



Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

The residual core microbiota in pasteurized milk mainly affect the qualities of products through pyruvate metabolism and specific amino acids biosynthesis pathways

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ABSTRACT: Pasteurized milk is highly regarded for its ability to effectively retain nutrients and sensory quality, but its shelf life is limited by the heat-resistant microorganisms and their metabolic products remaining during the mild sterilization process. To solve this problem, the key is to identify the core residual flora and its metabolic network that lead to quality degradation. In this study, pasteurized milk stored at 4 °C for 0, 6, 9, and 12 days was examined to explore the changes in physicochemical properties, microbial communities, and functional metabolism during storage. Physicochemical analysis showed a slight decrease in fat, protein, and lactose content and a significant increase in acidity, particle size, and yellowness during storage. Among three pasteurization treatments (65 °C/30 min, 75 °C/15 s, and 85 °C/15 s), the 75 °C/15 s protocol optimally preserved nutrients and sensory quality, providing critical parameters for process optimization. Metagenomic analysis by Illumina HiSeq sequencing platform showed that *Acinetobacter*, *Pseudomonas* and *Lactococcus* might be the main core microflora leading to the deterioration of pasteurized products during storage. Pasteurized milk processed for 75 °C/15 s can maintain good quality when stored at 4 °C for 0-9 days. Storing at 4°C for 9 days may be the key storage point for microbial dynamics. Functionally, genes related to carbohydrate metabolism, amino acid metabolism, and energy metabolism increased with storage time. These metabolic pathways are crucial for microbial growth and may affect the nutritional composition and quality of pasteurized milk. Core microbiota primarily impacted milk quality through pyruvate metabolism and valine, leucine, and isoleucine biosynthesis pathways, leading to changes in acidity, bitterness, and texture. This study provides a basis for the monitoring and quality safety management of the main microorganisms and active metabolites during the production, processing and storage of pasteurized milk, and lays a foundation for obtaining high-quality pasteurized milk with long shelf life.

Keywords: Pasteurized milk; Metagenomics; Metabolism; Storage; Core microbiota; Product quality;

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Received 9 August 2025

Received in revised form 27 October 2025

Accepted 21 January 2026

1. Introduction

To guarantee the removal of harmful organisms, such as mycobacterium tuberculosis, and to enhance preservation quality during storage, heat treatment is now the most crucial sterilization technique for milk^[1]. Compared with ultra-high-temperature sterilized milk, which has a longer shelf life, pasteurization is favored for its mild processing conditions and less impact on the sensory properties and nutrients of cow's milk. However, the shelf life of pasteurized milk is relatively short, and how to extend its shelf life and maintain its excellent quality has been a challenge for the dairy industry.

The shelf life of pasteurized milk has been found to be influenced by a number of factors. These variables include raw milk quality, pasteurization temperature and duration, psychrotrophic bacteria development, packing and storage conditions, and more^[2,3]. Studies have indicated that microbial metabolism impacts the quality of pasteurized milk, and microbial contamination throughout the dairy chain is thought to be a significant factor affecting the shelf life of pasteurized milk^[4]. Due to the characteristics of processing technology and inappropriate pasteurization, some psychrotrophic bacteria still persist in pasteurized milk. During storage, these microorganisms continue to grow and multiply, utilizing milk nutrients such as lactose, proteins, and lipids for metabolic activities while secreting a series of metabolites that degrade pasteurized milk quality. Furthermore, psychrophilic bacteria can produce heat-stable enzymes that remain active even after pasteurization. These enzymes trigger a series of hydrolysis reactions during storage, accelerating the deterioration of milk quality.

Recent studies have increasingly begun to focus on the changes in bacterial flora and their metabolic functions in pasteurized milk^[1,5,6]. The dominant genera reported in pasteurized milk are *Pseudomonas* and *Acinetobacter*^[7]. Previous studies have shown that psychrotrophic bacteria, such as *Pseudomonas*, produce heat-resistant proteases and lipases that are capable of degrading κ -casein and fat, leading to quality defects in milk during storage, including increased viscosity, sedimentation, and bitter taste^[8-10]. *Paenibacillus*^[6], *Streptococcus dysgalactiae*, and *Streptococcus uberis*^[3] have also been found in pasteurized milk in other investigations. Because of their resistance to pasteurization, *Paenibacillus* spores have the potential to withstand heat treatment and grow to be a significant population in the pasteurized milk's microbial community. *Streptococcus* is a common mastitis pathogen, and these bacteria not only cause milk spoilage but may also produce metabolites that are harmful to humans. Another limiting factor for pasteurized milk is that it must be stored under refrigeration, and proper storage temperature and duration are essential to maximize control of bacterial growth and metabolism. However, refrigeration alone is insufficient to completely inhibit bacterial growth, and prolonged storage may still lead to quality deterioration or even microbial spoilage. According to research by Ding et al.^[11], *Pseudomonas*, *Aeromonas*, *Paenibacillus*, and *Serratia* may still be present in pasteurized milk stored at 4 °C. Additionally, Li et al.^[12] also found that *Pseudomonas* and *Stenotrophomonas* persist in pasteurized milk stored at 4 °C, potentially affecting milk quality through key metabolic pathways such as glycerophospholipid and purine metabolism. Since the microbial composition during storage is an important factor affecting the shelf life of pasteurized milk, an in-depth understanding of

the microbial community succession pattern and its functional metabolism during pasteurized milk storage is important for optimizing dairy processing and improving product quality.

The study of microorganisms was previously limited because many bacteria could not be isolated by culture, and identification of microbial strains was often accomplished by time-consuming and tedious culture procedures. The rise of advanced high-throughput sequencing techniques has provided a more accurate method for obtaining microbial colonization information^[13]. Microbial diversity sequencing allows for the visualization of species composition and dynamics and has been used to identify most microbial strains in milk^[14]. Microbial diversity sequencing is performed by PCR amplification of a highly variable region (V4 or V3-V4 region), but there are some limitations in this sequencing method: although the highly variable region is highly specific, the specific fragments that can distinguish some very similar species may be outside the amplified region. In addition to accurately identifying microorganisms at the species level, restoring the actual state of microorganisms in the samples in a relatively objective manner^[15], and comprehending the species composition and dynamic changes of microorganisms in the samples, macro-genome sequencing compensates for this shortcoming by studying the microbial functions and metabolic pathways from the gene level, which can thoroughly explore the genome information of microorganisms. Furthermore, macro gene sequencing can be utilized as an efficient technical technique for a thorough investigation of functional genes and modifications in metabolic pathways since it can yield data on the interactions between various strains, thereby mining new functional genes^[16].

Previous studies have primarily focused on the microbial composition of pasteurized milk, while investigations into how bacterial community structure and metabolic activity during storage affect milk quality remain insufficient. This study systematically analyzed physicochemical properties, microbial communities, and functional metabolism in pasteurized milk across storage durations. Explore the main metabolic pathways by which the core microbiota affects milk quality, gain a more comprehensive understanding of the quality changes of pasteurized milk during storage and the reasons for these changes, and ensure the safety and stability of the product.

2. Material and method

2.1 Sample preparation and collection

According to Ding et al. ^[7], pasteurized milk samples produced on the same day were aseptically collected from a dairy processing plant in Shenyang, China. These samples were subjected to sterilization at three different conditions: 65 °C for 30 min (No. LT), 75 °C for 15 s (No. MT), and 85 °C for 15 s (No. HT). Samples were collected and transported to the laboratory and stored in an incubator at 4 °C. Samples were collected on the 0th, 6th, 9th, and 12th days. Each time samples were collected, the three repeated samples in the same group were thoroughly mixed to minimize the impact of individual differences on subsequent analysis. All samples were subsequently stored at -80 °C as backups for future analysis.

2.2 Quantitative analysis of main components

Basic nutrient composition (fat, protein, and solids-not-fat (SNF)) was determined using the 120 Integrated Dairy Comprehensive Component Index Analyzer (Flowserve, USA). The pH value was measured with the pH meter (Hanna, Italy), and color development was assessed using the Chroma-meter (HunterLab, Inc., USA). The average particle diameter of pasteurized milk was determined according to the method described by Yang et al.^[17].

2.3 Determination of protease activity

Protease activity was assayed using the Folin phenol method. In brief, 1 mL of sample was mixed with 1 mL of 1% casein solution and incubated at 40 °C for 10 min. Subsequently, terminate the reaction with 2 mL of 0.4 mol/L trichloroacetic acid, let stand for 10 min, and filter. Take 1 mL of the filtrate and add 5 mL of 0.4 mol/L sodium carbonate solution and 1 mL of Folin reagent. Incubate at 40 °C for 20 min, then measure the absorbance at 680 nm using a UV-visible spectrophotometer. A standard curve was constructed using tyrosine solutions at concentrations of 0, 10, 20, 30, 40, and 50 µg/mL. The unit of protease activity is defined as the amount of protease that produces 1 µg of tyrosine per minute.

2.4 Determination of taste sensation

The pasteurized milk sample (50 mL) was centrifuged at 10,000×g for 10 min to remove fat. The supernatant was transferred to a dedicated small cup and analyzed using the fully automated Insent SA402B electronic tongue (Insent, Japan) to measure sourness, bitterness, saltiness, astringency, umami, and sweetness. Each sample was subjected to five measurement cycles, with data from the three stable cycles used for subsequent analysis.

2.5 Metagenomic analysis

2.5.1 DNA extraction

Total microbial DNA was extracted from each sample using the PowerMax Soil DNA Isolation Kit (MoBio Laboratories, USA) according to the manufacturer's instructions. DNA concentration and purity were assessed by 1% agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometry^[18].

2.5.2 Sequencing and data quality control

Sequencing libraries were constructed with the NEXTFLEX™ Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, USA), targeting an insert size of ~350 bp. Each library was sequenced by the Illumina HiSeq X-ten platform (Illumina, USA). Raw reads were processed using Fastp (v0.23.0) to remove low-quality reads (length < 50 bp, quality score < 20, or having N bases) and adapter sequences. Host contamination was removed by the BWA (v0.7.17) based on published host cow DNA sequences at NCBI and Ensembl (<https://ensembl.org/index.html>).

2.5.3 Identification of Microbial Communities and Functional Metabolism

After quality filtering of the raw reads, good quality sequences were assembled using MEGAHIT (v1.1.2), and the open reading frame (ORF) prediction was performed using Prodigal software (v2.6.3). The predicted genes were clustered (90% identity, 90% coverage) using CD-HIT (v4.6.1) to construct

non-redundant gene sets. Then, gene abundance was estimated using SOAPaligner (soap2.21release, 95% identity). We translated the catalog of non-redundant genes into putative amino acid sequences and compared them with the non-redundant (NR) database and KEGG (<http://www.genome.jp/kegg/>) by blastp (v2.2.28+) with an e-value of $1e^{-5}$ to obtain the corresponding species and functional annotations. Carbohydrate-active enzyme (CAZyme) profiles were also obtained by matching protein sequences to the CAZyme database (<http://www.cazy.org/>) using HMMER software (v3.1b2).

2.6 Statistical analysis

One-way ANOVA of physical and chemical properties of the samples was performed using SPSS software (version 22.0, USA). Statistical significance of differences between groups was assessed using Duncan's test. Differences were considered statistically significant when the P -value < 0.05 .

3. Results and discussion

3.1 Physicochemical properties analysis

The main components and physicochemical properties are summarized in Table 1. Nutritional analysis indicated that fat, protein, and SNF content in pasteurized milk samples complied with the Chinese national standard for sterilized milk (GB 25190-2010: protein ≥ 2.9 g/100g, fat ≥ 3.1 g/100g, and SNF ≥ 8.1 g/100g) throughout the 12-day storage period. Protein, fat, and lactose contents decreased during storage at 4 °C, but the changes were not statistically significant ($P > 0.05$), which is in agreement with the results of Yu's study. Yu et al.^[19] observed changes in nutrients such as proteins and fats during storage at 4 °C in pasteurized milk (72 °C, 15 s), but the changes were minimal. Milk contains endogenous enzymes, including proteases and lipases capable of surviving pasteurization or even UHT processing^[20,21], which may induce protein and fat hydrolysis, thereby reducing their content. The pH of bovine milk (typically 6.6-6.8) decreased during storage (Table 1), potentially due to acidity elevation from milk spoilage and microbial growth. Acidity is often used as an indicator to measure bacterial contamination and growth, as it increases with the level of milk spoilage. The growth and metabolism of microorganisms utilize lactose to produce lactic acid and other acids, making milk tend to be acidic^[22,23], which explains the decrease in lactose content and pH during storage of pasteurized milk. At the same time, lipase produced by residual microorganisms in the milk hydrolyzes fat, releasing fatty acids that cause rancidity and also increase acidity. Chromaticity analysis revealed no significant alterations in L^* and a^* values, but significant increases in b^* values ($P < 0.05$). This color shift may be attributed to the effect of nutrients (fats, calcium caseinate), which led to alterations in the color and refractive index of the product^[11,16]. Notably, LT-treated samples (65 °C, 30 min) exhibited significantly higher b^* values than MT and HT groups ($P < 0.05$). Milk undergoes chemical transformations during heating, including Maillard reaction and oxidation^[24]. Continuous heat treatment of LT samples may result in a more severe Maillard reaction, which would impact the color and stability of milk and cause it to lose its nutritional value, as evidenced by changes in the content of protein, fat, and lactose. Particle size analysis demonstrated significant increases during storage ($P < 0.05$), potentially indicating micellar system

destabilization due to residual microbial activity^[22]. Li et al.^[25] investigated the parameters of particle size and protein micelles of pasteurized milk during 7 days of storage at 4 °C and found that the particle size increased significantly, with the most pronounced increase occurring on days 5-7. Sensory evaluation demonstrated progressive bitterness and astringency in LT samples, correlating with storage duration (Fig. 1). LT-treated samples (65 °C, 30 min) exhibited significantly higher bitterness than MT and HT groups. According to some studies, the bitter and astringent flavors of milk increase with the intensity of heat treatment and storage time^[26]. Protease hydrolysis of proteins produces peptides and amine compounds that increase the bitterness and astringency of milk^[27]; meanwhile, lipase hydrolysis produces free fatty acids, which give milk an unwanted rancid taste^[28]. The main source of proteolytic activity in milk is the growth of psychrotrophic bacteria at low temperatures (<15 °C). These bacteria are capable of secreting proteolytic enzymes, which degrade milk proteins and accelerate spoilage^[23,29-30]. Notably, *Pseudomonas*-derived AprX protease represents a well-documented cause of dairy quality deterioration. According to our data, protease activity persisted during pasteurization but remained relatively stable throughout storage (Table 1), suggesting that pasteurization limits protease production by partially inhibiting the growth of psychrotrophic bacteria.

All things considered, prolonged low-temperature heating increases pasteurized milk's bitterness and astringency while encouraging nutritional loss. Consequently, processing settings should favor higher-temperature, shorter-duration treatments in order to preserve flavor and maintain nutritional integrity. As seen in Table 1, MT processing (75 °C/15 s) maintains higher nutrient retention (protein, fat, and lactose). Previous studies have shown that when the heating temperature rises from 72 °C to 100 °C for 15 s the bioactive components in pasteurized milk will decrease^[31]. Meanwhile, Korhonen et al.^[32] found that 75 °C/15 s is the ideal temperature for preserving bioactive proteins such as lactoferrin, α -lactalbumin, and β -lactoglobulin. In order to limit pasteurization intensity without sacrificing safety, 75 °C/15 s was chosen for the ensuing metagenomic study.

Table 1. Composition and physical and chemical properties of samples. Different superscript letters represent significant differences ($P < 0.05$).

Samp le	Protein, %	Fat, %	SNF, %	Lactose, %	pH	L*	a*	b*	Particle size, nm	Protease ,U /mL
LT0	3.21±0.0 1 ^f	3.76±0.0 9 ^d	8.64±0.0 1 ^g	4.62±0.0 01 ^d	6.62±0.0 0 ^{def}	85.03±0.0 37 ^c	-2.72±0.0 02 ^{cd}	2.42±0.1 3 ^b	147.60±25.7 6 ^c	8.50±0.02 ^e
LT6	3.17±0.0 1 ^h	3.87±0.0 5 ^c	8.58±0.0 2 ^h	4.54±0.0 01 ^e	6.63±0.0 1 ^{cde}	84.81±0.0 32 ^c	-2.84±0.0 15 ^e	2.52±0.2 0 ^b	175.80±29.8 8 ^{bc}	8.77±0.01 ^b
LT9	3.20±0.0 1 ^{fg}	3.31±0.1 6 ^e	8.50±0.0 2 ⁱ	4.53±0.0 01 ^f	6.61±0.0 1 ^{fg}	86.92±0.0 08 ^a	-2.38±0.0 03 ^a	3.34±0.1 5 ^a	193.67±38.5 7 ^b	8.74±0.03 ^b
LT12	3.21±0.0 1 ^f	3.09±0.0 9 ^f	9.15±0.0 1 ^{cd}	4.52±0.0 02 ^f	6.58±0.0 1 ^h	85.52±0.0 30 ^b	-2.64±0.0 09 ^{bc}	3.37±0.1 4 ^a	203.90±7.85 ab	8.89±0.02 ^a
MT0	3.45±0.0 1 ^a	4.13±0.0 0 ^a	9.31±0.0 1 ^a	4.78±0.0 00 ^a	6.65±0.0 1b ^c	83.34±0.0 12 ^c	-2.57±0.0 02 ^b	1.89±0.1 5 ^{cd}	183.13±1.71 b	7.02±0.01 ^g
MT6	3.42±0.0 1 ^b	3.90±0.0 2 ^c	9.14±0.0 1 ^d	4.76±0.0 01 ^b	6.64±0.0 0 ^{cd}	83.38±0.0 10 ^c	-2.58±0.0 04 ^b	1.91±0.1 2 ^{cd}	191.33±1.89 b	7.22±0.03 ^f
MT9	3.18±0.0 1 ^{gh}	3.74±0.0 0 ^d	9.25±0.0 1 ^b	4.75±0.0 02 ^b	6.61±0.0 1 ^{efg}	83.78±0.0 11 ^d	-2.56±0.0 02 ^b	2.03±0.0 8 ^c	190.17±3.82 b	7.44±0.01 ^e
MT1 2	3.37±0.0 1 ^d	3.94±0.0 1 ^{bc}	9.17±0.0 2 ^c	4.75±0.0 01 ^b	6.58±0.0 1 ^h	83.41±0.0 12 ^c	-2.60±0.0 00 ^b	2.39±0.1 7 ^b	224.67±3.28 a	7.63±0.01 ^d
HT0	3.40±0.0	4.02±0.0	8.39±0.0	4.68±0.0	6.69±0.0	83.25±0.0	-2.75±0.0	1.68±0.1	173.46±6.27	6.89±0.02 ⁱ

	1 ^c	1 ^b	1 ^j	01 ^c	2 ^a	08 ^e	01 ^d	0 ^d	bc	
HT6	3.24±0.0	3.93±0.0	8.34±0.0	4.68±0.	6.66±0.0	83.36±0.	-2.72±0.	1.84±0.1	182.80±10.4	6.95±0.03 ^h
	1 ^e	1 ^{bc}	2 ^k	01 ^c	2 ^b	16 ^e	02 ^{cd}	7 ^{cd}	5 ^b	
HT9	3.18±0.0	3.84±0.0	8.91±0.0	4.68±0.	6.62±0.0	83.37±0.	-2.74±0.	1.90±0.2	189.43±4.29	7.22±0.02 ^f
	1 ^h	2 ^{cd}	0 ^e	01 ^c	1 ^{def}	06 ^e	02 ^d	0 ^{cd}	b	
HT1	3.13±0.0	3.77±0.0	8.78±0.0	4.67±0.	6.60±0.0	83.21±0.	-2.75±0.	2.00±0.0	192.70±2.98	7.45±0.02 ^e
2	0 ⁱ	1 ^d	1 ^f	01 ^c	2 ^{gh}	04 ^e	02 ^{de}	7 ^c	b	

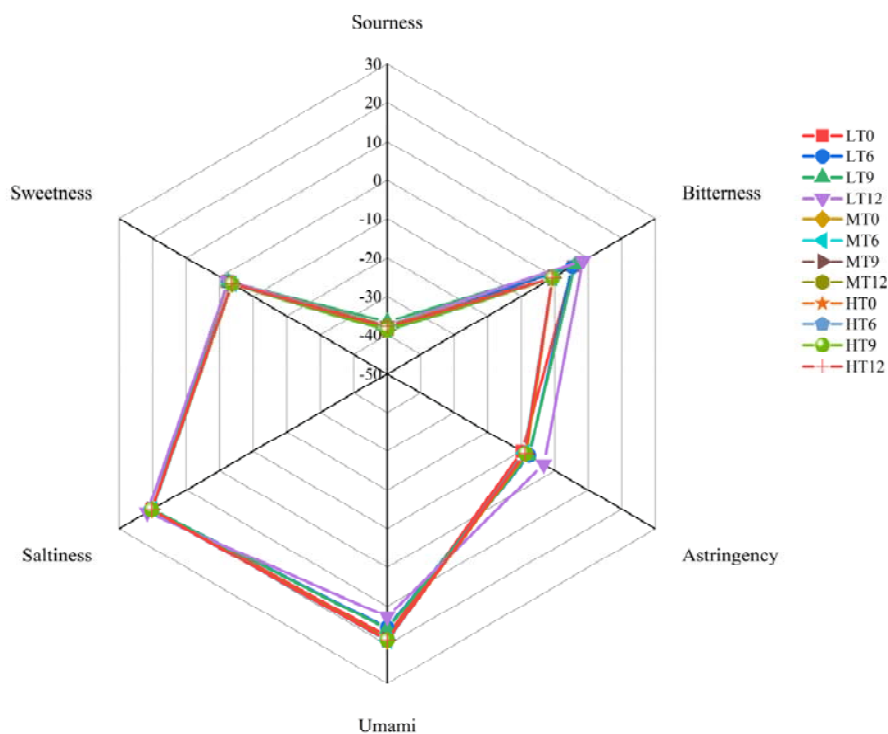


Fig. 1. Electronic tongue radargram of pasteurized milk samples.

3.2 Overview of metagenomic data

This study analyzed metagenomic datasets from pasteurized dairy samples stored for varying durations. Metagenomic sequencing was performed on the Illumina HiSeq X-ten platform (Illumina, USA), with an average sequencing depth of 6.36–7.11 Gbp across samples. Metagenomic sequencing generated 174,990,442 raw reads from pasteurized milk samples (Supplementary material). Following quality filtration (removing adapters, low-quality reads, and host contamination), 965,862 optimized reads were obtained. Assembly of the clean reads yielded a total of 3530 contigs with 1,778,030 bases and 3557 predicted ORFs.

3.3 Microbiota composition analysis

Gene-based taxonomic classification was conducted by aligning sequences to the NR database. 4 domains, 19 phyla, 32 classes, 59 orders, 97 families, and 137 genera were identified from pasteurized milk. The domains identified encompassed bacteria, eukaryotes, and viruses.

The main microbiota of pasteurized milk was composed of bacteria (92.79%) and eukaryotes (7.00%), with bacteria being the most prevalent species. *Proteobacteria*, *Firmicutes*, *Tenericutes*, *Actinobacteria*, and unclassified_d_Bacteria were the five major phyla within the bacterial community (Fig. 2A). *Proteobacteria* was found to be the most dominant phyla in pasteurized milk, accounting for over 61.5% of the population, followed by *Firmicutes* (2.2%–31.5%). This finding is consistent with previous research that also identified

Proteobacteria and *Firmicutes* as the dominant phyla in pasteurized milk^[7]. The *Proteobacteria* has been reported to include many known human pathogens, such as *Escherichia* and *Salmonella*. In contrast, the *Firmicutes* includes several probiotic bacteria^[33]. During storage, the relative abundance of the *Proteobacteria* increased, and the relative abundance of the *Firmicutes* decreased in MT9 and MT12 samples compared to MT0 samples. This result aligns with the findings of Ding et al.^[11], who observed that during storage of pasteurized milk at 4°C, 10°C, and 15°C, the relative abundance of *Proteobacteria* increased with storage duration, while that of *Firmicutes* progressively decreased. These findings may signal a potential safety risk for the samples during storage. Furthermore, *Actinobacteria* gradually become less abundant, which may be related to longer storage times. Given *Actinobacteria*'s role as producers of bioactive secondary metabolites, this reduction may diminish functional benefits in prolonged-stored milk^[34,35].

At the genus level, a total of 17 genera were identified in pasteurized milk with relative abundance greater than 1% (Fig. 2B), including *Pseudomonas*, *Acinetobacter*, *Mycoplasmopsis*, *Lactococcus*, and *Moraxella*. This composition is not entirely consistent with previous reports^[28,36], and may reflect differences in geographic location, rearing environment, processing and storage conditions, seasonal climates, and sampling protocols^[37]. Ding et al.^[11] and Du et al.^[36] collected pasteurized milk samples from two cities, Shenyang in northern China and Zhengzhou in central China, and analyzed their microbial composition using 16S rRNA sequencing technology. Results indicated that *Pseudomonas* and *Streptococcus* dominated pasteurized milk samples from Shenyang^[11], while *Acinetobacter*, *Macroccoccus*, *Pseudomonas*, and *Lactococcus* were predominant in Zhengzhou samples^[36]. A recent study further confirmed the geographic specificity of pasteurized milk microbiomes. Yi et al.^[38] employed metagenomic sequencing to identify *Pseudomonas* and *Ralstonia* as dominant genera in pasteurized milk from four distinct regions of South Korea. Seasonal fluctuations were also shown to significantly impact pasteurized milk microbial diversity. Porcellato et al.^[39] performed a study on Norwegian pasteurized milk, employing 16S rRNA gene sequencing to analyze seasonal variations in microbial communities. Results indicated that *Bacillus* was the dominant genus in spring and summer samples, whereas *Pseudomonas*, *Streptococcus*, and *Acinetobacter* predominated in winter samples. Processing and storage conditions also influenced the microbial composition of pasteurized milk. Zhao et al.^[40] employed a similar approach, finding higher abundances of *Pseudomonas*, *Flavobacterium*, and *Acinetobacter* in pasteurized milk stored for 1-5 days. In contrast, milk stored for 6-10 days and 11-15 days primarily harbored *Ralstonia*, *Caulobacter*, *Bradyrhizobium*, and *Sphingomonas*. These studies collectively confirm that the microbial diversity of pasteurized milk is jointly regulated by geographical, seasonal, and storage factors. Dominant bacterial genera identified across various studies, such as *Pseudomonas*, *Acinetobacter*, and *Bacillus*, are typical members of the dairy product microbial community. This study employed metagenomic sequencing to analyze the microbial composition and dynamic succession of pasteurized milk (75°C, 15s) during 12 days of refrigeration at 4 °C. Sequencing results indicated that *Acinetobacter* and *Pseudomonas* dominated during storage (Fig. 2B). In contrast to previous findings, we also detected genera such as *Mycoplasmopsis*, *Macroccoccus*, *Lactococcus*, and *Moraxella*. This discrepancy may

stem from variations in the initial microbial composition of raw milk, geographical location, and rearing environments^[41]. It should be noted that the detection characteristics of metagenomics, based on genetic material, make it unable to effectively distinguish DNA from living cells versus dead cells^[42]. Therefore, the detection of microorganisms in pasteurized milk via metagenomic analysis does not necessarily imply their viability. However, a growing body of literature supports the persistence of these taxonomic groups in pasteurized dairy products^[11,36], and the detection of these microorganisms continues to reflect potential food safety risks. Furthermore, although this study observed changes in bacterial composition during pasteurized milk storage, certain taxa were detected consistently across samples and were thus defined as the core microbiota of pasteurized milk (core microbial communities are defined as taxonomic units present in all samples with an average relative abundance $\geq 1\%$)^[39,43]. Most of these taxa, including *Acinetobacter*, *Pseudomonas*, *Macrococcus*, and *Lactococcus*, have also been classified as components of the core microbiota of pasteurized milk in previous studies^[7,11,36]. The dominant genera in sample MT0 were *Acinetobacter* (48.2%), *Mycoplasmopsis* (12.8%), and *Lactococcus* (6.1%). *Acinetobacter*, a common psychrotrophic bacteria in milk, can grow even under refrigeration, displaying high lipolytic activity leading to phenomena like fat flotation^[44-46]. In addition, *Lactococcus*, another psychrotrophic bacteria commonly found in milk, showed significant differences between samples (Fig. 2C, $P < 0.05$). *Lactococcus* as a leavening agent is beneficial in the production of fermented foods, but some undesirable strains are present, producing unwanted sourness, off-flavors, and curdling. The three most common genera after 6 days of storage were *Acinetobacter* (47.4%), *Macrococcus* (17.3%), and *Lactococcus* (8.4%). *Macrococcus* is a veterinary pathogen that has been linked to a variety of illnesses, including cattle mastitis. It is commonly found on the skin surface of mammals and in foods derived from them, such as raw and pasteurized milk^[47]. Interestingly, after 9 days of cold storage of pasteurized milk, the relative abundance of *Lactococcus* decreased to 1.3%, while *Pseudomonas* abundance rapidly increased quickly (81.5%), reaching 15-21 times the relative abundance observed in MT0, MT6, and MT12 samples. According to Lo et al.^[48], during the early storage phase (0-6d), rapidly growing *Lactococcus* break down lactose into glucose and galactose, producing organic acids like lactic acid. This acidified environment partially inhibits the growth and secretion of *Pseudomonas*, explaining why *Pseudomonas* did not dominate during the initial storage period. Previous studies have demonstrated that *Pseudomonas* species exhibit remarkable metabolic diversity in response to various environmental stresses, while the abundant nutrients in milk provide highly favorable conditions for their growth and biofilm formation^[49]. This pronounced shift in microbial composition suggests that 4 °C storage for 9 days may represent a critical storage point for microbial dynamics in pasteurized milk, suggesting that complex microbial succession may occur during cold storage of pasteurized milk. Our findings align with multiple prior studies^[1, 11, 41]. Ding et al.^[11] reported that pasteurized milk stored at 4 °C for 0-9 days was of better quality due to lower microbial abundance. Al-Farsi et al.^[1] also showed that 5 °C storage for up to 9 days was considered safe. In a more comparable study, Hu et al.^[41] observed *Pseudomonas* becoming the dominant genus in pasteurized milk (85 °C, 15s) by the eighth day of storage, suggesting that 8 days of

refrigeration at 4 °C represents a critical time point for microbial succession. Collectively, these findings confirm that the approximately 8-9 days refrigerated storage period constitutes a critical phase for microbial succession in pasteurized milk, potentially governing subsequent product stability and quality. In the MT12 sample, *Pseudomonas* relative abundance decreased to 5.3%, while *Acinetobacter* (66.2%) became dominant. The abundance of *Acinetobacter* decreased and then increased, suggesting that it may be involved in specific metabolic engagement. Under low-temperature storage, prolonged storage leads to population shifts in which cold-tolerant microorganisms, especially *Pseudomonas*, become more dominant. *Pseudomonas* is a prevalent psychrotrophic bacteria commonly found in milk, and its proliferation in significant quantities is widely regarded as a primary contributor to the reduced shelf life of liquid milk^[50,51]. It produces heat-stable proteases and lipases, leading to quality defects in milk, including increased viscosity, precipitation, aging gelation, fat separation, and heightened bitterness^[9,52]. Multiple studies have demonstrated that *Pseudomonas* and *Acinetobacter* maintain high abundance in raw milk and pasteurized milk, playing a dominant role in the spoilage process of dairy products^[8,53]. Additionally, a number of bacterial genera with low relative abundance were identified, including *Kaistia*, *Escherichia*, *Staphylococcus*, *Corynebacterium*, *Salmonella*, and *Streptococcus*. *Corynebacterium* is often recognized as a pathogen and is commonly found in mammary-infected dairy products^[54]. Additionally, various strains of *Staphylococcus*, especially *Staphylococcus aureus*, are recognized as one of the most important foodborne pathogens globally, and milk is considered an important source of contamination^[55]. *Streptococcus* has also been recognized as one of the potential pathogens in pasteurized milk in the Chinese region and is strongly associated with mastitis^[3,56]. Although pasteurization can eliminate pathogenic microorganisms to a certain extent, it cannot eradicate all spoilage microorganisms. Studies have shown that thermotolerant microorganisms resist the pasteurization process utilize components such as proteins and fats as nutrient sources, and form biofilms in dairy processing equipment, leading to changes in the quality of the product^[57,58]. Notably, the thermotolerance of *Macrococcus* and *Streptococcus* demonstrates pasteurization's limited efficacy against heat-adapted spoilage bacteria. Based on the between-group analysis of variance, we also found that the other microorganism that differed significantly in the other three samples compared to MT 9 was *Salmonella* (Fig. 2C), which is recognized as a pathogen associated with bacterial contamination in milk^[59]. The coexistence of potential pathogens with beneficial microorganisms in microbial communities suggests that individual pathogens, when present in microbial communities, may not have deleterious effects on consumer health. The pathogenicity of *Salmonella* and *Staphylococcus* has the potential to be attenuated, blocked, or prevented by surrounding beneficial microorganisms. This highlights the complexity of the various microbial populations in pasteurized milk and the importance of maintaining an ecological balance.

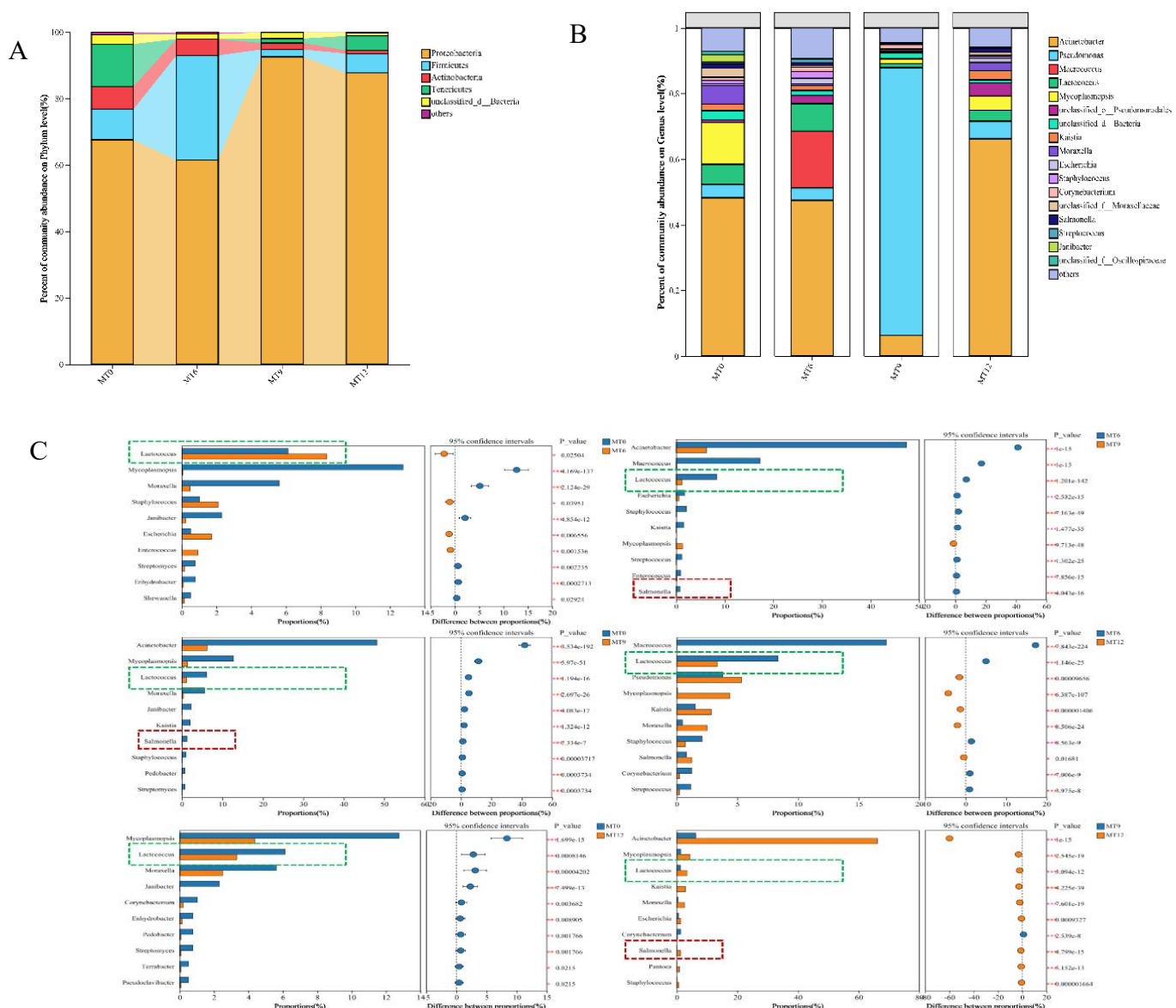


Fig. 2. Relative abundance of bacterial phylum (A)/genus (B) in pasteurized milk. Bacterial genera with a relative abundance of <1% were categorized as "others". Significance of differences between groups at genus level (C). * $0.01 < P < 0.05$, ** $0.001 < P < 0.01$, *** $P < 0.001$.

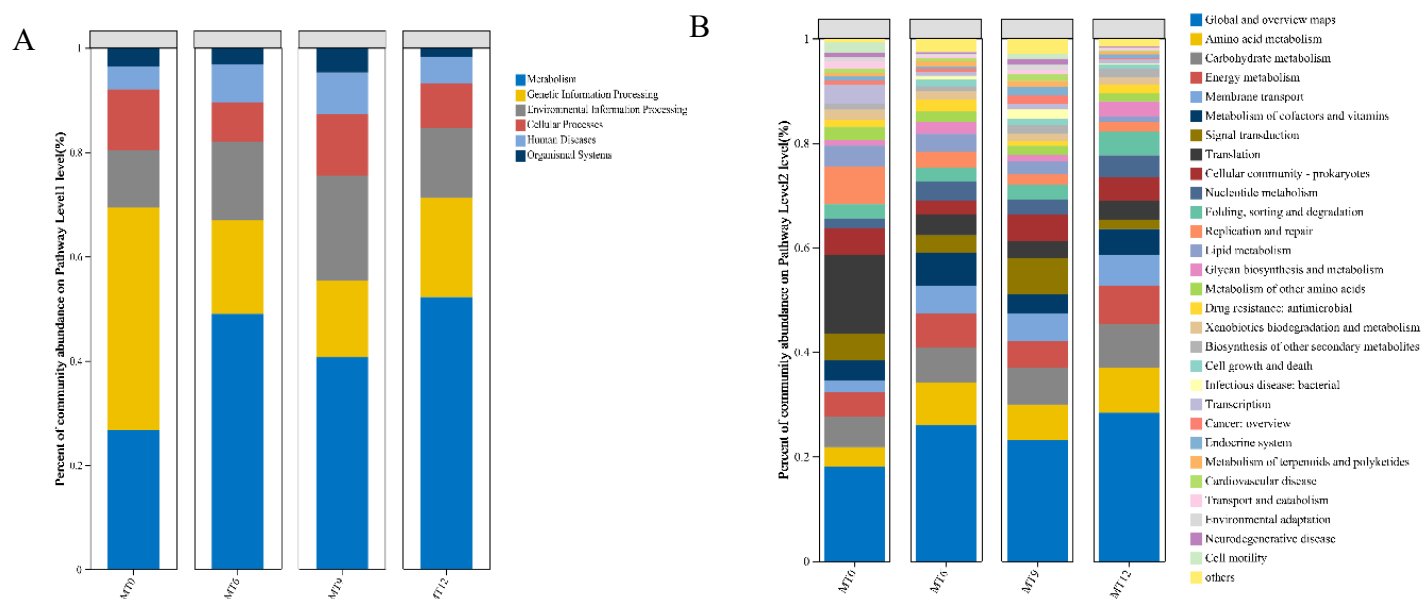
3.4 Annotation of microbial functional genes

The sequenced genome sequences are compared to the KEGG database to determine gene functions and pathways in order to obtain a better understanding of the vital role played by the microbial populations found in pasteurized milk samples. The functional genes of bacteria in pasteurized milk primarily involve six major categories: Metabolism, Genetic Information Processing, Environmental Information Processing, Human Diseases, Cellular Processes, and Organismal Systems (Fig. 3A). The results showed that genes related to metabolism (including carbohydrate metabolism, amino acid metabolism, and energy metabolism) were found to be more active during storage. This was followed by genetic information processing (including translation, folding, sorting degradation, and transcription) and environmental information processing (including membrane translocation and signal transduction). With increasing storage time (Fig. 3B), the relative abundance of genes associated with carbohydrate metabolism (5.7%–8.2%), amino acid metabolism

(3.8%-8.7%), and energy metabolism (4.8%-7.4%) increased in the samples. This finding aligns with the research by Hu et al.^[41], which also identified amino acid and carbohydrate metabolism as the primary metabolic pathways in refrigerated pasteurized milk. These results suggest that microbial proliferation during pasteurized milk storage may deplete nutrients in milk through pathways such as carbohydrate and amino acid metabolism, thereby accelerating product quality deterioration.

The degradation of proteins and lipids, along with the accumulation of organic acids, are key biochemical processes leading to the deterioration of pasteurized milk products^[60]. Carbohydrates provide the material basis for other metabolisms as a major component of cellular structure and energy supply. Microorganisms primarily assist in the digestion of carbohydrates in dairy products, providing the host with substrates for metabolism and energy conversion^[18]. Notably, the glycolytic/gluconeogenesis pathway gradually became more active during storage. *Lactococcus* can use lactose decomposition to produce glucose and galactose, which are then converted by the glycolysis pathway to lactic acid or other organic acids^[61], resulting in pasteurization in the storage process of the increase in acidity (Table 1). When the pasteurization of lactic acid is too high, it may lead to casein, lactoglobulin, and other precipitation, clots, and even whey precipitation phenomenon. Zhu et al.^[62] employed untargeted metabolomics to reveal that after 8 days of storage, pasteurized milk exhibited significantly elevated concentrations of certain fatty acids, organic acids, peptides, and ketone bodies. Another study on yak milk and cattle-yak milk in the Gannan region observed increased organic acid content during storage, primarily attributed to the metabolic activity of *Lactococcus*^[60]. Microorganisms can efficiently break down carbohydrates through metabolic pathways such as glycolysis to produce ATP, providing energy for their growth and reproduction^[63]. During this process, microorganisms consume large amounts of nutrients in milk, thereby reducing its nutritional quality. Amino acids, as important components of protein structure, have a significant impact on protein composition and function. Protein hydrolysis caused by heat-resistant proteases produced by microorganisms is the main cause of changes in amino acid metabolites during storage^[64]. As can be seen in Fig. 3C, the metabolic pathways of alanine, aspartate, and glutamate metabolism became progressively more active during storage. This observation aligns with the findings reported by Zhang et al.^[65], who documented increased activity in pathways such as alanine, aspartate, and glutamate metabolism, as well as glyoxylate and dicarboxylate metabolism (carbohydrate metabolism) in refrigerated pasteurized milk. Previous studies have shown that glutamate plays a key role in the Maillard reaction, reacting with glucose to produce a distinctive bitter almond flavor. Bitter peptides produced by enzymatic hydrolysis of proteins as well as bitter almond flavors from the Maillard reaction may contribute to the increase in bitter values during the later stages of pasteurized milk storage^[56,66]. Changes in the microbiota are the main reason for the enhanced amino acid metabolism during pasteurized milk storage^[65]. Previous studies found that genes related to metabolism, especially amino acid metabolism, were significantly up-regulated in psychrotrophic bacteria such as *Pseudomonas* and *Acinetobacter* at low temperatures (4 °C), suggesting that cryophilic bacteria can adapt to low temperatures by increasing their metabolism and thus maintain continuous bacterial growth and reproduction during storage^[67,68]. The

percentage of genes linked to energy metabolism was high because all microbial metabolic processes involve the consumption and production of energy (Fig. 3B). Membrane transportation-related genes were also upregulated (Fig. 3B), which can promote the transmembrane transport of enzymes, nutrients, and metabolic products, ensuring that microorganisms efficiently obtain substrates and excrete waste in the nutritionally limited dairy environment, thereby maintaining metabolic activity. In this study, lipid metabolism was relatively active during the pre-storage period (MT0 and MT6), and *Acinetobacter* had a higher relative abundance during this period. Ma et al.^[68] investigated *Acinetobacter* genes under low-temperature conditions and found that genes associated with amino acid metabolism, lipid metabolism, and carbohydrate metabolism were significantly upregulated. This gene upregulation provides a molecular basis for the enhanced metabolic activity of *Acinetobacter* during the pre-storage phase. Zhuang et al.^[64] also identified significant positive correlations between *Acinetobacter* and lipid-related metabolites, as well as certain amino acids and carbohydrates through correlation analysis. *Acinetobacter* exhibits stronger lipolytic than proteolytic activity, leading to the accumulation of triglycerides, free fatty acids, and other compounds^[69]. More critically, the lipase produced by *Acinetobacter* promotes fat oxidation, releasing fatty acids that are further degraded into carbonyl compounds and other volatile substances, imparting a pronounced bitter taste and rancid odor to milk^[69]. At 9 days of storage, the microorganisms had a significant succession phenomenon, and the dominant genus became *Pseudomonas* (Fig. 2B). *Pseudomonas* has been extensively demonstrated to secrete thermostable extracellular proteases that degrade milk proteins. By synthesizing heat-resistant enzymes, *Pseudomonas* ensures protein degradation even under harsh conditions, thereby enabling efficient nutrient utilization. This process not only generates bitter peptides but also disrupts casein micelle structures, leading to irreversible textural defects in milk such as gelation, thickening, and whey separation^[51-53]. This shift in microbial functions underscores the pivotal role of microbial community dynamics in shaping the temporal quality changes of pasteurized milk during refrigerated storage.



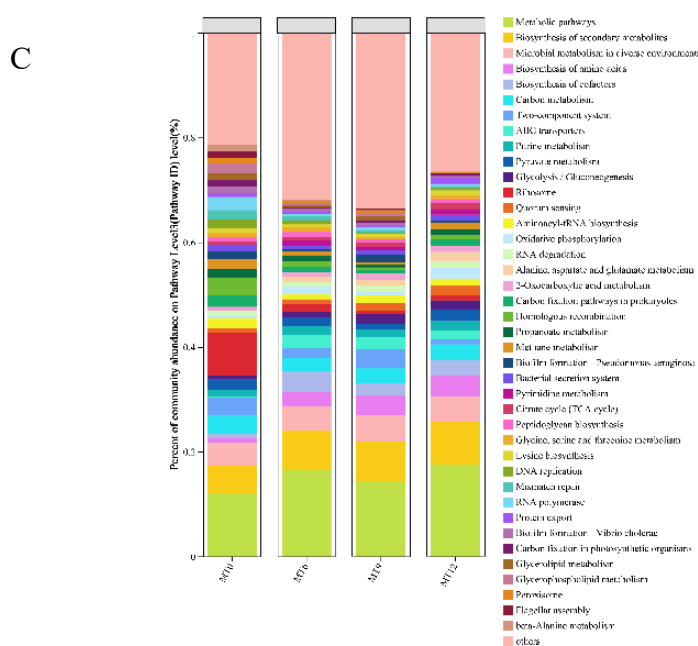


Fig. 3. Functional annotation of KEGG in pasteurized milk(level 1 (A),level 2 (B), level 3 (C)).

Since polysaccharides are the primary source of carbon and energy for microorganisms, we investigated the abundance of carbohydrate-active enzymes (CAZymes) to learn more about the role of these microbiomes. CAZymes analysis revealed that carbohydrate esterases (CEs) and glycoside hydrolases (GHs) were the most predicted abundance of carbohydrate-active enzymes in the macrogenomes (Fig. 4A). These enzymes play a crucial role in the breakdown and modification of complex carbohydrates, influencing the flavor and quality of pasteurized milk^[41].

Compared with the MT0 sample, the quantity and diversity of CAZymes in the MT6, MT9, and MT12 samples were higher (Fig. 4B), suggesting that the microbiota in the MT6, MT9, and MT12 samples may have higher carbohydrate degradation activity^[70]. GHs involved in the hydrolysis of several polysaccharide types were found in the pasteurized milk samples. GHs are primarily associated with pyruvate metabolism and glycolysis. Among them, the GH1 and GH2 families encode β -galactosidases. The carbohydrates in milk are predominantly lactose-based^[71,72], and *Lactococcus* in pasteurized milk can hydrolyze lactose into glucose and galactose via β -galactosidase. Subsequently, *Lactococcus* further utilizes glucose to generate lactic acid from pyruvate via lactate dehydrogenase, thereby increasing the acidity of pasteurized milk. In addition, a large number of genes encoding enzymes of the GH13, GH23, and GH32 families, which participate in the breakdown of carbohydrates, have been identified. The most prevalent family found among them is GH13, which includes GH13_16, GH13_20, GH13_29, and GH13_31. GH13 is the basis of starch hydrolysis, which reflects the important role of starch/glycogen as a source of nutrients^[73]. GH23 is a common family known to be involved in glycogen degradation^[74]. Glycoside hydrolase activity significantly increased after storage (Fig. 4A), indicating that bacteria may use polysaccharides extensively and that their metabolism of carbohydrates is active. Furthermore, other glycosyl transferases, such as GT2, GT4, GT5, GT20, GT41, and

GT51, were identified (Fig. 4B). The GT2 and GT4 families have activities to degrade both oligosaccharides and polysaccharides^[75]. The activities of GT2 and GT4 in MT12 samples were relatively high, indicating that the degradation of carbohydrates might be more active during prolonged storage^[76]. Additionally, carbohydrate-binding modules (CBMs) were identified in the metagenomic analysis (Fig. 4B). Among these, CBM50 was particularly abundant in the bovine rumen. It specifically binds to polysaccharides, enhancing the catalytic activity of carbohydrate-active enzymes and thereby promoting carbohydrate metabolism^[77]. Most CAZymes were found in *Acinetobacter* and *Pseudomonas*, followed by *Lactococcus* and *Macroccoccus* (Fig. 4C). Previous studies have shown that *Pseudomonas* members are rich in genes involved in carbohydrate metabolism^[78]. Hu et al.^[41] also reported a significant positive correlation between carbohydrate metabolism-related genes and *Pseudomonas*. In summary, the microbial community in pasteurization, capable of producing enzymes, plays a role in the storage process, and, in turn, the carbohydrates in pasteurized milk provide the necessary conditions for microbial growth and multiplication.

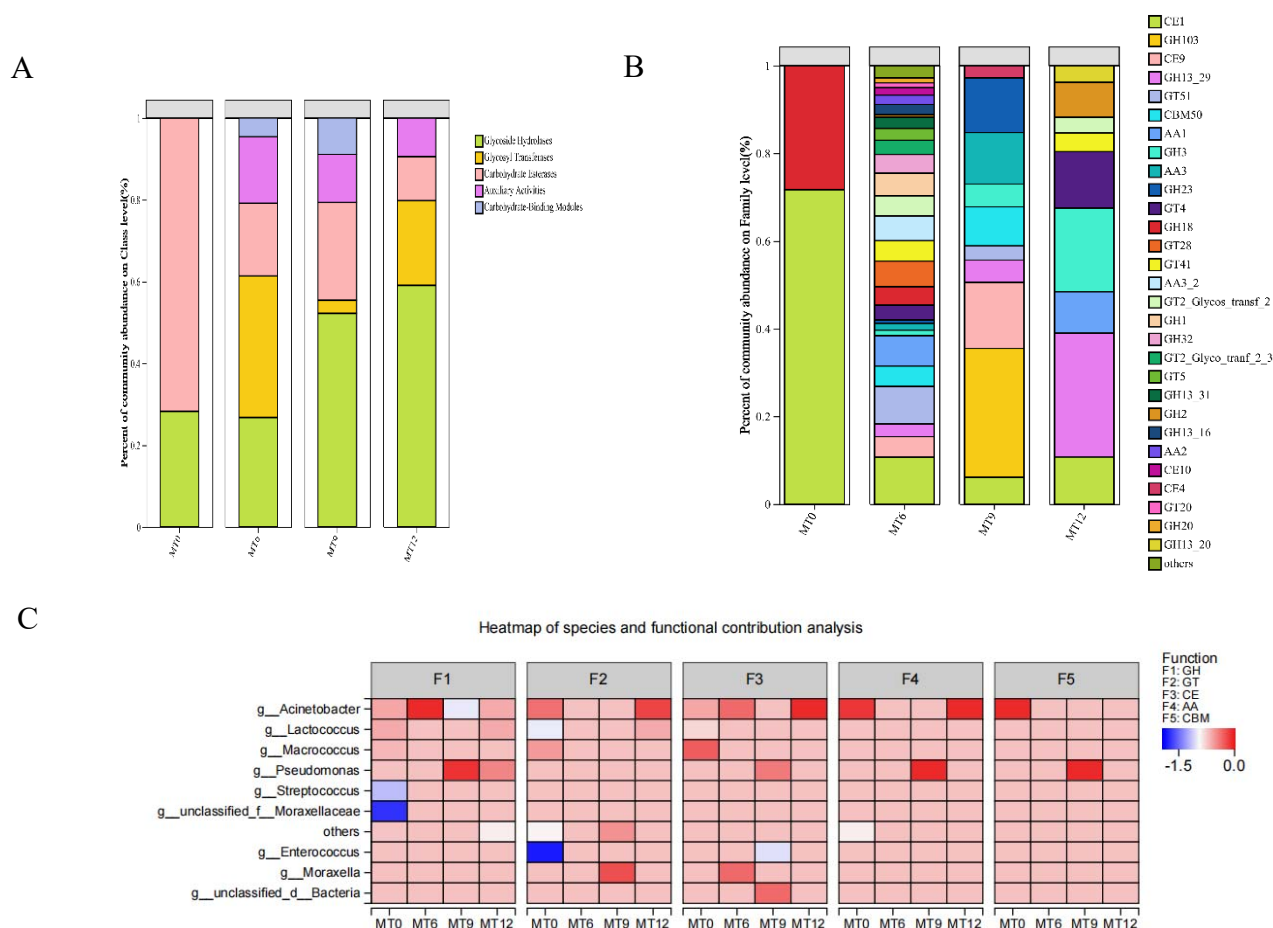


Fig. 4. Carbohydrate-active enzyme activity (A) and composition (B) in pasteurized milk. Analysis of microbial and carbohydrate-active Enzyme Contributions (C).

3.6 Sources of enzymes in pyruvate metabolic pathway

Based on the previous analysis that microbial activities are closely related to carbohydrate metabolism, we then analyzed the carbohydrate metabolic pathways in different samples. The way microbes use carbohydrates varies greatly, and different samples may exhibit notable variations in particular metabolic pathways, which could result in the creation and buildup of distinct compounds. Metabolic pathways such as

pyruvate metabolism, glycolysis/gluconeogenesis, and the citrate cycle TCA cycle were more active in MT6, MT9, and MT12 samples compared to MT0 samples (Fig. 5A). Pyruvate is the hub of various metabolic pathways, including glycolysis, TCA cycle, amino acid, and fatty acid biosynthesis, and is an intermediate product of carbohydrate catabolism^[79]. Differences in pathways such as pyruvate metabolism may be the main reason for the changes in the corresponding components in pasteurized milk. Bioinformatics annotations indicate that pyruvate metabolism during storage of pasteurized milk involves a variety of kinases and dehydrogenases. The β -galactosidase enzyme secreted by microorganisms remaining in pasteurized milk breaks down lactose to glucose and galactose and then enters glycolysis to produce pyruvate. Pyruvate is a crucial component for further metabolism, and this process supplies energy for microbial development. Pyruvate, a product of glycolysis, can be further converted into acetic acid and lactic acid, producing undesirable flavors such as sourness, astringency, and bitterness^[80]. The accumulation of these organic acids is a key factor in the increased acidity and flavor changes observed in pasteurized milk during storage. The pyruvate dehydrogenase complex ([EC:1.2.4.1], [EC:2.3.1.12]) facilitates the conversion of pyruvate to acetyl coenzyme A and its entry into the tricarboxylic acid (TCA) cycle. Pyruvate, orthophosphate dikinase ([EC:2.7.9.1]), and pyruvate kinase ([EC:2.7.1.40]) can catalyze the production of pyruvate from phosphoenolpyruvate and release ATP for energy. Furthermore, pyruvate carboxylase ([EC:6.4.1.1]) catalyzes the conversion of pyruvate with ATP, water, and CO₂ into oxaloacetate, which is then recycled throughout the TCA cycle to yield a variety of organic acids, including succinate and malate^[81]. Succinic acid, as an intermediate in the TCA cycle, possesses sour, salty, and bitter flavors that may affect the sensory quality of milk^[82]. Meanwhile, L-lactate dehydrogenase ([EC:1.1.1.27]) catalyzes the reduction of pyruvate to L-lactate and D-lactate dehydrogenase ([EC:1.1.1.28]) catalyzes the oxidation of D-lactate to pyruvate. Decarboxylation of pyruvate produces acetaldehyde, which in turn produces ethanol and acetic acid by the action of ethanol dehydrogenase ([EC:1.1.1.1], [EC:1.1.1.1], [EC:1.1.1.2]) and acetaldehyde dehydrogenase ([EC:1.2.1.3], [EC:1.2.1.10], [EC:1.2.1.10]), respectively^[83]. Extensive research has confirmed the accumulation of these metabolites during the storage of pasteurized milk. Rashid et al.^[84] analyzed the changes in pasteurized milk under different storage conditions using gas chromatography coupled with flame ionization detection (GC-FID). The results showed that the levels of pentanal and ethanol significantly increased with prolonged storage time. Ethanol is a byproduct produced by many bacteria, including *Lactococcus* (Table 2), during lactose fermentation. Concurrently, Zhu et al.^[62] investigated metabolic changes in pasteurized milk under refrigerated storage using LC-QTOF-MS, identifying increased levels of organic acids (succinic acid, ribonic acid, and galactonic acid) in the later stages of storage. This increase in organic acids is believed to be associated with lipase-mediated lipolysis during storage, which leads to elevated free fatty acid levels and may contribute to the development of off-flavors^[68]. Another study^[5] exploring metabolite changes in pasteurized milk under different storage conditions also found that metabolites such as succinic acid, ethanol, acetic acid, and lactic acid increased with extended storage time. As the storage time of pasteurized milk was extended, the microbial activity gradually increased and its mediated

metabolic processes, such as lipolysis and protein hydrolysis, were activated synergistically with the carbohydrate metabolic pathway, such as pyruvate metabolism and the TCA cycle. The accumulation of metabolic by-products like ethanol, acetic acid, and lactic acid further supported the idea that the increase in microbial metabolic activity in storage was correlated with the enhancement of the carbohydrate metabolic pathway. The production and conversion of pyruvate involves the catalysis of several enzymes (Table 2), which are mainly derived from different microbial genera, including *Acinetobacter*, *Pseudomonas*, *Lactococcus*, and *Moraxella* (Fig. 5B). Currently, research on changes in metabolite concentrations in milk remains relatively scarce^[85]. In the future, more precise quantification of these metabolic products and analysis of their correlation with indicators of milk quality deterioration will help shed deeper light on the mechanisms underlying metabolic and quality changes in milk during storage.

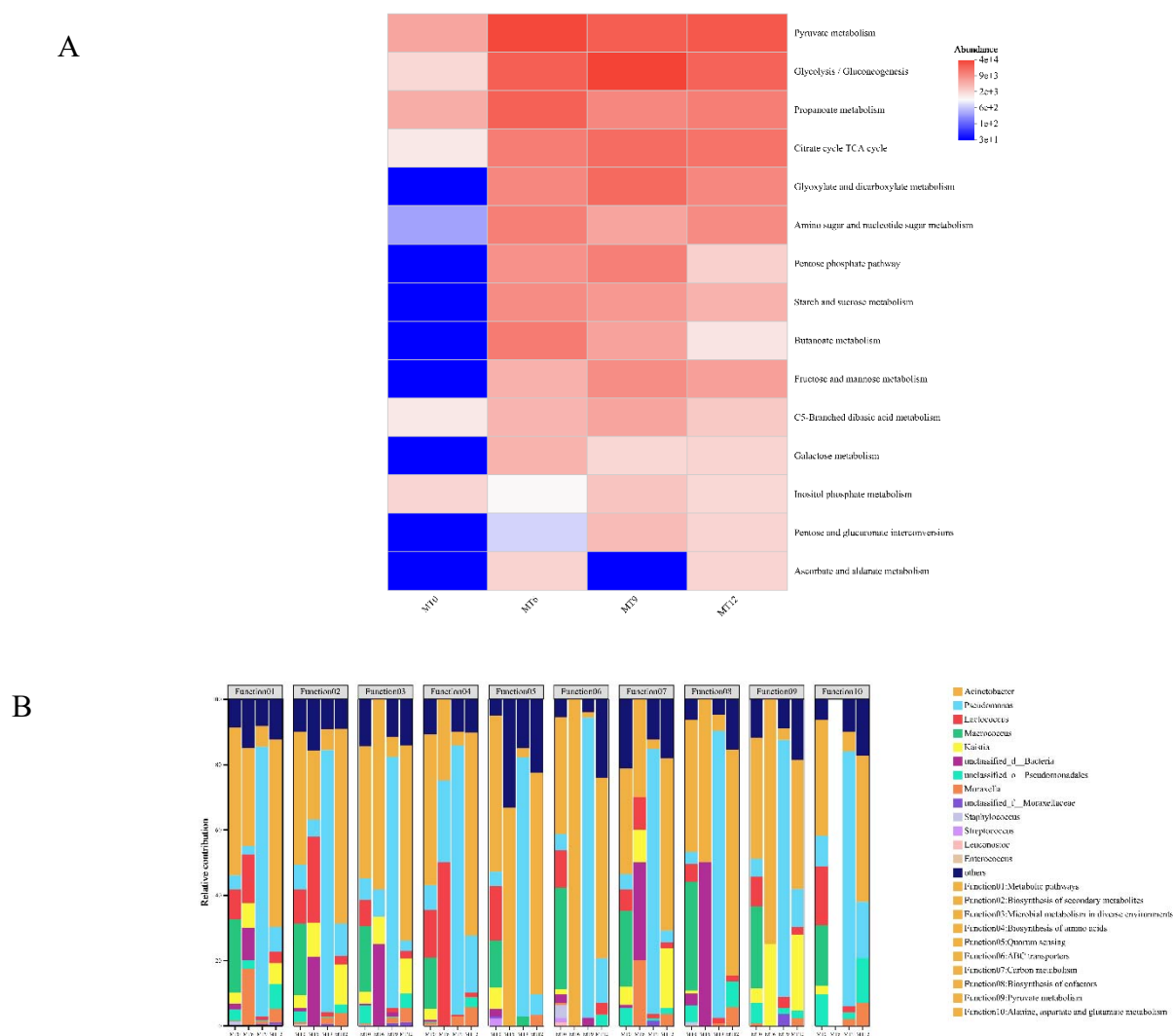


Fig. 5. Carbohydrate metabolism in pasteurized milk (A). Analysis of microbial and functional contribution in pasteurized milk samples (B).

Table 2. Statistics on the source of enzymes in the pyruvate metabolic pathway.

Enzyme number	Enzyme Name	Source of genes involved in enzyme synthesis
[EC:1.1.1.1]	alcohol dehydrogenase, propanol-prefering	<i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Amycolatopsis</i>
[EC:1.1.1.2]	alcohol dehydrogenase (NADP ⁺)	<i>Rothia</i>

[EC:1.1.1.27]	L-lactate dehydrogenase	<i>Lactococcus</i> , <i>Listeria monocytogenes</i>
[EC:1.1.1.28]	D-lactate dehydrogenase	<i>Enterococcus</i>
[EC:1.1.1.37]	malate dehydrogenase	<i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Pseudoclavibacter</i>
[EC:1.1.1.38]	malate dehydrogenase (oxaloacetate-decarboxylating)	<i>Acinetobacter</i> , <i>Enterococcus</i>
[EC:1.1.1.40]	malate dehydrogenase (oxaloacetate-decarboxylating)(NADP+)	<i>Acinetobacter</i> , <i>streptomyces</i>
[EC:1.1.1.283 1.1.1.-]	methylglyoxal/glyoxal reductase	<i>Lactococcus</i>
[EC:1.1.2.4]	D-lactate dehydrogenase (cytochrome)	<i>Moraxella fulton</i>
[EC:1.1.5.4]	malate dehydrogenase (quinone)	<i>Megasphaera</i> , <i>Kocuria</i> , <i>Pseudoclavibacter</i> , <i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Corynebacterium</i> , <i>Pseudoclavibacter</i> , <i>Enhydrobacter</i> , <i>Rothia</i> , <i>Kocuria</i>
[EC:1.1.5.12]	D-lactate dehydrogenase (quinone)	<i>Acinetobacter</i> , <i>Enterococcus</i> , <i>Megasphaera</i> , <i>Gallibacterium</i>
[EC:1.2.1.3]	aldehyde dehydrogenase (NAD+)	<i>Enterococcus</i>
[EC:1.2.1.10]	acetaldehyde dehydrogenase (acetylating)	<i>Lactococcus</i> , <i>Enterococcus</i> , <i>Staphylococcus</i> , <i>Listeria monocytogenes</i>
[EC:1.2.1.10 1.1.1.1]	acetaldehyde dehydrogenase / alcohol dehydrogenase	<i>Lactococcus</i>
[EC:1.2.3.3]	pyruvate oxidase	<i>Pseudomonas</i> , <i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Salmonella</i> , <i>Pseudoclavibacter</i> , <i>Kaistia</i> , <i>Enterococcus</i> , <i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Enterococcus</i> , <i>Corynebacterium</i> , <i>Kocuria</i> , <i>Rothia</i>
[EC:1.2.4.1]	pyruvate dehydrogenase E1 component beta subunit	<i>Pseudomonas</i> , <i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Salmonella</i> , <i>Pseudoclavibacter</i> , <i>Kaistia</i> , <i>Enterococcus</i> , <i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Enterococcus</i> , <i>Pseudoclavibacter</i>
[EC:2.3.1.8]	phosphate acetyltransferase	<i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Enterococcus</i> , <i>Pseudoclavibacter</i>
[EC:2.3.1.9]	acetyl-CoA C-acetyltransferase	<i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i>
[EC:2.3.1.12]	pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase)	<i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Enterococcus</i> , <i>Pseudoclavibacter</i>
[EC:2.3.3.9]	malate synthase	<i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Salmonella</i> , <i>Kocuria</i>
[EC:2.3.3.13]	2-isopropylmalate synthase	<i>Pseudomonas</i> , <i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i>
[EC:2.7.1.40]	pyruvate kinase	<i>Pseudomonas</i> , <i>Lactococcus</i> , <i>Salmonella</i> , <i>Kocuria</i>
[EC:2.7.2.1]	acetate kinase	<i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Salmonella</i> , <i>Megasphaera</i> , <i>Kocuria</i> , <i>Enhydrobacter</i>
[EC:2.7.9.1]	pyruvate, orthophosphate dikinase	<i>Enterococcus</i> , <i>Megasphaera</i>
[EC:3.1.2.6]	hydroxyacylglutathione hydrolase	<i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Enterococcus</i>
[EC:4.1.1.31]	phosphoenolpyruvate carboxylase	<i>Pseudomonas</i> , <i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Salmonella</i> , <i>Shewanella</i> , <i>Kaistia</i>
[EC:4.1.1.32]	phosphoenolpyruvate carboxykinase (GTP)	<i>Acinetobacter</i> , <i>Pseudoclavibacter</i>
[EC:4.1.1.101]	malolactic enzyme	<i>Lactococcus</i>
[EC:4.1.1.112]	oxaloacetate decarboxylase	<i>Pseudomonas</i>
[EC:4.2.1.2]	fumarate hydratase, class II	<i>Pseudomonas</i> , <i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Staphylococcus</i> , <i>Microbacterium</i> , <i>Dermacoccus</i> , <i>Pseudoclavibacter</i>
[EC:4.4.1.5]	lactoylglutathione lyase	<i>Moraxella fulton</i> , <i>Lactococcus</i> , <i>Enterococcus</i>
[EC:6.2.1.1]	acetyl-CoA synthetase	<i>Acinetobacter</i> , <i>Escherichia coli</i> , <i>Salmonella</i> , <i>Kocuria</i>
[EC:6.4.1.1]	pyruvate carboxylase	<i>Rothia</i> , <i>Lactococcus</i> , <i>Microbacterium</i> , <i>Kocuria</i>
[EC:7.2.4.2]	oxaloacetate decarboxylase (Na ⁺ extruding) subunit alpha	<i>Listeria monocytogenes</i>

3.7 Sources of enzymes in the biosynthetic metabolic pathways of valine, leucine and isoleucine

Long-term storage has been shown to adversely affect the content and composition of milk proteins in pasteurized milk. Amino acids are one of the crucial substrates produced during the degradation of milk proteins. According to the results of the metagenomic study, a sizable fraction of the genes are involved in the metabolism of amino acids. The metabolism of alanine, aspartate, and glutamate, as well as lysine biosynthesis, valine, leucine, and isoleucine, are all more active during storage, as shown in Fig. 6. Microorganisms, enzymes, and other biochemical reactions cause the free amino acid concentration of pasteurized milk to fluctuate constantly throughout storage, which also impacts the milk's flavor and nutritional value. Biosynthesis of free amino acids (such as lysine) increases during storage, which may indicate enhanced hydrolytic activity of proteins during storage^[86]. Furthermore, there was a strong association between bitterness and total hydrophobicity, N-terminal hydrophilic amino acids, and C-terminal hydrophobic amino acid residues^[87,88]. One of the possible causes of the development of bitterness in pasteurized milk during storage is the increase in free amino acids like lysine. In order to identify changes in milk metabolites, Zhu et al.^[62] stored pasteurized milk at 4 °C. They discovered that valine, leucine, and isoleucine rose about 12-fold after 9 days of storage, demonstrating that valine, leucine, and isoleucine biosynthesis is gradually active throughout storage. Leucine and isoleucine can be converted into substances with fruity and alcoholic flavors, as well as acidic compounds with sour notes, which may adversely affect the quality of pasteurized milk to some extent^[80]. The valine, leucine, and isoleucine synthesis pathway belongs to the pyruvate derivative type, and pyruvate metabolism provides the carbon source for valine, leucine, and isoleucine biosynthesis. Table 5 shows the enzymes involved in the valine, leucine and isoleucine biosynthesis pathway involving valine-pyruvate aminotransferase [EC:2.6.1.66], alanine-synthesizing transaminase [EC:2.6.1.66 2.6.1.2], ketol-acid reductoisomerase [EC:1.1.1.86], branched-chain amino acid aminotransferase [EC:2.6.1.42], and threonine dehydratase [EC:4.3.1.19], etc. By tracing the source of the enzymes in the database, it was found that they are mainly produced by *Moraxella*, *Lactococcus*, *Acinetobacter*, and *Pseudomonas*. Additionally, pathways annotated in amino acid metabolism include arginine biosynthesis and phenylalanine, tyrosine, and tryptophan biosynthesis pathways (Fig. 6). Arginine and phenylalanine, associated with bitterness, showed greater pathway enrichment after 9 days of refrigeration^[89]. *Pseudomonas* exhibited the highest relative abundance on the 9th day of storage (Fig. 2B). The proteases secreted by these bacteria remained active even after pasteurization, further breaking down milk proteins into small peptide molecules and free amino acids, thereby promoting the formation of bitter compounds. Additionally, prior studies have reported that *Lactococcus* may upregulate phenylalanine and tryptophan concentrations during milk storage, and the accumulation of these amino acids is also a cause of unpleasant odors in milk^[80]. This discovery further indicates that microbial functional characteristics promote carbohydrate metabolism, protein hydrolysis, and the production of essential metabolites.

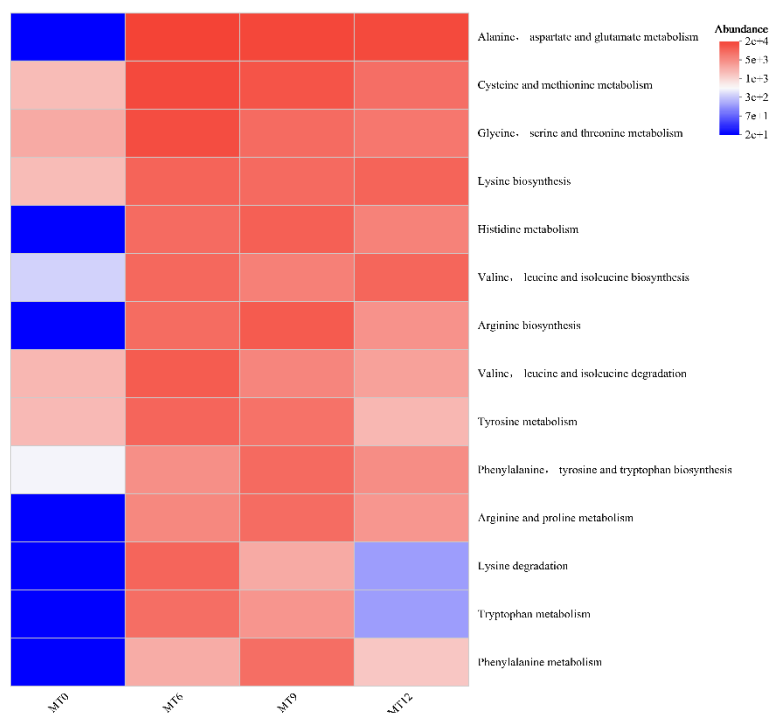


Fig. 6. Amino acid metabolism in pasteurized milk.

Table 3. Statistics on the source of enzymes in the biosynthetic metabolic pathways of valine, leucine and isoleucine.

Enzyme number	Enzyme Name	Source of genes involved in enzyme synthesis
[EC:1.1.1.85]	3-isopropylmalate dehydrogenase	<i>Moraxella fulton</i> , <i>Lactococcus</i> , <i>Acinetobacter</i>
[EC:1.1.1.86]	ketol-acid reductoisomerase	<i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Enterococcus</i> , <i>Pseudoclavibacter</i>
[EC:2.2.1.6]	acetolactate synthase I/II/III large subunit	<i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Enterococcus</i> , <i>Megasphaera</i> , <i>Pseudoclavibacter</i> , <i>Kocuria</i> , <i>Rothia</i> , <i>Janibacter</i>
[EC:2.3.3.13]	2-isopropylmalate synthase	<i>Pseudomonas</i> , <i>Moraxella fulton</i> , <i>Lactococcus</i> , <i>Acinetobacter</i> , <i>Kocuria</i>
[EC:2.6.1.42]	branched-chain amino acid aminotransferase	<i>Pseudomonas</i> , <i>Moraxella fulton</i> , <i>Acinetobacter</i> , <i>Lactococcus</i> , <i>Megasphaera</i> , <i>Pseudoclavibacter</i>
[EC:2.6.1.66]	alanine-synthesizing transaminase	Unclassified- <i>Moraxella fulton</i> , <i>Enhydrobacter</i>
[EC:2.6.1.66 2.6.1.2]	alanine-synthesizing transaminase	<i>Pseudomonas</i> , <i>Lactococcus</i> , <i>Acinetobacter</i>
[EC:4.2.1.9]	dihydroxy-acid dehydratase	<i>Pseudomonas</i> , <i>Moraxella fulton</i> , <i>Lactococcus</i> , <i>Acinetobacter</i> , <i>Pseudoclavibacter</i>
[EC:4.3.1.19]	threonine dehydratase	<i>Moraxella fulton</i> , <i>Lactococcus</i> , <i>Enterococcus</i> , <i>Notodromas</i>

3.8 Core functional microbiota associated with pasteurized milk quality

Correlation heatmaps were used to analyze the correlation between microbial community composition in pasteurized milk and milk quality. The results indicated that the protein content, SNF content, and b* in pasteurized milk were related to the microbial bacterial community (Fig. 7). *Pseudomonas*, as the recognized dominant genus in pasteurized milk, shows a significant negative correlation with fat content. *Pseudomonas* not only secretes protease to degrade κ -casein, but also secretes lipase, generating a putrid taste and smell, as well as a bitter taste, which shortens the shelf life of milk during storage and transportation^[90]. *Streptococcus*, *Enterococcus*, *Escherichia*, and *Klebsiella* were significantly negatively correlated with SNF content. During the dairy production process, *Streptococcus*, *Enterococcus*, *Escherichia*, and *Klebsiella* mainly originate from the farm environment, including bedding, soil, and water, and they can serve as pathogens causing mastitis

infection^[91,92]. *Streptococcus*, *Enterococcus*, *Escherichia*, and *Klebsiella* need to continuously utilize nutrients during the storage of pasteurized milk, resulting in a decrease in SNF content. This finding further indicates that the microbial metabolism in pasteurized milk samples promotes the metabolism of sugar, the hydrolysis of fat and protein, and the production of essential metabolites^[7]. Overall, microbial succession is closely associated with the depletion of nutrients in the milk environment and the accumulation of metabolites. Our correlation analysis indicates that dominant bacterial genera are key determinants of milk quality changes during refrigeration, which may be attributed to the specific physiological and metabolic activities of these microorganisms, particularly *Acinetobacter* and *Pseudomonas*.

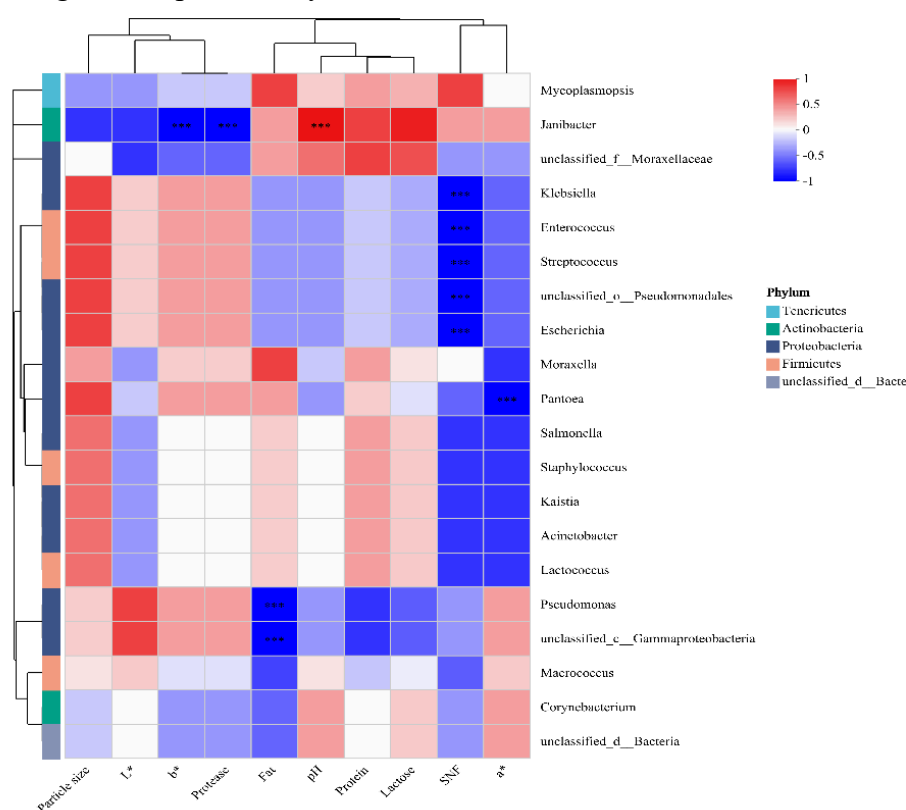


Fig. 7. Heat map analysis between physicochemical parameters and associated core bacterial communities

4. Conclusion

This study investigated dynamic changes in nutrients and dominant bacterial communities in pasteurized milk during 12-day storage at 4 °C. The results showed that the fat, protein, and lactose contents of pasteurized milk decreased slightly during storage, while acidity, particle size, and b* value (yellowness) increased significantly. Sensory analysis revealed pronounced bitterness and astringency in LT samples (65 °C/30 min), whereas MT treatment (75 °C/15 s) better preserved nutritional and sensory qualities. Microbial profiling identified *Firmicutes* and *Actinobacteria* as dominant phyla, with *Pseudomonas*, *Acinetobacter*, and *Lactococcus* constituting core genera. Microbial succession was found in pasteurized milk during storage. *Acinetobacter* dominated during the initial storage phase (0–6 days), displaying strong lipolytic activity. *Pseudomonas* peaked on day 9, exhibiting potent proteolytic capabilities. Day 9 of storage may represent a critical quality control phase for pasteurized milk. Pathogens such as *Salmonella* and *Staphylococcus* were also detected but may be suppressed by beneficial microorganisms, which makes storage from 0–9 days safer.

Functional annotation demonstrated increased gene abundance related to carbohydrate, amino acid, and energy metabolism over time. Annotations based on CAZy reveal that GEs and GHs were the most annotated families within CAZyme. The core microorganisms primarily influenced milk quality through pyruvate metabolism and branched-chain amino acid (valine, leucine, and isoleucine) biosynthesis pathways. Among them, the pyruvate metabolism as the core hub of carbohydrate decomposition, by generating lactic acid, acetic acid, succinic acid, ethanol, and other metabolites, led to rising acidity and a quality of texture change. The key enzymes involved in pyruvate metabolism and branched-chain amino acid synthesis mainly originate from *Acinetobacter*, *Pseudomonas*, *Lactococcus*, and *Moraxella*. The dynamic changes in core microbial community succession and dairy product quality likely result from the interaction between bacterial metabolism and milk components. These findings elucidate microbial community dynamics and functional metabolic pathways, providing theoretical foundations and practical strategies for quality control and shelf-life extension of pasteurized dairy products. However, the study has certain limitations. Future research will integrate metabolomics to quantify specific metabolite changes in pasteurized milk and clarify their correlations with microbial metabolism and dairy quality.

Appendix A. Supplementary data

The following is the Supplementary data to this article.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No.31972047); Mengniu Dairy Horizontal Cooperation Project (202309186027032916); Shenyang Science and Technology Innovation Platform Project (21-103-0-14, 21-104-0-28).

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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