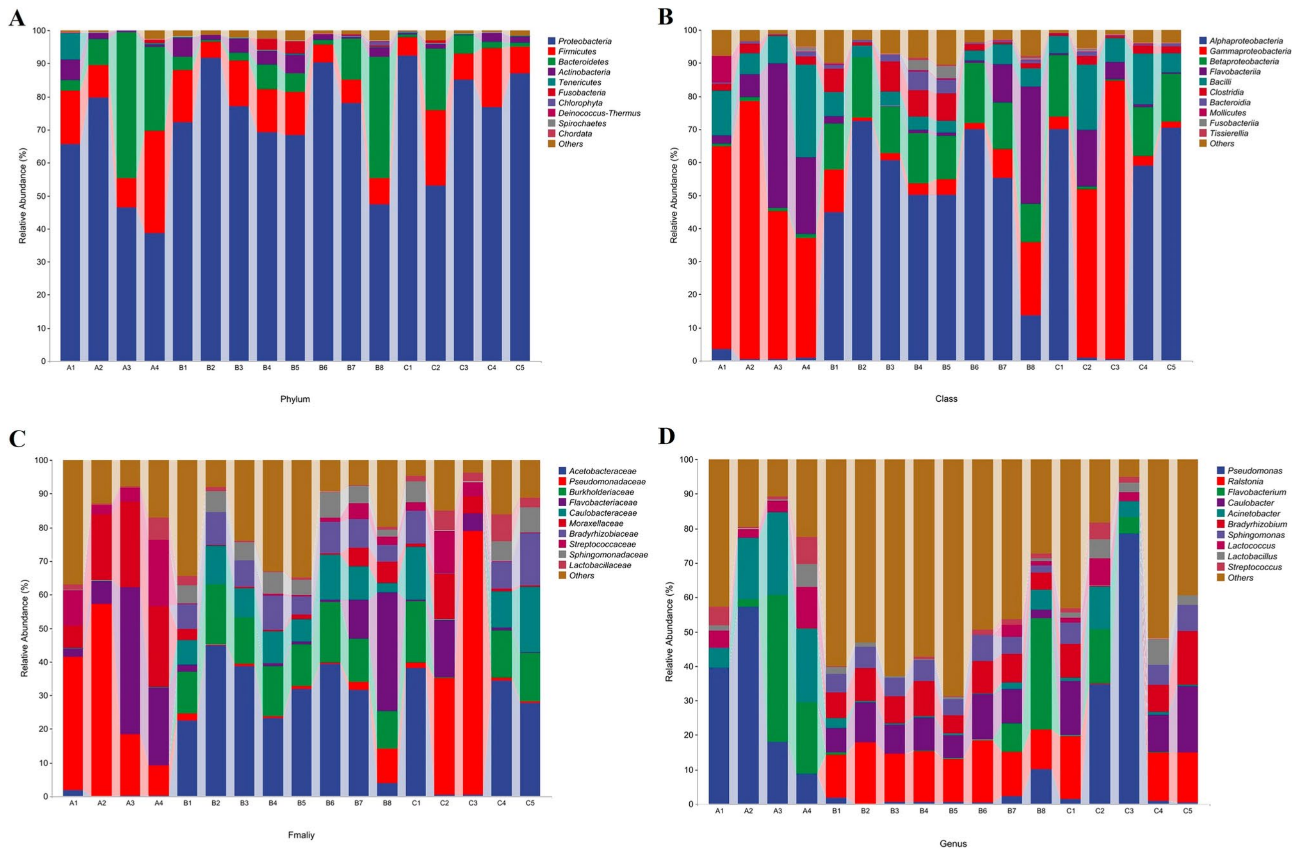

Fig. 4. Relative abundance of bacteria in pasteurized milk at the level of phylum (**A**), class (**B**), family (**C**), and genus (**D**). Genera of the top ten abundant microorganisms were listed and unclassified were marked as others.

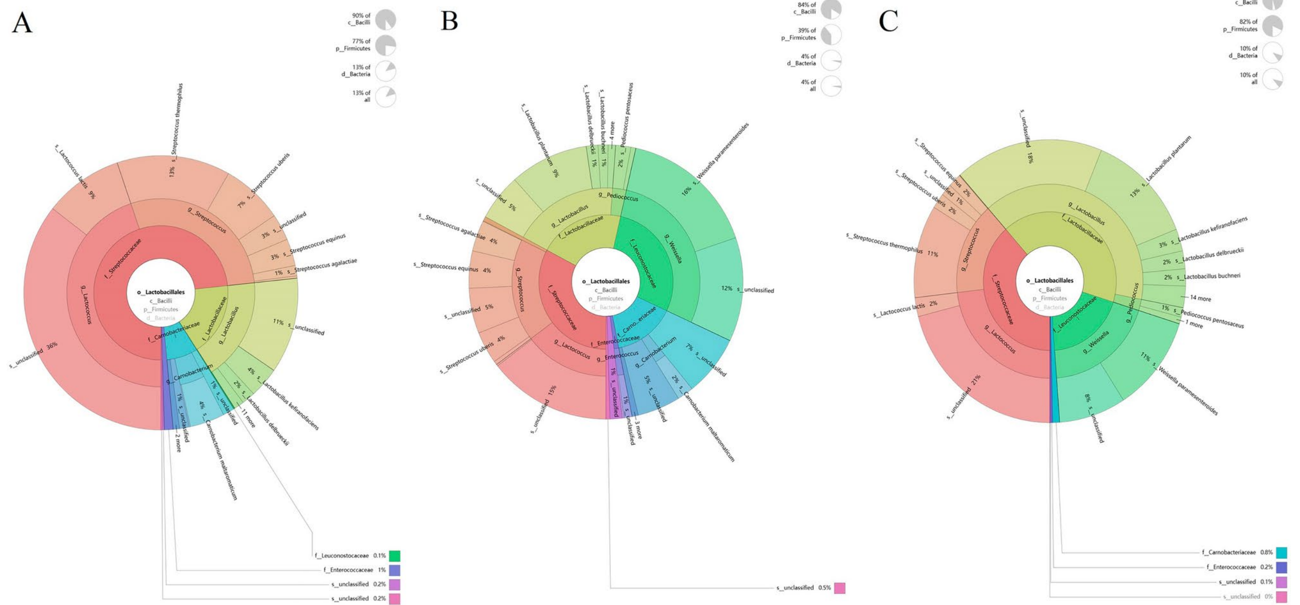

Fig. 5. The Krona species composition map of LAB in group A (**A**), group B (**B**), and group C (**C**) based on different shelf lives.

Staphylococcus saprophyticus, *Streptococcus parauberis*, *Enterococcus faecium*, and *Bacillus subtilis* were isolated from group A. *Staphylococcus*, *Enterobacter* and *Enterococcus* were mostly isolated from group B. In group C, *Enterobacter cloacae, Exiguobacterium acetylicum*, and *Microbacterium oxydans* were isolated (Table 1).

Heat resistance of *Enterococcus faecium*

In this study, a strain of *E. faecium* SFM2 was isolated from the A2 sample. Furthermore, the optimum temperature and temperature tolerance of *E. faecium* SFM2 were explored and compared with the human oral source *E. faecium* SFM1 preserved in our laboratory. The optimal temperature of *E. faecium* SFM1 and *E. faecium* SFM2 was 35 °C, and the viable cell counts of *E. faecium* SFM2 were higher than those of *E. faecium* SFM1 at different temperatures (Fig. 6A). The temperature tolerance of these two strains was determined at 50 °C. After incubation at 50 °C for 2 h, the survival rates of *E. faecium* SFM1 and *E. faecium* SFM2 reached 28.20 ± 0.04% and 82.58 ± 0.01%, respectively (Fig. 6B).

Safety evaluation and assessment of probiotic potential of *Enterococcus faecium*

The safety evaluation results showed that both *E. faecium* SFM1 and *E. faecium* SFM2 presented no halo in Columbia CNA blood agar plate, and were classified as γ-hemolytic (Fig. 7). Another important safety concern is the presence of mobile antimicrobial resistance genes. The antibiotic sensitivity of *E. faecium* to 7 commonly used antibiotics was examined. As shown in Table 2, *E. faecium* SFM1 was sensitive to penicillin, chloramphenicol, vancomycin, rifampicin, ciprofloxacin, and tetracycline, while *E. faecium* SFM2 was sensitive to chloramphenicol, vancomycin, and rifampicin.

Furthermore, the tolerance of *E. faecium* SFM1 and *E. faecium* SFM2 in simulated gastric fluid and simulated intestinal fluid was shown in Fig. 6C,D. The survival rates of *E. faecium* SFM1 and *E. faecium* SFM2 were 21.88 ± 0.03% and 79.31 ± 0.03% ($p < 0.001$) after gastric fluid treatment for 2 h. After 8 h of intestinal fluid treatment, the survival rates of *E. faecium* SFM1 and *E. faecium* SFM2 reached 67.19 ± 0.03% and 71.57 ± 0.02% ($p < 0.001$), respectively. Therefore, *E. faecium* SFM2 isolated from pasteurized milk represented better temperature as well as gastrointestinal tolerance.

The carbon sources of *Enterococcus faecium*

The utilization of different carbon sources by *E. faecium* SFM1 and SFM2 was shown in Table 3. The carbon source spectra of the two strains were similar, both showing the highest growth in sucrose. After cultivation for 24 h, the OD_{600} value of *E. faecium* SFM1 and *E. faecium* SFM2 reached 1.58 ± 0.00 and 1.77 ± 0.00 (< 0.05), respectively. In addition, compared with *E. faecium* SFM1, *E. faecium* SFM2 exhibited higher biomass in glucose, fructose, lactose, and galactose.

Discussion

Metagenomic sequencing is a commonly used method to analyze species diversity in samples. Nowadays, microbial diversity analysis is used to explore the microbiota of the milk environment, which provides the latest information on the changes in microbiota, the control of food-borne pathogens, and the discovery of beneficial microorganisms. Yuan et al.[1] used 16S rRNA amplicon sequencing to analyze the variation in raw milk microbiota throughout the year from three farms in China, providing new information on the ecology of the raw milk microbiota in pastures and contributing to understanding the changes in the Chinese milk microbiota. Luoyizha et al.[2] used high-throughput sequencing technology to determine bacterial communities in donkey milk from two counties in eastern and western China, providing the microbial profiles of pathogens and spoilage

Samples	Strains isolated in LB medium	Strains isolated in MRS medium
A1	*Staphylococcus saprophyticus*	*Streptococcus parauberis*
A2	*Enterococcus faecium*	–
A3	–	–
A4	*Bacillus subtilis*	–
B1	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*
B2	–	–
B3	*Enterobacter ludwigii*	*Enterobacter cloacae*
B4	*Enterobacter cloacae*	*Enterobacter cloacae*
B5	*Enterococcus gallinarum*	*Enterococcus casseliflavus*
B6	*Enterobacter cloacae*	–
B7	–	–
B8	*Enterobacter hormaechei*	*Enterobacter hormaechei*
C1	*Enterobacter cloacae*	–
C2	–	–
C3	–	–
C4	–	–
C5	*Exiguobacterium acetylicum*	*Microbacterium oxydans*

Table 1. The strains isolated from LB medium and MRS medium. "–" indicated that data was not detected.

Fig. 6. (**A**) The viable cell counts of *E. faecium* cultured at different temperatures for 24 h. (**B**) The survival rates of *E. faecium* treated at different temperatures for 2 h. (**C**) The survival rates of *E. faecium* in simulated gastric fluid. (**D**) The survival rates of *E. faecium* in simulated intestinal fluid. *E. faecium* SFM1 was represented by black solid lines, and *E. faecium* SFM2 was represented by red solid lines.

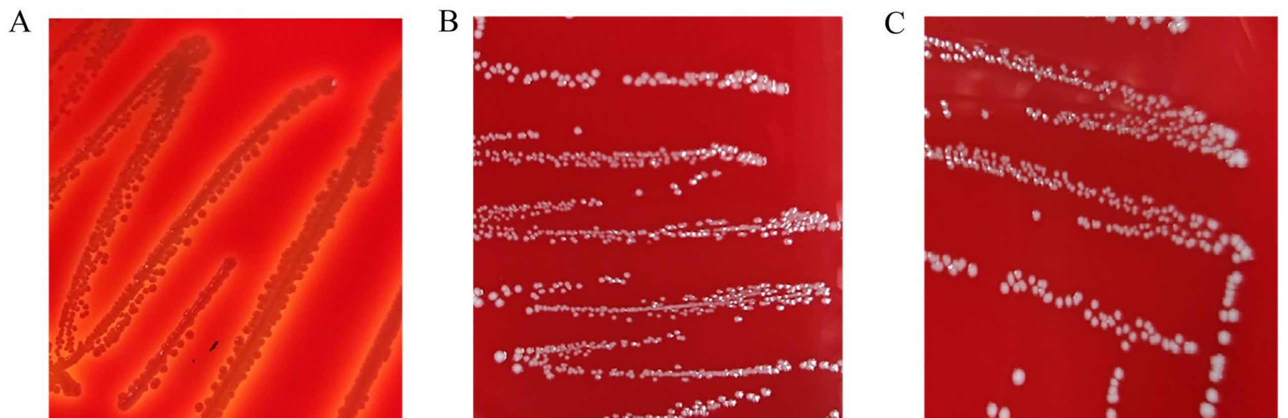

Fig. 7. Hemolytic activity of *S. aureus* ATCC6538 (**A**), *E. faecium* SFM1 (**B**), and *E. faecium* SFM2 (**C**).

Strains	Antibiotic	Bacteriostatic zone diameter (mm)	Antibiotic susceptibility
E. faecium SFM1	Penicillin	27.67 ± 0.58	S
	Chloramphenicol	28.67 ± 1.53	S
	Erythromycin	13.67 ± 1.53	R
	Vancomycin	22.67 ± 1.53	S
	Rifampicin	31.67 ± 1.15	S
	Ciprofloxacin	22.67 ± 1.15	S
	Tetracycline	28.67 ± 0.58	S
E. faecium SFM2	Penicillin	18.00 ± 1.00	I
	Chloramphenicol	26.67 ± 1.53	S
	Erythromycin	11.67 ± 0.58	R
	Vancomycin	22.67 ± 1.53	S
	Rifampicin	30.33 ± 0.58	S
	Ciprofloxacin	17.33 ± 0.58	I
	Tetracycline	12.33 ± 0.58	R

Table 2. Antibiotic susceptibility profile of *E. faecium* SFM1 and *E. faecium* SFM2. Values are presented as mean ± standard deviation of three independent experiments (n = 3). "S" represented sensitive, "I" represented intermediate, and "R" represented resistance.

	E. faecium SFM1	*E. faecium* SFM2
Control	0.79 ± 0.02^{i}	0.77 ± 0.02^{i}
Glucose	1.44 ± 0.02^{d}	1.67 ± 0.02^{b}
Fructose	1.21 ± 0.03^{f}	1.40 ± 0.00^{d}
Sucrose	1.58 ± 0.00^{c}	1.77 ± 0.00^{a}
Lactose	1.41 ± 0.06^{d}	1.69 ± 0.02^{b}
Galactose	1.06 ± 0.00^{g}	1.34 ± 0.05^{e}
GOS	1.36 ± 0.02^{e}	1.35 ± 0.01^{e}
Stevioside	0.98 ± 0.01^{h}	0.96 ± 0.03^{h}

Table 3. The utilization of different carbon sources by *E. faecium* SFM1 and *E. faecium* SFM2. Values are presented as mean ± standard deviation of three independent experiments (n = 3). Different lowercase superscript letters indicated statistically differences at $P < 0.05$.

bacteria that need to be controlled, and proposing the possible utilization of beneficial microbial resources for the future. Delgado et al.[8] assessed the microbial diversity of raw milk treated by pasteurization (63 °C, 30 min), and sequencing results showed that *S. thermophilus* accounted for more than 98% of the reads in IPM samples (incubation at 42 °C for 24 h after pasteurization). This study provided the possibility to isolate new thermophilic LAB strains from pasteurized milk. In our study, 16S rRNA amplification sequencing was performed on 17 commercial pasteurized milk samples with different shelf lives, and the distribution of microorganisms in pasteurized milk with different shelf lives provided effective information for analyzing the potential microbiota of pasteurized milk and controlling foodborne pathogenic microorganisms.

Pasteurization is usually heated at 62.5 °C for 30 min or at 72–75 °C for 15 s, and the shelf life of the sample may be related to the sterilization time and temperature. Therefore, we thought that the shorter the shelf life, the lower the pasteurization temperature or the shorter the processing time. The heat treatment temperature of group A samples with the shortest shelf life may be lower, and the viable cell counts of sample A4 were the highest, so some thermoresistant LAB (such as *E. faecium* SFM2) could survive. The shelf life of group C was the longest, and the content of pathogenic bacteria such as *Staphylococcus epidermidis* was significantly lower than that of group B. The results of microbial isolation and identification showed that the number of viable cell counts in 17 kinds of commercial pasteurized milk was less than 10^4 CFU/mL. In addition, there were two samples that did not meet the conditions for building a library and did not participate in the diversity analysis, which may be the samples that also used ceramic filtration technology after pasteurization, so that the number of bacteria in the product was lower than the standard of the database (data not shown).

The diversity of microorganisms in raw milk may come from a variety of sources, such as milk teats, milking equipment, feeding environment, feed and manure, and even after a short period of high-temperature treatment, a small amount of microorganisms may remain in the finished product[23]. *S. epidermidis* was isolated both in group A and group B. *S. epidermidis* is a normal human microbiota and is widely distributed on the skin surface and intestinal tract of humans and other animals, but it is also a conditional pathogen, easy to cause cross infection in hospitals, and a pathogen that causes mastitis in dairy cows[24], Fernandes[25]. The microorganisms isolated from group B samples mostly belong to the genera of *Enterobacter* and *Enterococcus*, such as *Ec.*

gallinarum and *Eb. cloacae*, which are widely present in human and animal feces and soil. Besides, they are a common contaminating strain of dairy products and are also pathogens causing mastitis in cattle[26,27].

LAB is a natural and important microbial community in the milk environment. Different studies have reported LAB in the milk environment. For example, camel milk is typically dominated by *Lactobacillus helveticus*, *Lactiplantibacillus plantarum* and *Lacticaseibacillus casei*[28], cow milk contains a significant LAB population that includes *Lactobacillus, Streptococcus* and *Enterococcus*[1], goat milk is also typically dominated by LAB, including genera of *Lactococcus* and *Lactobacillus*[29], buffalo milk contains a large population of LAB, including *Lactococci* and *Lactobacilli*[30], and LAB are also present in breast milk, including *Lactobacillus* and *Bifidobacterium*[23]. In this study, metagenomic sequencing results also showed that the cow milk contained a high abundance of LAB. In addition, LAB strains were also screened in culture experiments.

This study also provided a new idea for expanding the resource pool of thermoresistant LAB strains. The application range of thermoresistant LAB is very wide, leading researchers to obtain thermoresistant LAB strains through different methods. For example, Dorau et al.[31] used a setup where the temperature was gradually increased over time, and isolated two evolved *Lactococcus lactis* strains (RD01 and RD07) better able to tolerate high growth temperatures. Jeon et al.[32] developed a new *Lactobacillus acidophilus* strain with improved heat resistance while retaining the existing beneficial properties through the adaptive laboratory evolution (ALE) method. Yang et al.[33] found that Mg^{2+} at 10–50 mmol/L could improve the survival rate of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* under high-temperature environments. Gong et al.[13] and Moreira et al.[34] used skim milk powder, sucrose, etc. as protective agents to increase cell viability. In addition, gene editing could also improve the stress resistance of the strain. Desmond et al.[35] overexpressed *groEL* in *Lactobacillus paracasei* and *Lactococcus lactis,* and the recombinant strains were cultured at 60 °C and 54 °C, the heat-adapted cultures maintained the highest level of viability (5-log-unit increase, approximately). Through the results of this study, pasteurized milk is a good source for isolating thermoresistant LAB strains, and *E. faecium* SFM2 was obtained.

E. faecium is a type of LAB, widely present in the feces of healthy infants and long-lived elderly people, and it could be used as a starter culture or probiotic culture in animal feed additives[36]. Aspri et al.[37] isolated *E. faecium* from donkey milk, which showed good antibacterial activity against *S. aureus* and *Listeria monocytogenes*. Quintela-Baluja et al.[38] performed a proteomic analysis of two bacteriocin-producing *E. faecium* strains and found that they hold great promise in terms of bioengineering and biotechnology properties. Rajput et al.[39] isolated *E. faecium* GMB16 from goat and sheep milk, which showed significant probiotic potential and immunological properties. In this study, a strain of *E. faecium* SFM2 was isolated from the A2 sample. The safety evaluation results showed that *E. faecium* SFM2 presented no halo in Columbia CNA blood agar plate and was classified as γ-hemolytic. Hemolytic ability is a relevant virulence factor that can be present in pathogenic microorganisms. To be safe for use, probiotic strains must be nonhemolytic. Besides, *E. faecium* SFM2 was sensitive to chloramphenicol, vancomycin, and rifampicin. Furthermore, the physiological and biochemical characteristics of *E. faecium* SFM2 were explored and compared with the human oral source *E. faecium* SFM1 preserved in the laboratory, including sugar utilization, optimal temperature, temperature tolerance, and the survival rates in a simulated gastrointestinal environment. The results showed that *E. faecium* SFM1 and *E. faecium* SFM2 had better utilization capacity for sucrose, glucose, and lactose. The optimal temperature of *E. faecium* SFM1 and *E. faecium* SFM2 was 35 °C, and the OD_{600} of *E. faecium* SFM2 was higher than that of *E. faecium* SFM1. After incubation at different temperatures for 2 h, *E. faecium* SFM2 showed higher survival rates in the temperature range of 30–50 °C. Besides, *E. faecium* SFM2 showed stronger gastrointestinal tolerance than *E. faecium* SFM1. This study provided a new idea for enriching the resource library of high-temperature resistant LAB strains.

Conclusion

According to the shelf life, all samples were divided into three groups: group A (1–5 d), group B (6–10 d), and group C (11–15 d). The analysis of bacterial diversity in pasteurized milk showed that *Proteobacteria*, *Firmicutes* and *Bacteroidetes* accounted for the highest proportion in the three groups. The species composition of group A was significantly different from that of group B and group C, while the latter two groups were similar. Most of the LAB in the three groups were *Lactobacillus* and *Lactococcus*, and the top five LAB all contained *Lc. raffinosa* and *Lb. salivarius*. Furthermore, a strain of *E. faecium* SFM2 was isolated from the A2 sample, which showed better temperature tolerance compared to the *E. faecium* SFM1 of oral origin. After treatment at 50 °C for 2 h, the survival rates of *E. faecium* SFM1 and SFM2 were 28.20 ± 0.04% and 82.58 ± 0.01%, respectively. Through this study, we provided some information on the microbial diversity in pasteurized milk, and pointed out that pasteurized milk is an important source of thermoresistant LAB. In the future, we can try to isolate more thermoresistant LAB from pasteurized milk and study their probiotic-related properties.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Declarations

Competing interests
The authors declare no competing interests.

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