Effects of Different Cold Storage Temperatures on Microbial Changes in Raw Milk

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Abstract [**Objectives**] This study was conducted to investigate the influence mechanisms of microbial succession in raw milk under cold storage at different temperatures. [**Methods**] A raw milk sample was collected from a local large-scale farm in Tangshan and divided into four treatment gradients: a control group (M) rapidly frozen at −80 ℃, and three experimental groups stored at 4 ℃ (T), 6 ℃ (F), and 8 ℃ (Y), respectively. A time series experiment was carried out according to time intervals of 24, 48 and 72 h in each experimental group. Traditional microbial culture methods and 16S rRNA high-throughput sequencing were combined to analyze the dynamic changes in microbial abundance and structural variation. [**Results**] Plate counting revealed significantly lower total bacterial count and psychrotrophic bacteria in the 4 ℃ storage group within 24 h compared with other treatment groups ($P<0.01$), confirming that maintaining low-temperature cold chain integrity and controlling treatment time (<24 h) can effectively inhibit microbial metabolic activity. 16S rRNA sequencing analysis revealed high initial microbial diversity in raw milk, with dominant genera being *Lactococcus*, *Acinetobacter*, and *Pseudomonas*. Low-temperature treatment effectively reduced the α diversity index of the microbial community. During the later stage of cold storage at 4 ℃, the relative abundance of *Pseudomonas* increased to over 90%, making it the dominant bacterial genus. [**Conclusions**] This study has significant application value for maintaining the quality of milk and dairy products and prolonging their shelf life.

Key words Raw milk; Refrigeration temperature; Psychrotrophic bacterium; Microbial community structure; Diversity

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The quality of raw milk is fundamental to ensuring dairy product quality[1]. During cold chain storage and transportation, microorganisms consume nutrients in raw milk, leading to changes in microbial community structure and psychrotrophic bacterium diversity[2], thereby affecting dairy quality. Furthermore, even if raw milk meets current national standards before processing, that is, the total bacterial count is below 2.0×10^6 CFU/g (ml)[3], the proliferation of dominant psychrotrophic bacteria during refrigeration can still influence raw milk quality. Studies have shown that storage temperature and duration are key factors determining the diversity of psychrotrophic bacteria[4-5]. The heat-resistant proteases and lipases produced by psychrotrophic bacteria can still degrade fats and proteins even after high-temperature sterilization, ultimately leading to spoilage of milk and dairy products[6-7]. In recent years, scholars at home and abroad have conducted sequencing analyses on microorganisms in raw milk, particularly psychrotrophic bacteria. Common psychrotrophic bacteria mainly include *Pseudomonas*, *Acinetobacter*, and *Flavobacterium*[8].

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Some studies have pointed out that the temperature for cold chain storage and transportation of raw milk should be maintained below 6 ℃. With prolonged cold storage, the nutrient content in raw milk decreases, while total bacterial count and psychrotrophic bacterium count increase significantly[9]. To investigate the effect of cold chain storage on raw milk quality, in this study, the cold chain storage and transportation environment of raw milk was simulated to investigate total bacterial count and the diversity/differences of psychrotrophic bacteria under varying low-temperature conditions and storage durations, and the correlation between milk quality changes and temperature fluctuations and storage time during cold chain storage was analyzed, aiming to provide a theoretical basis for optimizing cold storage conditions for raw milk.

Materials and Methods

Materials

Sampling was conducted in a raw milk production enterprise in Tangshan, Hebei Province, and 20 kg of freshly raw milk was aseptically collected. After cooling in a refrigeration chamber, the sample were transported to the laboratory within 1 h. The sample was divided into 10 treatment groups: control raw milk (M.1-M.3), 4 ℃ storage for 24 h (T1.1-T1.3), 48 h (T2.1-T2.3), and 72 h (T3.1-T3.3), 6 ℃ storage for 24 h (F1.1-F1.3), 48 h (F2.1-F2.3), and 72 h (F3.1-F3.3), and 8 ℃ storage for 24 h (Y1.1-Y1.3), 48 h (Y2.1-Y2.3), and 72 h (Y3.1-Y3.3). Samples were collected at predetermined intervals for plate culture and high-throughput sequencing experiments.

Methods

Determination of total bacterial count Total bacterial count

was determined according to *National Food Safety Standard-Food Microbiological Examination: Determination of Aerobic Plate Count* (GB 4789.2-2016).

Detection of psychrotrophic bacteria Psychrotrophic bacteria were determined with reference to *Enumeration of Colony of Psychrotrophic Microorganisms*, *Total Aerobic Bacterial Spores and Thermophilic Aerobic Bacterial Spores in Milk and Dairy Products* (NY/T 1331-2007).

Library construction and sequencing The PCR amplification products were purified using Agencourt AMPure XP beads and dissolved in an elution buffer for library construction. The fragment size distribution and concentration of the libraries were assessed using the Agilent 2100 Bioanalyzer. Qualified libraries were subjected to sequencing, and the resulting data were analyzed using bioinformatics methods.

Data processing Raw data from the Illumina platform were processed by removing barcode and primer sequences, followed by trimming and splicing to obtain high-quality effective sequences for subsequent analysis.

Results and Analysis

Changes in total bacterial count and psychrotrophic bacteria in raw milk at different storage temperatures

The changes in total bacterial count in raw milk during storage at different temperatures are shown in Fig. 1. Fig. 1(a) illustrates the changes in total bacterial count in raw milk stored at 4, 6, and 8 ℃, while Fig. 1(b) shows the changes in psychrotrophic bacterium count in raw milk stored at 4, 6, and 8 ℃.

The monitoring results showed that when stored at 4 ℃, raw milk maintained relatively low total bacterial and psychrotrophic bacterium counts within 24 h. After 24 h, psychrotrophic bacteria increased significantly, accounting for over 90% of total bacterial count. At storage temperatures of 6 and 8 ℃, total bacterial counts reached $(2.3 \pm 0.5) \times 10^6$ CFU/ml and $(3.8 \pm 0.6) \times 10^6$ CFU/ml respectively after 24 h, exceeding the national standard limit. It is recommended to maintain a storage temperature ≤4 ℃ throughout transportation and reduce pretreatment time to within 18 h.

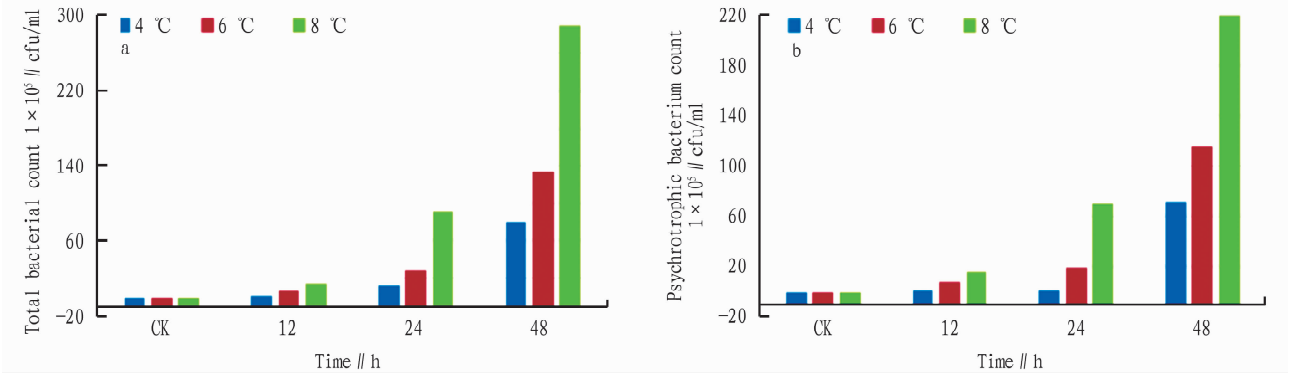

Fig. 1 Total bacterial count and psychrotrophic bacterium count in raw milk

Analysis of microbial diversity among different treatment groups based on venn diagram

Fig. 2 shows that among all samples, the 4 ℃ cold storage group had the highest number of overlapping OTUs with the control group (CK), indicating the greatest similarity in microbial community structure. The CK group (M) exhibited the richest microbial diversity, with high-throughput sequencing of 16S rRNA revealing 1 240 OTUs. After cold storage at 4, 6, and 8 ℃, microbial species richness significantly decreased ($P < 0.01$), and only a few cold-tolerant bacteria survived and gradually became dominant populations in the microbial community. More complex microbial species in raw milk increase the risk of quality deterioration during transportation.

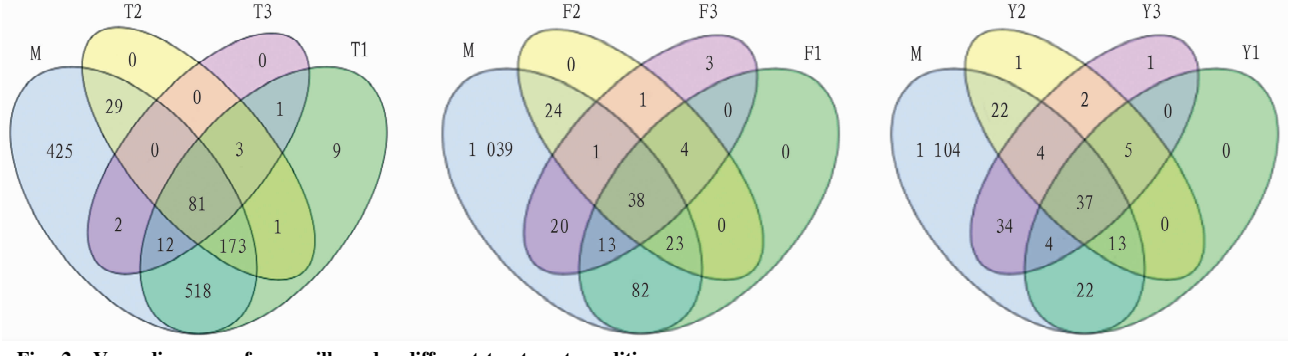

Fig. 2 Venn diagrams of raw milk under different treatment conditions

Principal component analysis of raw milk under different cold storage temperatures

In the PCA plot, closer distances between samples indicate greater similarity in microbial composition. Fig. 3 effectively characterizes the β-diversity features of microbial communities among the control group (M) and three cold storage treatment groups (4, 6, 8 ℃) at different sampling time points (T0, T24, T48). The results indicated that low-temperature treatment posed significant selective pressure on the microbial community structure. At T24 and subsequent time points, samples from the 6 and 8 ℃ treatment groups exhibited a clustering trend along the PC2 axis, suggesting convergent evolution in microbial community structure. In contrast, samples from the 4 ℃ treatment group showed significant niche separation from other groups, which might be closely related to the competitive advantage of psychrophilic bacteria under low-temperature conditions.

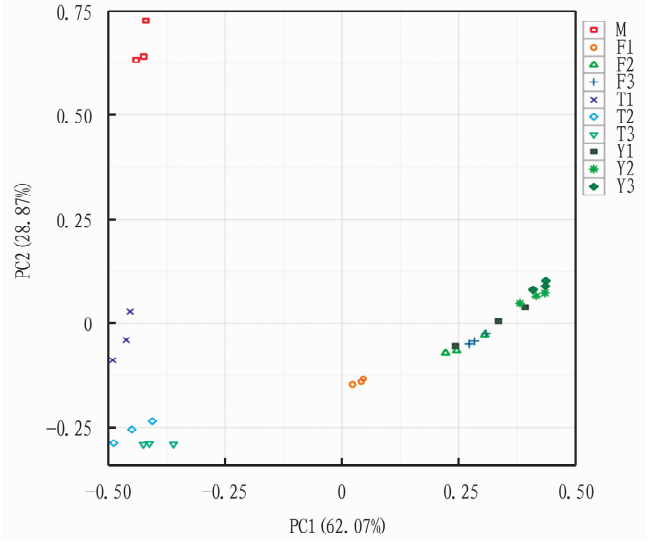

Fig. 3 Principal component analysis on bacterial OTUs in raw milk of different cold storage treatment groups

Bacterial community distribution at the genus level in various treatment groups

Microbial diversity analysis based on 16S rRNA high-throughput sequencing revealed that the bacterial populations in the 30 samples were annotated into 412 species, 385 genera, 202 families, 116 orders, 52 classes, and 20 phyla. At the genus level, the dominant bacterial genera included *Lactococcus*, *Acinetobacter*, *Citrobacter*, *Pseudomonas*, *Gluconacetobacter*, *Serratia*, and *Chryseobacterium*. Low-temperature treatment effectively reduced the α diversity index of microbial community.

The bacterial community distribution characteristics at the genus level in various treatment groups are shown in Fig. 4. *Acinetobacter* dominated significantly in fresh raw milk (M) and the 4 ℃ cold storage group (T). Notably, *Pseudomonas* exhibited a high peak abundance only in the 4 ℃ treatment group during cold storage. In contrast, *Lactococcus* became the absolutely dominant bacterial population under 6 ℃ (F) and 8 ℃ (Y) storage conditions. Further analysis revealed that in raw milk samples without low-temperature treatment, the known species *Acinetobacter* and *Chryseobacterium* jointly constituted the core microbial community.

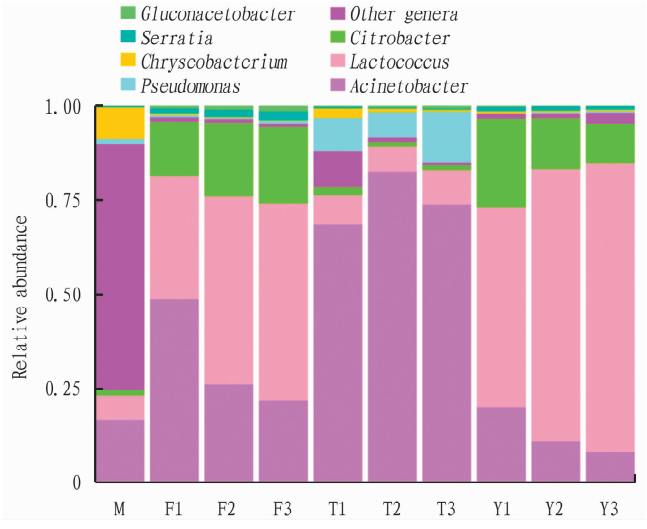

Fig. 4 Relative abundance of bacterial community under different treatment conditions at the genus level

Conclusions and Discussion

Effects of temperature on microbial proliferation

Compared with fresh raw milk, the total bacterial count and psychrotrophic bacterium count remained low within the first 24 h of storage at 4 ℃. However, after 24 h, the proportion of psychrotrophic bacteria increased significantly, exceeding 90%. Storage at 6 and 8 ℃ resulted in total bacterial counts surpassing the national standard limit (2×10^6 CFU/ml) after 24 h. Temperature increase showed a significant positive correlation with the growth rate of psychrotrophic bacteria ($P < 0.01$). Maintaining the cold chain at ≤4 ℃ and reducing the pre-processing period to within 18 h are critical for controlling microbial risks.

Changes in microbial community diversity

The raw milk exhibited the highest initial microbial diversity (with OTUs reaching 1 240), while low-temperature treatment significantly reduced α diversity ($P < 0.01$). Storage at 8 ℃ for 72 h (Y3) led to decreased species evenness and increased abundance of dominant populations, whereas the 4 ℃ treatment group maintained relatively higher species evenness. Although low temperatures inhibit most microorganisms, psychrotolerant bacteria (such as *Pseudomonas* and *Lactococcus*) become dominant, increasing the risk of dairy deterioration.

Microbial community structure differences and dominant genera

Principal component analysis (PCA) revealed significant separation ($P < 0.01$) in microbial community structure between low-temperature treatment groups and the control group. The 6 and 8 ℃ groups showed convergent community structures, while the 4 ℃ group exhibited distinct niche separation. *Pseudomonas* became the dominant core genus after 24 h of cold storage at 4 ℃, and its secreted proteases and lipases were identified as primary factors causing milk protein degradation, fat oxidation, and off-flavor development.

In summary, maintaining the integrity of the low-temperature cold chain (≤4 ℃) and reducing processing time are the core strategies for controlling the microbial safety of raw milk. The adaptive growth and metabolic activity of psychrotrophic bacteria are the primary causes of dairy deterioration. Maintaining the temperature at ≤4 ℃ throughout storage and transportation and reducing the processing interval to within 18 h can effectively inhibit the proliferation of psychrotrophic bacteria. Special attention should be given to the metabolic activity of dominant psychrotrophic bacteria (such as *Pseudomonas*). Optimizing sterilization processes to deactivate their heat-stable hydrolytic enzymes is crucial for extending the shelf life of dairy products.

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Meanwhile, it should be noted that the detection rate of nasopharyngeal swabs does not necessarily reflect the incidence rate[8]. In sows, it might indicate a carrying state, while in piglets, it could signify either carrying or infection. Additionally, there were differences in sampling methods before and after vaccination. There was a tendency of increasing difficulty in collecting pathological samples, leading to a greater reliance on swab testing and isolation. Therefore, variations in sampling methods might introduce certain biases in positive rate. These represent potential operational and statistical limitations in this study.

The use of Hps vaccines has always been controversial, and many farms prefer to control the disease through medication. It is because users perceive Hps vaccines as having low protective efficacy, coupled with the fact that drug-based prevention and control are effective. Based on the data from the farms in this study, Hps infection still occurred in the nursery stage after vaccination, with typical clinical symptoms of Glässer's disease observed. PCR testing confirmed the presence of the pathogen, and some farms even reported relatively high incidence rates. It seems to confirm the perceived low efficacy of Hps vaccines. However, serotyping data reveals a different reality. Under the selective pressure of vaccination, the prevalence of serotypes 4 and 5 significantly decreased, and the dominant prevalent serotypes shifted to others. Infection caused by these alternative serotypes resulted in milder lesions compared with serotypes 4 and 5, which explained the overall reduction in nursery mortality observed in the big data analysis. The results unequivocally demonstrate that Hps vaccination is indeed effective.

If properly matched Hps vaccines are proved to be effective, can we anticipate that by enhancing surveillance to track prevalent serotype shifts and subsequently updating vaccine formulations to replace serotypes or include more prevalent serotypes, we could achieve better control of Glässer's disease? This hypothesis requires further investigation for validation.

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