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Non-pasteurized milk enhances early colonization and persistence of *Lactobacillus* species in dairy calves promoting a stable hindgut microbiome

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Abstract

Pasteurization is a standard method to minimize pathogen transmission from milk to calves, and its impact on calf morbidity and growth performance has been well-documented. However, its long-term effects on the development of the gut microbiota and the underlying mechanisms remain poorly understood. In this study, a longitudinal analysis of 15 newborn calves was conducted over their first month, employing high-throughput sequencing and microbial cultivation to explore the impact of continuous feeding with non-pasteurized vs pasteurized milk on the calf growth performance and hindgut microbiome. Fecal and milk samples were collected, and microbial communities were analyzed using 16S rRNA gene sequencing. Additionally, microbial isolates were cultivated and phylogenetically characterized. Non-pasteurized milk has no significant impact on calf body weight, but it preserves microbial resources and facilitates the transfer of beneficial microbes such as *Limosilactobacillus reuteri* (*L. reuteri*) from milk to the hindgut. Extensive cultivation of *Lactobacillus* from milk and feces identified 40 distinct clusters out of 218 isolates using Rep-PCR. Whole-genome sequencing of 40 *L. reuteri* strains confirmed the presence of shared isolates in milk and the hindgut of calves. These *L. reuteri* isolates exhibited a remarkable ability to metabolize diverse oligosaccharides, including FOS, GOS, and inulin, contributing to early carbohydrate metabolism in dairy calves. By demonstrating how non-pasteurized milk fosters early microbial colonization and promotes a functionally stable gut microbiota, this study provides critical insights into the microbial and functional dynamics of the calf GIT. These findings highlight the potential of raw milk's microbial resources for optimizing calf development.

Introduction

The establishment and maturation of the gut microbiota during early life have far-reaching implications for growth performance and health later in life[1]. Shortly after birth, the gut microbiota undergoes a transition from being dominated by facultative anaerobes to strict anaerobes[2]. This process is influenced by numerous factors, among which diet is a crucial determinant in shaping the gastrointestinal microbiota of young animals[3,4]. Considering the high susceptibility during this critical period from birth to weaning, early interventions are crucial for regulating a healthy microbial ecosystem, thereby enhancing the health and productivity of adult dairy cows.

Liquid diet serves as the primary nutrient source for calf growth and development, as well as the substrate for microbial growth, primarily including whole milk, milk replacer, acidified milk, and waste milk[5]. Pasteurization technology is widely used in many countries, primarily in the treatment of colostrum and waste milk in modern dairy farms[6,7]. It ensures milk quality and biosafety, particularly by preventing the transmission of microbial pathogens from milk to suckling calves[6]. Early studies have demonstrated that feeding pasteurized colostrum and waste milk can enhance growth performance, improve health status, and increase economic returns in calf rearing[6,7]. While pasteurization effectively eliminates harmful pathogens in milk, such as *Salmonella*, *Listeria*, and pathogenic *Escherichia coli*, thereby reducing disease incidence in calves, it also eliminates beneficial probiotics, which may negatively impact the early gut microbiota. Thus, pasteurization may act as a double-edged sword. Its impact on gut probiotics and the underlying mechanisms warrant further in-depth investigation. To resolve this trade-off between safety and microbial health, the effects of pasteurized milk feeding on calf gut microbiota were systematically evaluated. Prior investigations have predominantly focused on the effect of heat treatment colostrum and waste milk on neonatal dairy calves[8,9]. The information on the effects of continuous feeding pasteurized milk on gut microbiome maturation in dairy calves is extremely limited. Consequently, the objective of this study was to explore the effect of continuous administration of pasteurized milk on the early intestinal microbial colonization of calves and identify crucial absent microorganisms after pasteurization.

In this study, 15 newborn dairy calves were enrolled to systematically investigate the effects of feeding pasteurized vs non-pasteurized milk on calf growth performance and gut microbiota development.

Materials and methods

Animal management

The experiment was conducted on a dairy farm in Zhenjiang, China. Holstein male calves were selected as experimental subjects based on the initial body weights of 40.65 ± 1.85 kg (mean ± SEM) and a serum total protein level of ≥ 5.5 g/dL. Calves were separated from their dams soon after birth and housed in individual hutches. Within the first 12 h, calves received two colostrum servings, equivalent to 10% of their body weight for the initial feeding and 5% for the subsequent feeding, administered with a nursing bottle. During the test period, the amount of milk fed per meal varies with the calf's body weight, and according to the feeding standards of the dairy farm, it is specifically as follows: 10 L of milk/d until 14 d old; 12 L of milk/d until 28 d old. The milk composition includes 3.28% protein, 3.54% fats, 5.01% lactose, 12.68% total solids, and 9.21% nonfat solids. Water was consistently available to the calves *ad libitum*. Treatments were non-pasteurized milk (CON; n = 7; colostrum and milk without pasteurization) and pasteurized milk (HT; n = 8; colostrum and milk pasteurized). The milk processing for the pasteurized group is explained below. The trial period is 28 d (Fig. 1a).

Colostrum and milk management

Colostrum was collected by farm staff within 2 h after calving to ensure optimal quality. The quality of colostrum was tested by a Brix refractometer (Brix value ≥ 23). The collected colostrum was mixed into a single homogeneous batch to eliminate individual variations among dams. The batch was thoroughly blended and then divided into two equal portions. One portion of the colostrum remained untreated and was used as the control. The other portion was pasteurized at 65 °C for 30 min using a Commercial Pasteurizer (FLDJL, China) to ensure consistent heat treatment. After processing, the pasteurized colostrum was stored at −20 °C to maintain its quality. Before feeding to the calves, the frozen colostrum was thawed and gently heated to 40 °C to ensure optimal temperature for calf consumption.

Raw milk was collected from the bulk tank and divided into two equal portions. One portion of the milk remained untreated and served as the control. The other portion was pasteurized at 65 °C for 30 min using the same Commercial Pasteurizer (FLDJL, China) to ensure consistency with the colostrum treatment. Throughout the processing of both colostrum and milk, strict adherence to the specified temperature and duration was maintained to ensure the effectiveness of pasteurization and the reliability of the experimental results. Detailed procedures for the treatment process are illustrated in Fig. 1b.

Health monitoring of calves

During the experiment, all calves were examined for their mental state and rectal temperature, respiratory rate, limb coordination, and umbilical area. The fecal standards (0 to 3 scales) referred to published literature[1,10]. If a calf is sick, it needs to be isolated promptly to prevent it from infecting other cattle with contagious diseases. In cases of severe diarrhea, antibiotics were administered for treatment; however, these instances led to the exclusion of affected calves from the experimental group.

Calf fecal samples

Fresh fecal samples were collected from all calves on days 1, 3, 5, 7, 10, 13, 16, 19, 23, and 28 before the morning feeding. Aseptic techniques involving anal stimulation with sterile gloves were employed to obtain fresh feces. A part of the fecal samples was promptly transferred on ice, incorporating a 50% glycerine solution in equal proportions. The remaining samples were stored at −80 °C until analysis.

Milk samples

Colostrum samples were collected before feeding calves. Milk samples were collected from a bulk tank on days 3, 5, 7, 10, 13, 16, 19, 23, and 28 before feeding and stored in 50 mL centrifuge tubes at −80 °C until analysis.

Analysis of carbohydrates in raw milk and colostrum

Sample analysis was conducted utilizing liquid bonding with the Class-Xevo TQ-XS instrument, and the chromatographic column employed was ACQUITY UPLC BEH Amid (e) with dimensions 2.1 mm × 150 mm and a particle size of 1.7 μm (Serial Number: 01733918918659). The content of the two sialyllactose, 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL), was quantified in the samples using an external quantitative method. For sample preparation, a moderate amount of milk sample was obtained through low-temperature centrifugation. Following centrifugation, the middle layer was taken out, and an appropriate volume of ethanol was added. After homogenization and subsequent decentralization, the upper layer was removed. The remaining solution was diluted with 50% ethylene, and the resultant mixture underwent turbulence before membrane organic testing. In the standard preparation process, varying concentrations of corrosion solution were created using a standard quantity and 50% ethyl ethanol. These solutions served as standards for comparison during the analysis of the sample. Each concentration was thoroughly tested using the same liquid bonding system to ensure accurate and reliable results.

Thin-layer chromatography (TLC)

The qualitative analysis of carbohydrate composition in bovine milk was conducted via TLC. Approximately 2 mL of vortexed colostrum and milk samples underwent centrifugation at 10,000 × g for 30 min to effectively separate cells and fat from the whey fraction. Following this, two times the volume of anhydrous ethanol was added, and the reaction was initiated at −20 °C for 4 h, achieving a protein removal efficiency of 88.55%. The treated milk samples (8 μL) were subsequently spotted four times onto TLC aluminum silica-gel sheets using microhematocrit capillary tubes. Glucose, lactose, galactose, 3'-SL, and 6'-SL were employed as controls. The TLC sheets underwent development twice, utilizing a mixture of n-butanol, acetic acid, and water in a ratio of 2:1:1 (vol/vol). For color development, 0.6 g of N-(1-naphthyl) ethylene-diamine dihydrochloride was dissolved by adding 10 mL of sulphonic acid to 190 mL of methanol. The resulting solution was then air-dried at 105 °C until discernible bands became visible.

The SCFAs in calf feces

The pre-treatment of filtered feces is initiated, and the determination of short-chain fatty acids (SCFAs) content is conducted using a gas phosphate spectrometer (Shimadzu GC-14B, Japan). The spectrometry columns utilized in this analysis are configured as DB-FFAP (Agilent, USA), with nitrogen serving as the carrier gas at a flow rate of 1.1 mL/min and a combustion gas composition of hydrogen (40 mL/min) and air (450 mL/min) at a flow ratio of 20:1. The temperature settings for the column box, input point, and detection point are established at 110 °C, 180 °C, and 180 °C, respectively.

16S rRNA gene sequencing

For fecal DNA extraction, fecal samples were homogenized in a FastPrep-24 (MP Biomedicals) for lysis. After mechanical lysis, DNA extraction, and purification were performed using the TIANamp Stool DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The microbial DNA from cow milk was extracted using the Takara kit (TakaRa MiniBEST Bacterial Genomic DNA Extraction Kit). The V3–V4 region of the 16S rRNA gene was amplified with the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGGTATCTAAT-3'). PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the

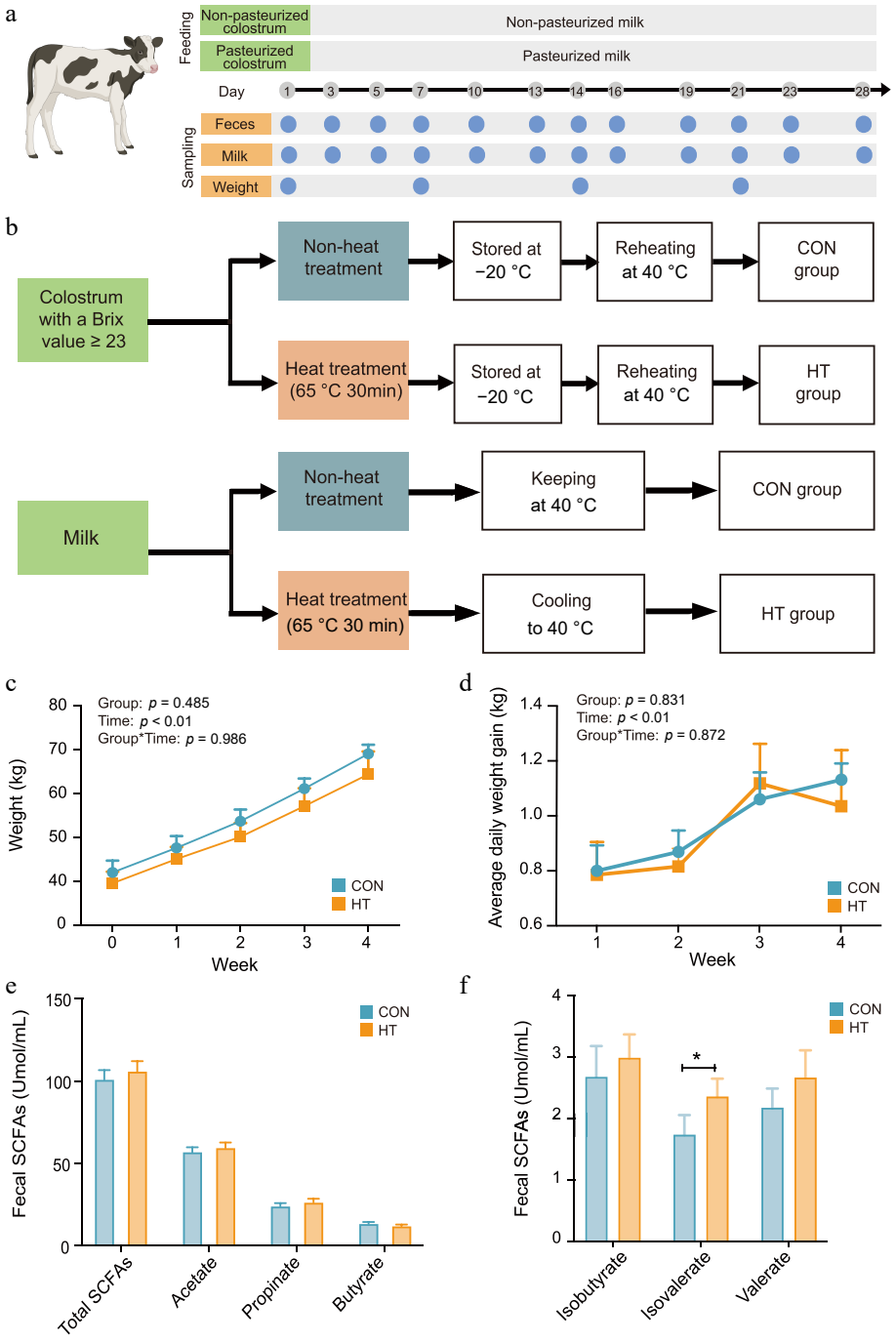

Fig. 1 Effect of feeding pasteurized milk on dairy calf growth performance. (a) Schedule of feeding and sampling throughout the whole experiment period. (b) The pasteurization flow process. (c), (d) Weekly weight and weekly ADG of calves. (e), (f) Changes in fecal total SCFAs in both groups throughout the test period. CON, control group; HT, pasteurization group.

manufacturer's instructions. The amplicons were paired-end sequenced (2 × 250) using the Illumina MiSeq platform (Shanghai BIOZERON Co., Ltd, Shanghai, China). Amplicon Sequence Variants (ASVs) were generated using the DADA2 pipeline (QIIME2-2020.11.1), which involves denoising raw sequencing reads to correct errors and remove chimeras, resulting in high-resolution, single-nucleotide sequence variants. The ASVs were determined by the RDP Classifier (http://rdp.cme.msu.edu/) against the Silva (SSU132) 16S rRNA database, achieving a species-level annotation rate of 89.9%.

Quantitative real-time PCR (qPCR)

The abundances of total bacteria, *Lactobacillus*, *L. reuteri*, and *L. johnsonii* in calf feces were determined by quantitative real-time PCR. The pure cultures of *Lactobacillus plantarum* YT041, *L reuteri*, and *L. johnsonii* as standard strains, and employed the traditional plate method to determine their colony-forming units per gram (CFU/g). These cultures were subjected to serial dilutions to obtain a known range of CFU/g values. Then, genomic DNA was extracted from the standard strains and purified. PCRs were performed in triplicate with SYBR Green chemistry (Fast SYBR Green Master Mix) on the StepOnePlus real-time PCR system from Applied Biosystems, employing bacterial primers as detailed in Supplementary Table S1. The qPCR program commenced with an initial denaturation step at 95 °C for 5 min, followed by 40 cycles comprising denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 30 s. Subsequently, a melting curve analysis was conducted at

95 °C for 15 s, followed by 60 °C for 1 min, and concluding with 95 °C for 15 min. A standard curve was generated for each target by plotting the cycle threshold (Ct) values against the log-transformed CFU/g values of the corresponding standard strain dilutions. This allowed us to establish a linear relationship between Ct values and bacterial load (CFU/g). Then, total DNA was extracted from fecal samples, and qPCR was performed using the same primers and conditions as for the standard curves. The Ct values obtained from the fecal samples were interpolated into the respective standard curves to determine the absolute quantities of total bacteria, *Lactobacillus*, *L. reuteri*, and *L. johnsonii* in CFU/g. The information on the standard curve is in Supplementary Table S2.

Isolation, purification, and identification of *Lactobacillus*

Stool aliquots were retrieved from storage and allowed to thaw at room temperature before being resuspended in 0.9% saline (1:9, w/v). The samples were thoroughly resuspended by vortexing and then serially diluted (10-fold) in prereduced 0.9% saline before being evenly spread on the agar plates of LAMVAB containing vancomycin for incubation at 37 °C for 48 h, aerobically. After an initial aerobic incubation, isolates were re-streaked to achieve purity. Presumptive colonies with distinct morphologies (ranging from 4 to 16 per sample) were selected and streaked onto pre-prepared MRS agar twice consecutively to obtain pure bacterial isolates. These pure colonies were then inoculated into MRS broth and incubated overnight at 37 °C. An equal volume of 50% glycerol (500 µl) was added to the overnight culture to achieve a final concentration of 25%. The mixture was thoroughly mixed and stored at −80 °C for subsequent analysis.

To confirm bacterial identity, the 16S rRNA gene was examined using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') via Sanger sequencing and searched for close relatives using the National Center for Biotechnology Information (NCBI) database.

Rep-PCR

The Rep-PCR method, known for its simplicity and efficiency, was selected for bacterial typing to distinguish between strains. Genomic DNA extracted from 207 *L. reuteri* strains served as the template for PCR amplification. The PCR reactions were conducted in a 25 µL volume, including 2 × Taq Mix, 2 pmol of the BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3'), 50–100 ng of bacterial DNA, and deionized water. The BOX-PCR protocol initiated with an initial denaturation step at 95 °C for 7 min, followed by 30 cycles comprising denaturation at 94 °C for 3 s, annealing at 92 °C for 30 s, and extension at 65 °C for 8 min. The process concluded with a final extension step at 65 °C for 8 min. Subsequently, the PCR products were electrophoresed on a 2% agarose gel (1,520 cm) for 2 h at a constant voltage of 90 V in 1TAE buffer. The gel electrophoresis results for *Lactobacillus* strains, identified using BOXA1R primers, displayed less variability for bands up to 2,000 bp. The GelJ.V.2.0 software was then employed for the analysis of the electrophoresis results. Similarity analyses were conducted using the Pearson product-moment correlation coefficient, and dendrograms were generated using the UPGMA method. Strains with a Pearson similarity of 90% or greater were defined as identical clones. This detailed procedure facilitated the identification and visualization of various carbohydrate components within cow's milk, offering valuable qualitative insights into the sample composition.

Assessment of growth curve and acidogenic potential of *L. reuteri*

The bacteria were picked from the glycerol tubes kept at −80 °C and streaked on MRS medium plates for activation and incubated at 37 °C for 24–48 h. The single colonies were then picked with an inoculating loop and streaked repeatedly until a single colony was isolated. Inoculate the single colony in MRS broth and incubate at 37 °C for 12 h. Prepare sterile 50 mL centrifuge tubes with 45 mL of MRS broth, inoculate 500 µL of overnight culture (1%), and incubate at 37 °C for 48 h. Take the fermentation broth at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h, and measure the OD value of *Lactobacillus* bacterial broth with an enzyme labeling instrument at the wavelength of 600 nm, respectively. The pH of the fermentation broth was also determined by a pH meter. Each group was repeated twice.

Measurement of the carbohydrate metabolism ability of *L. reuteri*

A basic medium bMRS (without carbon source) was prepared and separately supplemented with 1% glucose, 1% lactose, 1% galactose, 1% fructo-oligosaccharide (FOS), 1% galacto-oligosaccharide (GOS), 1% Inulin, 1% 3'-SL, and 1% 6'-SL to examine the bacterial fermentability of different carbohydrate. To prevent contamination, vancomycin hydrochloride (4 mg/mL) is added to the medium. The culture system consists of 200 µL (including 180 µL of bMRS, 20 µL of 1% carbohydrate solution, and 2 µL of bacterial suspension). These cultures were sequentially added to a 96-well plate and were incubated at 37 °C for 48 h. The optical density at 600 nm (OD_{600}) is measured at 0 and 48 h, respectively. 'Metabolic ability' is defined as the difference between the OD_{600} at 48 h and the OD_{600} at 0 h. Only the results with an OD_{600} increase greater than 0.1 in two replicates are considered as growth. All experiments are repeated three times.

Whole-genome sequencing of *L. reuteri*

Thirty-seven strains isolated from the hindgut of calves and three strains isolated from bovine milk were cultured on MRS agar plates at 37 °C. Single colonies were picked and propagated in MRS broth medium. Bacterial DNA was extracted using a bacterial DNA extraction kit (OMEGA) following the manufacturer's instructions. Subsequently, the purified DNA was quality-controlled using a TBS-380 fluorometer (Turner BioSystems Inc., CA). High-quality DNA samples ($OD_{260}/_{280}$ = 1.8–2.0, > 6 µg) were selected for library construction. The sequencing was performed by Shanghai Biozeron Biotechnology Co., Ltd. (Shanghai, China). The raw paired-end reads were trimmed and quality-controlled using Trimmomatic (v 0.36) with the parameters (SLIDINGWINDOW:4:15 MINLEN:75). This quality control process yielded high-quality clean data for subsequent analysis. CAZyme profiles were annotated by aligning high-quality reads to the dbCAN2 database (v20230802) using DIAMOND (v2.0.15.153). ABySS was used for genome assembly with multiple Kmer parameters, and the optimal result of the assembly was obtained. Subsequently, GapCloser software was applied to fill in the remaining local internal gaps and correct single-base polymorphisms in the final assembly result. For prokaryotic organisms, an *ab initio* prediction method was employed to obtain gene models for 26 strains. The gene models were identified using GeneMark. Then, all gene models were subjected to blastp against the non-redundant (NR) database in NCBI for functional annotation through the blastp module. In addition, tRNAscan-SE (v1.23) was used to identify tRNAs, and RNAmmer (v1.2) was employed to detect rRNAs. To construct a phylogenetic tree for the 26 high-quality strains, phyloXML was used for tree building, and iTOL was employed for the visualization and beautification of the phylogenetic tree.

Statistical analysis

In R (v4.1.2), Alpha-diversity metrics, including Shannon diversity, and Beta-diversity metrics (PCoA, Bray Curtis distances) were calculated using normalized data. The calculations were performed with the vegan package. For the significance testing of alpha diversity, a Linear Mixed Model (LMM) was employed from the lmerTest package. In this model, the calf served as the random effect, while Group and Time were treated as fixed effects. Differences in beta diversity were assessed using Adonis 2 (PERMANOVA test) with the vegan package in R. To investigate differential abundance at the genus taxonomic level and unrarefied data were subjected to analysis of compositions of microbiomes with bias

correction (ANCOM-BC) method. This analysis was executed using the ANCOMBC R package (v.1.4.0). For ADG and weight, a Linear Mixed Model (LMM) was employed, while the other indicators were tested using Mann-Whitney U-tests between each time point. These statistical analyses provide a comprehensive understanding of microbial diversity and abundance dynamics, allowing for robust assessments of the effects of group and time on various microbiome-related parameters.

Results

The effect of pasteurized milk on the growth performance of preweaning calves

To investigate the potential mechanisms underlying feeding pasteurized milk on calf health, the growth performance of 15 newborn dairy calves was continuously monitored over a month (Fig. 1a, b). During the whole experiment, calves fed non-pasteurized milk showed a slight numerical increase in growth compared to those fed pasteurized milk, although the difference was not statistically significant (Fig. 1c, d, $p = 0.396$, two-sided Mann-Whitney test). Short-chain fatty acids (SCFAs) are the principal metabolites of the microbiota. Analysis of the concentration of SCFAs in calf feces revealed that most of the SCFAs in feces were acetate, propionate, and butyrate, accounting for about 80%. Among all the SCFAs tested, isovaleric acid was the only one showing a significant difference, higher in the HT group than in the CON group (Fig. 1e, f, $p = 0.02$).

The *Lactobacillus* in non-pasteurized milk contributed to a more stable microbial community of calves

To further compare the fecal microbiota between the two groups and investigate the impact of feeding pasteurized milk on hindgut microbial colonization and maturation, we have conducted an in-depth exploration through 16S rRNA sequencing analysis on fecal samples. The results illustrated that the Shannon index (Fig. 2a) of the fecal microbiota increased with calf age, and there was no significant difference between the two groups. The ordination analysis based on Bray-Curtis distances revealed a significant difference in fecal microbiota in the HT group compared to the CON group (Fig. 2b, $p = 0.003$), suggesting microbial structure variances. Further investigation into the inter-subject variability revealed a more stable microbial community in the CON group (Fig. 2c), indicating that non-pasteurized milk plays a dominant role in shaping the calf gut microbiota. A generalized linear model (GLM) was applied to the microbial genera (ranked top ten) to evaluate the impact of pasteurization on microbial composition (Fig. 2d, Supplementary Fig. S1a, b). Using a logistic regression model with group designation (HT vs CON) as the dependent variable and the relative abundances of ten genera as independent variables, the study identified *Lactobacillus* ($p = 0.00187$)

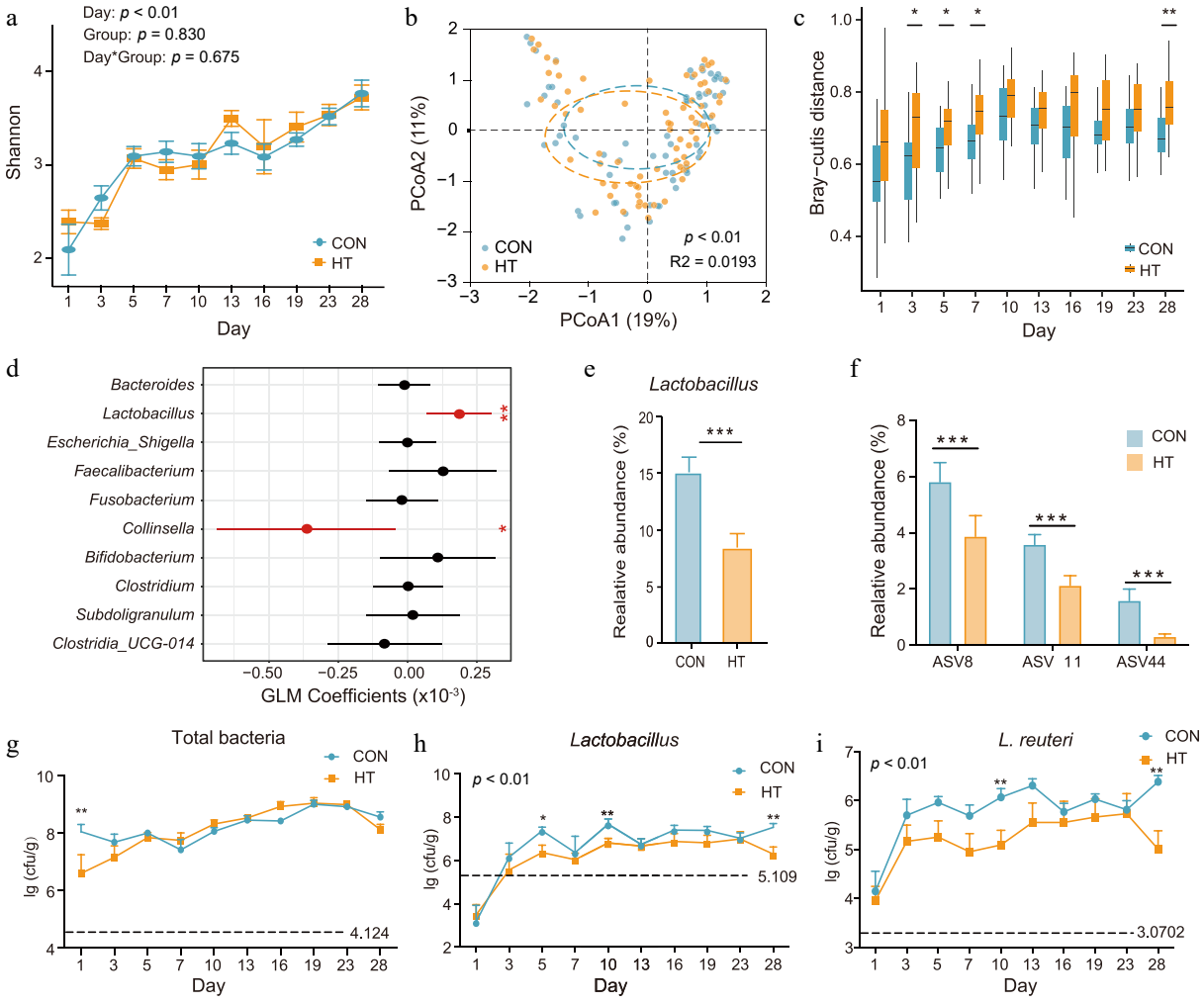

Fig. 2 Fecal microbial composition of the CON and HT groups over time. (a) Alpha diversity as measured by Shannon diversity index (LMM, $p > 0.05$). (b) Bacterial composition of CON and HT calves based on genus level visualized using principal coordinate analysis (PCoA). (c) Boxplot showing beta diversities (inter-community Bray-Curtis distance based on relative abundances) of bacterial communities between samples within each time point. (d) Estimates from the GLM analysis for quantifying the bacterial contribution on feeding pasteurized milk. (e), (f) Differences in relative abundance of *Lactobacillus* in calf feces (%). (g)–(i) Absolute abundance of total bacteria, *Lactobacillus* (U. test, $p < 0.01$) and *L. reuteri* (U. test, $p < 0.01$). CON, control group; HT, pasteurization group.

and *Collinsella* (p = 0.02626) as the primary genera significantly associated with group classification ($p < 0.05$). Further analysis using analysis of microbiome composition for bias correction (ANCOM-BC) indicated that *Lactobacillus* was significantly more abundant in the CON group ($p < 0.01$, Fig. 2e), particularly ASV8 (*L. joshnii*), ASV11 (*L. reuteri*), and ASV44 (*L. amylovorus*) ($p < 0.01$, Fig. 2f).

There was little distinction in the overall bacterial density between the two groups, except on the first day after birth when the microbial density in the CON group was significantly higher than that in the HT group (Fig. 2g, $p < 0.01$). With the maturation of the gut microbiota, there is no difference in the total bacterial density in the hindgut of calves between the two groups after the third day of age (Fig. 2g). Overall, the density of *Lactobacillus* increased with time in calf feces, consistent with the ANCOM-BC analysis, which demonstrated significant differences in *Lactobacillus* abundance between the two groups. qPCR analysis revealed that the density of total *Lactobacillus*, *L. reuteri*, and *L. joshnii* was higher ($p < 0.01$) in the CON group (Fig. 2h, i and Supplementary Fig. S1c). Additionally, *L. reuteri* was more abundant than *L. joshnii* in fecal samples (Fig. 2i and Supplementary Fig. S1c).

Pasteurization has no effect on the content of key carbohydrates in milk

The changes in key carbohydrates in milk before and after pasteurization were detected. Qualitative detection results via TLC assay revealed that pasteurization has no effect on the structure of key carbohydrates such as 3'-SL, 6'-SL, lactose, and others in colostrum and milk (Fig. 3a, b). In this study, the carbohydrates in milk were quantitatively tested. The concentrations of carbohydrates between the two groups were comparable, with no appreciable variations observed (Fig. 3c–g).

The inactivation of *Lactobacillus* in pasteurized milk likely leads to a decrease in the abundance of *Lactobacillus* in the hindgut of dairy calves

To examine the primary factors influencing variations in microbial colonization, a 16S rRNA sequencing analysis was conducted on colostrum and milk samples. Alpha diversity of colostrum and raw milk microbes did not vary significantly between the HT and CON groups (Fig. 4a). However, colostrum displayed higher diversity in microbial composition than raw milk (Fig. 4a). Beta-diversity analysis showed that the microbial structure had no significant differences between the two groups (Fig. 4b). *Proteobacteria*, *Bacteroides*, *Firmicutes*, and *Actinobacteria* as the four most abundant phyla in both colostrum and raw milk (Supplementary Fig. S2a, b). The core and abundant genera in colostrum included *Bradyrhizobium*, *Vibrionimonas*, *Mycobacterium*, and *Lactococcus* (Fig. 4c). At the genus level, the bacterial flora of cow's milk is mainly composed of *Vibrionimonas*, *Bradyrhizobium*, *Mycobacterium*, *Methylovirgula*, *Mesorhizobium*, *Burkholderia-Caballeronia-Paraburkholderia*, and *Rhodanobacter*, accounting for more than 80% of the total bacteria (Fig. 4d).

Despite the low relative abundance and presence of inter-population differences, potential probiotic bacteria, especially *Lactobacillus*, were found in colostrum and raw milk (Supplementary Fig. S2d). Sequencing does not identify the inactivation of microorganisms, especially after heat treatment. Therefore, we performed extensive cultivation and found that pasteurization treatment could inactivate most microorganisms in milk, including *Lactobacillus* (Supplementary Fig. S2c, d).

Whole-genome sequencing confirmed the transmission of *L. reuteri* from feeding milk to calf hindgut

A total of 333 *Lactobacillus* isolates were identified in calf feces (Fig. 5a). Among these isolates, *L. reuteri* (N = 215), *Lactobacillus salivarius* (*L. salivarius*) (N = 54), and *Lactobacillus rhamnosu* (*L. rhamnosus*) (N = 22) were the most frequently observed species, representing approximately 87.38% (excluding unclassified *Lactobacillus*, 291 out of 333). Additionally, *Lactobacillus amylolyticus* (*L. amylolyticus*) (N = 9), *Lacticaseibacillus paracasei* (*L. paracasei*) (N = 10), *Lactobacillus plantarum* (*L. plantarum*) (N = 3), and *Lactobacillus johnsonii* (*L. johnsonii*) (N = 1) were isolated within this cohort. Furthermore, 236 *Lactobacillus* strains were isolated and identified from samples of unpasteurized milk. The most frequently isolated species from milk were *L. paracasei* (N = 67), *L. amylolyticus* (N = 55), *Lactobacillus fermentum (L. fermentum)* (N = 41), *Lactobacillus pentosus* (*L. pentosus*) (N = 25), *and L. salivarius* (N = 23). *L. reuteri* (N = 3) was a rarely isolated species from milk samples. *L. reuteri* was the most dominant *Lactobacillus* in

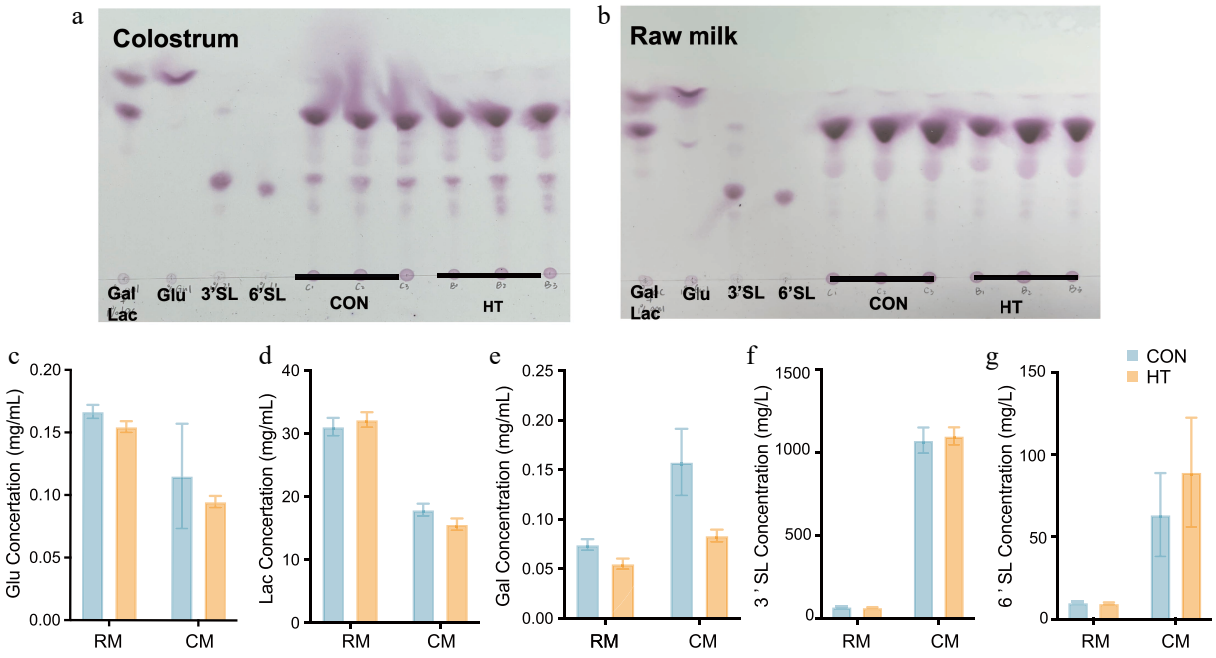

Fig. 3 The impact of pasteurization on key carbohydrates in milk. (a), (b) Results of TLC of key carbohydrates in raw milk and colostrum. (c)–(g) Carbohydrate content of raw milk and colostrum ($p > 0.05$). RM, raw milk; CM, colostrum; CON, control group; HT, pasteurization group.

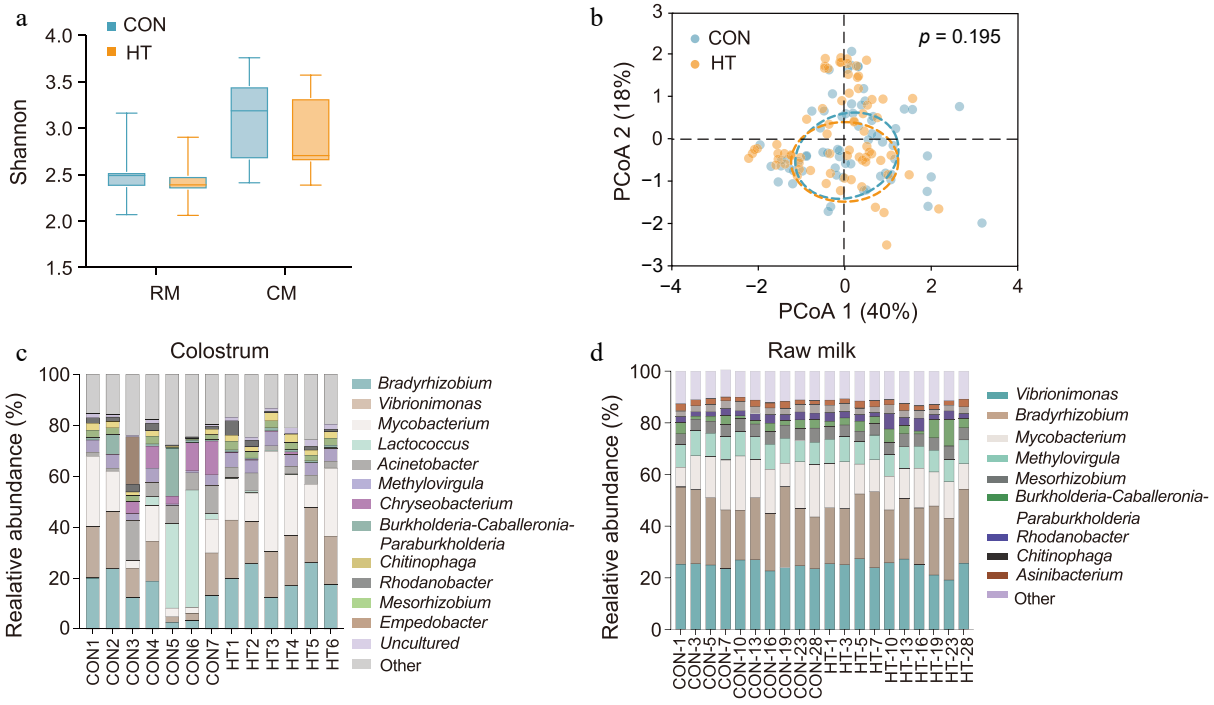

Fig. 4 Effect of pasteurization on the microbiota of milk. (a) Alpha diversity of milk and colostrum microorganisms ($p > 0.05$). (b) Bacterial composition of milk before and after pasteurization visualized using principal-coordinate analysis (PCoA). (c), (d) Composition of colostrum and raw milk microorganisms at the genus level. RM, raw milk; CM, colostrum; CON, control group; HT, pasteurization group.

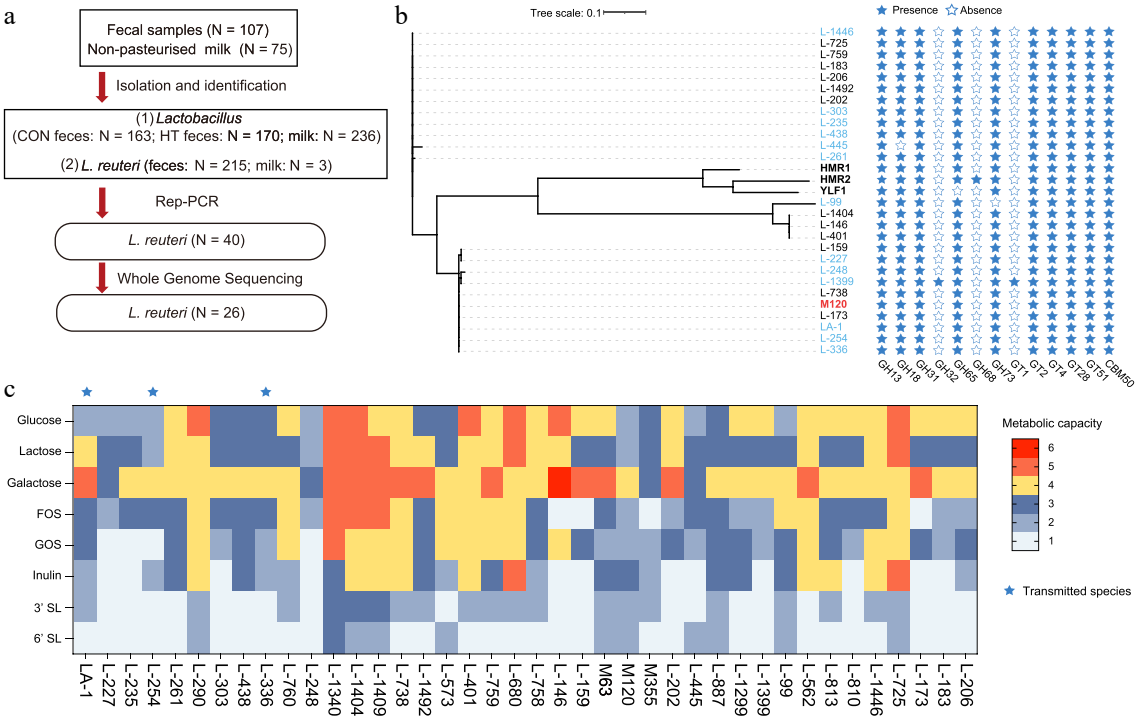

Fig. 5 Results of *Lactobacillus* isolation and identification. (a) Processes for strain screening and identification. (b) Phylogenetic analysis and the predicted glycosyl hydrolases in *L. reuteri*. Phylogenetic supertree showing the relationship in 29 *L. reuteri* strains. HMR1 and HMR2 are human-derived *L. reuteri* strains. YLF1 is a Yak-derived *L. reuteri* strain. Strains isolated from milk are marked in red. Strains isolated from CON group feces are marked in blue. Strains isolated from HT group feces are marked in black. (c) The metabolic capacity of *L. reuteri* for different carbon sources.

feces in terms of abundance. Since the accuracy of PCR amplification and sequence analysis based on the 16S rRNA gene can only be achieved at the species level to precisely identify and distinguish the strains, BOX-PCR fingerprinting has been carried out on 218 strains of *L. reuteri*. Analysis of the banding patterns obtained by PCR amplification using the BOX primers revealed that the 218 *Lactobacillus* strains were classified into 40 clusters, with a 90% similarity.

To analyze the phylogenetic relationships among *L. reuteri* strains, genome sequencing of 40 selected isolates was performed, resulting in 26 high-quality genome assemblies after rigorous quality filtering.

Subsequently, the iTOL software was utilized to construct a phylogenetic tree. The resulting phylogenetic tree (Fig. 5b) reveals the presence of three major branches among the analyzed members of the *L. reuteri* species. Based on the topological structure of the phylogenetic tree, it was observed that LA-1, L-254, and L-336 strains (isolated from the feces of calves in the CON group) clustered together with the bovine milk-derived M120 strain on the same branch, demonstrating high genetic similarity and possible homology. The three reference strains (HMR1, HMR2, and YLF1) showed a relatively far genetic distance from the strains isolated in this experiment on the phylogenetic tree, indicating their genetic relationships were relatively far apart.

To assess the carbohydrate fermentation capabilities of the *L. reuteri* strains, a prediction of carbohydrate-active enzymes (CAZymes) encoded in their genomes was performed using the CAZy database. This analysis revealed that CAZymes predicted in *L. reuteri* consisted mainly of glycoside hydrolases (GHs), glycosyl transferases (GTs), and carbohydrate-binding modules (CBMs). Carbohydrate esterases (CEs), polysaccharide lyases (PLs), and auxiliary activity (AA) family proteins were absent. The presence of CAZymes largely overlapped among the 26 strains of *L. reuteri*. It is noteworthy that there is no obvious difference in CAZymes between M120 (milk source) and *L. reuteri* derived from feces.

Transmitted *L. reuteri* exhibit a stronger capacity for galactose utilization

To further investigate the growth characteristics and capabilities of this subset of *L. reuteri*, the growth capacity and acid production capacity of 40 strains of *L. reuteri* was determined. The OD_{600} (bacterial growth) peaked at approximately 12 h and then entered a plateau period (Supplementary Fig. S3a–c). Notably, L-562 had the most prominent growth ability, and the growth abilities of other strains were equivalent. The acid-producing abilities of 40 strains of *L. reuteri* are shown in Supplementary Fig. S3d–f. Correspondingly with the growth curve results, L-562 had a more prominent acid-producing performance.

FOS, GOS, and inulin are common dietary prebiotics that are employed in the diet to modulate the neonatal gut microbiota of livestock. Meanwhile, 3'SL and 6'SL are the most representative oligosaccharide components in bovine milk. Therefore, the metabolic capacity of 40 strains of *L. reuteri* was tested on different carbon sources, and the results are shown in Fig. 5c. Specifically, the metabolic activities of *L. reuteri* strains are mainly concentrated on simple sugars such as glucose, lactose, and galactose. However, the number of strains that can respond to these two specific carbohydrates, 3'-SL and 6'-SL, is relatively small. Interestingly, the five transmitted species – LA-1, L-254, and L-336 – exhibited a stronger capacity for galactose utilization compared to other sugars. This distinctive ability may highlight the role of diet in shaping the microbial community to sustain a stable gut ecosystem.

Discussion

The early gut microbiota plays a crucial role in the long-term health of the host. Because of the immature intestinal function, newborn calves are highly susceptible to infections, leading to diarrhea, which is one of the primary causes of high morbidity and mortality among calves[11]. Considering the need to control the transmission of pathogens to calves, dairy farms commonly adopt pasteurization to treat milk. While pasteurization effectively reduces the risk of pathogens, it also leads to significant losses of natural probiotics in the milk. To evaluate the impact of pasteurized and unpasteurized milk on the gut microbial colonization of calves and understand the underlying reasons for this difference, the study selected 15 newborn Holstein calves as experimental subjects. Through a combination of sequencing techniques and cultivation methods, the changes in gut microbial communities in calves were systematically examined following the consumption of pasteurized milk.

In general, feeding pasteurized colostrum has no adverse effect on the performance of calves, and previous studies have confirmed that the production performance of calves fed with pasteurized colostrum before weaning is better than that of calves fed with non-pasteurized colostrum[12]. Likewise, no negative impact of continuous feeding of pasteurized milk on BW or ADG of calves was observed. The results of previous studies provide evidence that the benefits of feeding milk with a lower bacterial count for calves persist for at least three months after weaning[12]. By counting the viable bacteria in milk before and after pasteurization, it was found that heat treatment resulted in microbial loss. Therefore, pasteurization may not have a significant impact on the health of calves. As gut microbes break down carbohydrates, they produce gases, organic acids, and many SCFAs. Some bacteria are capable of fermenting amino acids and proteins as alternative energy sources[13], with protein decomposition typically resulting in the production of branched-chain fatty acids (BCFAs), such as isovaleric acid and isobutyric acid. In this study, higher levels of isovaleric acid were observed in heat-treated (HT) milk, potentially due to protein denaturation caused by the heat treatment; however, the study did not conduct a more in-depth analysis of the protein composition in milk. Notably, BSCFAs (such as isobutyric acid and isovaleric acid) are primarily produced through the fermentation of branched-chain amino acids (such as valine and leucine). A significant increase in the abundance of amplicon sequence variants (ASVs) from the genus *Bacteroides* was observed in the feces of calves fed pasteurized milk (not displayed), whereas previous studies have shown that the fermentation of branched-chain amino acids is mainly carried out by bacteria from the genera *Clostridium*, *Peptostreptococcus*, and *Bacteroides*[14]. Although this study did not directly measure protein denaturation or changes in composition, this finding supports the hypothesis that pasteurization may alter protein structure, prompting a shift in the gut microbiota towards protein hydrolytic metabolism, similar to the increased levels of branched-chain fatty acids (IVFAs) observed in calves fed milk subjected to high-temperature treatment. Therefore, future research could further explore the impact of pasteurization on protein denaturation and its association with functional shifts in the microbiota.

Pioneering bacterial species wield significant and enduring effects on immunological responses and metabolic processes, particularly during the early stages of development[15]. As the gut matures, the diversity and overall quantity of microbes are maintained at a healthy level, achieving a balanced state. The qPCR results showed a significant difference in the total bacteria in feces between the two groups on the first day. However, starting from the fifth day, the absolute abundance of total bacteria in the two groups reached a balanced state and exhibited similar changing trends with the increase in age. Overall, compared with calves fed with unpasteurized milk, the microbial diversity of feces from calves fed with pasteurized milk did not change significantly. Investigating the fecal microbiotas of 28 d old calves revealed that the hindgut of calves in the HT group lacked the colonization of *Lactobacillus* (especially *L. joshnii*, *L. reuteri*, and *L. amylovorus*) compared with the CON group and qPCR results also support this finding.

To investigate the differences between the above, the problem was approached from two aspects. Firstly, heat treatment may change the content of key components in milk[16]. Both qualitative and quantitative analyses were conducted to determine the content of key carbohydrates in milk before and after pasteurization. However, no significant difference was observed in the content of key carbohydrates in milk. Although carbohydrates may increase the abundance of intestinal *Lactobacillus*, the changes in *Lactobacillus* abundance observed in this study are not directly attributed to carbohydrates. As a

critical factor influencing the intestinal microbiota, the variation in the species and quantity of milk-borne microbes has a significant impact on the intestinal microbial environment of calves[17]. Thus, the study examined differences in microbial composition between pasteurized and unpasteurized milk. There is no significant difference in the composition and diversity of microorganisms in milk before and after pasteurization. Sequencing analysis could not identify the inactivation of microorganisms, especially after heat treatment. Therefore, LAMVAB was used to selectively culture *Lactobacillus* in milk and colostrum. The culture result indicated that *Lactobacillus* lost activation after pasteurization. Many studies have confirmed that pasteurization can effectively reduce the total live bacteria in colostrum[6], and our microbial culture results also support this finding.

Given that the probiotic capacity is strain-dependent, it is crucial to investigate its physiological function at the strain level. Therefore, *Lactobacillus* was selectively screened in calf feces and unpasteurized milk. *Lactobacillus* is a well-recognized probiotic, and preparations containing *Lactobacillus* have proven effective in reducing diarrhea in young animals. *L. reuteri* is the dominant *Lactobacillus* species in the feces of healthy calves in the first week after birth[18]. A variety of *Lactobacillus* strains, such as *L. reuteri*, *L. salivarius*, *L. rhamnosus*, *L. pentosus*, *L. plantarum*, *L. fermentum*, *L. jensenii*, *L. amylolyticus*, and *L. paracasei*, were screened from both non-pasteurized milk and calf feces with non-pasteurized milk. The coexistence of the same *Lactobacillus* genus in the hindgut and cow's milk provides the possibility for *Lactobacillus* in milk to colonize in the gut. Further differentiation of the *L. reuteri* isolates using Rep-PCR revealed 40 different band profile types, indicating gene variation within the species. The whole-genome sequencing analysis showed the presence of shared strains in both bovine milk and feces, and these strains exhibited differences in gene function from strains from other hosts. An increasing number of studies have indicated that gut microbiota colonization in newborn calves is largely related to vertical transmission from cows[19–21]. As a critical factor influencing the intestinal microbiota, the variation in the species and quantity of milk-borne microbes has a significant impact on the intestinal microbial environment of calves[17]. Martín et al. suggested that *Lactobacillus* in breast milk serves as the primary source of *Lactobacillus* in infant guts[22]. It was reported that isolated and identified identical *Lactobacillus* strains from multiple mother-infant pairs[23] provided evidence that *Lactobacillus* can vertically transmit to the intestinal tract of infants through breastfeeding. Therefore, it is inferred that the colonization of *Lactobacillus* in calf guts is closely associated with bovine milk, and pasteurization treatment may disrupt this process, subsequently reducing the abundance of *Lactobacillus* in the intestine.

Carbohydrate metabolism is also the main source of energy metabolism for *Lactobacillus*, thus contributing to their ecological adaptability[24]. On one hand, common prebiotics such as FOS, GOS, and inulin were selected to test the fermentation ability of *L. reuteri*. The results showed that 40 strains of *L. reuteri* had certain metabolic abilities toward different carbon sources. Studies have shown that *L. reuteri* has a high level of utilization of GOS. In the lactose operon in *L. reuteri*, the lac gene can encode hydrolases, transporters, or galactoside substrates necessary for GOS metabolism, enabling *L. reuteri* to fully utilize GOS[25]. On the other hand, it has genetically been demonstrated that *L. reuteri* may possess a broad range of carbohydrate metabolic capabilities. Consistent with the sourdough isolates and the rodent isolates, members of GH13, GH31, and GH65 were also annotated in the genome of *L. reuteri* isolated from milk and calf feces[26]. The presence of GH32 (β-fructofuranosidase) suggests that *L. reuteri* may be able to metabolize FOS. These results indicate that *L. reuteri* possesses a certain capability of carbohydrate metabolism, providing a basis for future research on the combination of probiotics with different prebiotics.

Conclusions

This study compares pasteurized vs non-pasteurized milk's effects on calf hindgut microbiota, showing that non-pasteurized milk preserved microbial resources, facilitating the transfer of beneficial microbes such as *L.reuteri* from milk to the hindgut. Although raw milk poses pathogen risks (e.g., diarrhea, mortality), justifying pasteurization for safety, this process sacrifices functional probiotics. This finding is globally relevant, as dairy systems vary (e.g., China's pasteurized whole milk vs other countries' pasteurized colostrum or waste milk). While the study did not assess pathogen inactivation, its findings highlight the need to balance microbial safety with probiotic loss. Future research should explore probiotic supplementation in pasteurized milk to restore gut health benefits. This work advances understanding of calf microbiota and offers practical insights for optimizing feeding protocols.

Ethical statements

The protocols used in this study were approved by the Nanjing Agricultural University Institutional Animal Care and Use Committee; Identification number: NJAU. No20210920N08; Approval date: 2021-9-20. All calves were treated in compliance with the Ethical and Animal Welfare Committee of Jiangsu Province, China. The research followed the 'Replacement, Reduction, and Refinement' principles to minimize harm to animals. This article provides details on the housing conditions, care, and pain management for the animals, ensuring that the impact on the animals is minimized during the experiment.

Author contributions

The authors confirm their contributions to the paper as follows: study conception and design: Liu J; sample collection and experiment conduction: Yu Y, Zhang W, Gao Y, Zhang T, Shi S, Zhan P; statistical analyses and draft manuscript preparation: Qiu S, Zhang W; manuscript revision: Zhang X, Liu J. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The sequences described in this article have been submitted to the NCBI Sequence Read Archive under the following accession number: PRJNA1149344. All other data are contained within the main manuscript and its supplementary information files.

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Conflict of interest

The authors declare that they have no conflict of interest. Jinxin Liu is an Editorial Board member of *Animal Advances* who was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer review handled independently of this Editorial Board member and the research groups.

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