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Processing-Associated Taxonomic Profiles of Microbiota in Liquid and Powdered Horse Milk Revealed by Full-Length 16S rRNA Sequencing

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ABSTRACT

Horse milk contains diverse microbial communities whose composition may be altered by processing and the addition of carrier ingredients. This study evaluated the effects of milk form and dextrin supplementation on the microbiota of horse milk prepared as pasteurized liquid milk (SKC), powdered milk supplemented with 15% dextrin (SKD), and powdered milk without dextrin (SKT). Microbial diversity and composition were analyzed using full-length 16S rRNA gene sequencing covering the V1–V9 regions. SKT showed the highest microbial richness, with 1185 observed taxa and a Shannon index of 3.43 ± 0.23, compared with 963 taxa and 2.40 ± 0.99 in SKD and 190 taxa and 2.02 ± 0.06 in SKC. SKC and SKD were dominated by lactic acid bacteria, particularly *Lactobacillus gallinarum* and *Lactobacillus helveticus*, whereas SKT contained higher proportions of stress-tolerant and spore-forming taxa such as *Brevibacillus parabrevis* (26%), *Thalassoporum komareki* (12%), and *Lysinibacillus boronitolerans* (10%). Sankey diagrams and principal component analysis further demonstrated distinct microbial community structures among the treatments. Overall, dextrin supplementation appeared to act as a protective carrier during drying, helping maintain the predominance of lactic acid bacteria, whereas powdered milk produced without dextrin was associated with a broader microbial profile.

Keywords: Dextrin; full-length 16S rRNA; horse milk; microbiota; powdered milk

INTRODUCTION

Processing methods and the use *of* additives are known to play a crucial role in shaping the microbial taxonomic profiles of dairy products (Abdul Hakim *et al.*, 2023). In mare milk, the natural microbiota is important from both technological and safety perspectives because it contributes to fermentation characteristics, product stability, and the presence of beneficial microorganisms, while also influencing the risk of contamination by undesirable taxa (İstanbullugil *et al.*, 2025; Boranbayeva *et al.*, 2025). However, information regarding the dynamics of its microbial community in relation to processing conditions remains limited (Kossaliyeva *et al.*, 2025).

Mare milk is reported to contain relatively high levels of lysozyme and lactoferrin compared with cow milk, which may contribute to its natural antimicrobial properties and longer shelf life (Kossaliyeva *et al.*, 2025). In addition, the high lactose content of mare milk provides a favorable substrate for the growth of lactic acid bacteria, which are commonly reported as dominant members of the mare milk microbiota (Kudaibergenova *et al.*, 2025). Like other dairy products, however, mare milk remains susceptible to microbial contamination during handling and distribution (Ljubojević Pelić *et al.*, 2024; Shalini & Gurunathan, 2025). To improve stability and facilitate transportation, milk is often converted into powder by drying, sometimes with the addition of dextrin as a carrier compound (Wu *et al.*, 2024). Although these approaches improve storage stability, they may also modify the microbial community by reducing heat-sensitive microorganisms and favoring stress-tolerant taxa (Lohita & Srijaya, 2024; Gao *et al.*, 2021).

Previous studies on cow, goat, and camel milk have shown that processing treatments such as pasteurization, fermentation, and drying can alter microbial diversity and community structure (Bao *et al.*, 2022; Monareng *et al.*, 2025). Heat treatment and food additives may reduce the abundance of lactic acid bacteria, whereas spore-forming taxa such as *Bacillus* and *Paenibacillus* can persist in dried milk products (Dash *et al.*, 2022; Grujović *et al.*, 2022; Liu *et al.*, 2025). In mare milk, lactic acid bacteria, particularly *Lactobacillus* and *Streptococcus*, are generally reported as dominant taxa (Monareng *et al.*, 2025). Nevertheless, little is known about how drying and dextrin addition affect the diversity and taxonomic composition of the mare milk microbiota during powder production (Blanco-Doval *et al.*, 2024; Wei *et al.*, 2021).

To the best of our knowledge, no previous study has systematically compared the microbial communities of mare milk in liquid form, powdered form, and powdered form supplemented with dextrin under the same processing conditions. We hypothesized that drying would modify the microbial community structure of mare milk and that dextrin addition, through its role as a protective carrier during dehydration, might help preserve lactic acid bacteria during powder production, thereby resulting in a microbial profile different from that of milk powder produced without dextrin (Kong *et al.*, 2025; Hamed *et al.*, 2025). The application of metagenomic approaches, such as full-length 16S rRNA gene sequencing to investigate and compare processing-associated microbiota profiles across these three milk forms has also been rarely reported in the literature (De Melo Pereira *et al.*, 2022; Supamri *et al.*, 2025). Such an approach can provide more detailed information on microbial taxa that persist, decline, or emerge following different treatments and may help identify microbial groups with potential technological relevance (Nam *et al.*, 2023).

Accordingly, this study compared the microbiota of pasteurized liquid milk, powdered milk supplemented with 15% dextrin, and powdered milk without dextrin using full-length 16S rRNA gene sequencing. The objective was to evaluate treatment-related changes in microbial diversity and composition at the phylum, genus, and species levels and to identify taxa shared among, or specific to, the different milk forms. The results provide descriptive information relevant to food microbiology, particularly for horse milk–based products, and serve as a reference for future studies exploring microbial stability and potential functional roles following powder production and dextrin supplementation.

MATERIALS AND METHODS

Mare's Milk Sampling

Approximately 3 liters of raw Sumbawa mare's milk were purchased directly from local producers located near the Curi Mori Karamabura partner site. The milk was obtained during routine morning milking and immediately placed into sterile containers in accordance with standard hygiene and sanitation procedures. After collection, the milk was portioned into sterile breast milk storage bags with a capacity of 150 mL using aseptic handling techniques. Throughout collection and initial handling, the temperature of the milk was maintained at approximately 4 ± 1 °C. The samples were then transported to the laboratory within about 2 hours using dry ice and subsequently stored at −80 °C until further processing and analysis.

Preparation of Mare's Milk Samples

Sumbawa mare's milk was processed into three different forms: pasteurized liquid milk (SKC), powdered milk supplemented with 15% dextrin (SKD), and powdered milk without dextrin (SKT). Before treatment preparation, raw mare's milk was pasteurized using the low-temperature long-time (LTLT) method at 63 °C for 30 min in a thermostatically controlled water bath (Memmert WNB 14, Memmert GmbH, Germany), followed by immediate cooling to 4 °C. For each treatment, 500 mL of pasteurized milk was used. The powdered products were prepared separately using a vacuum-drying technique. A digital vacuum drying oven was selected because it is more suitable for laboratory-scale processing and provides lower oxygen exposure and milder thermal conditions than spray drying, which may help preserve the detectable microbial profile prior to sequencing. In the SKD treatment, the milk was supplemented with 15% dextrin and whipped to produce stable foam prior to the drying process. The procedure followed the method described in our previous study (Sari *et al*., 2025). Prior to processing, the milk was weighed and mixed with 1% Tween 80 as a food-grade emulsifier, while dextrin at a concentration of 15% was added according to the treatment design.

The use of 15% dextrin was determined based on preliminary optimization experiments carried out in our laboratory. These trials indicated that this concentration achieved a suitable balance between drying performance, powder stability, and the prevention of protein aggregation during dehydration. Similar carrier concentrations have also been reported in dairy powder processing applications (Tița *et al.*, 2024). In addition, Tween 80 (1%) was added to improve the dispersion of milk components, support stable foam formation prior to drying, and reduce phase separation during dehydration. The use of such emulsifiers is commonly reported in vacuum- and spray-drying systems involving carbohydrate–protein matrices (Exarhopoulos *et al.*, 2025).

Drying was carried out using a digital vacuum drying oven (VOV-91, B-One Scientific, Korea) operated at 70 °C under a vacuum pressure of −66 cmHg for 7 hours. The resulting powders had final moisture contents of 4.33 ± 0.14% for powdered milk supplemented with 15% dextrin (SKD) and 4.54 ± 0.13% for powdered milk without dextrin (SKT). After drying, the resulting flakes were milled using a laboratory grinder (FGD-20, FOMAC, Indonesia) for 1–2 minutes to obtain a fine and homogeneous powder. Three milk batches were prepared, and each treatment was produced in triplicate to ensure consistency during the drying process. One representative sample from each treatment was subsequently selected for sequencing. Each selected sample was analyzed twice during the sequencing workflow to confirm technical consistency. The powdered samples were then stored in a desiccator at room temperature and kept protected from light until further analysis.

Microbial Cell Preparation Prior to DNA Extraction

Prior to DNA extraction, the three horse milk samples—SKC (pasteurized liquid milk), SKD (powdered horse milk with dextrin), and SKT

(powdered horse milk without dextrin)—were gently homogenized to ensure an even distribution of microbial cells. An aliquot of 1–2 mL from each sample was then transferred into sterile microcentrifuge tubes and centrifuged at 10,000 × g for 10 minutes to concentrate the microbial biomass. After centrifugation, the supernatant was carefully removed, while the pellet containing microbial cells was retained for genomic DNA extraction. All procedures were carried out under sterile conditions to minimize the risk of external contamination.

16S rRNA Gene Sequencing and Taxonomic Analysis

Three types of Sumbawa mare's milk samples—SKC, SKD, and SKT—were analyzed to characterize their microbial communities. For each treatment, one independently prepared milk batch was selected for full-length 16S rRNA gene sequencing. To improve sequencing reliability and maintain technical consistency, two technical replicates were included during the next-generation sequencing (NGS) workflow. These replicates served exclusively for quality-control purposes and were not treated as independent biological replicates.

Genomic DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, D4300) following the manufacturer's instructions, which included mechanical lysis through bead-beating to facilitate effective disruption of microbial cells. All extraction steps were carried out in a sterilized laminar flow hood using DNA-free consumables to reduce the risk of contamination. A negative extraction control (blank control) was included in each extraction batch and processed through library preparation and sequencing to detect any potential background contamination. DNA concentration and purity were evaluated using a Qubit fluorometer and a NanoDrop spectrophotometer, respectively.

Sequencing was performed using the Oxford Nanopore Technologies (ONT) platform, which enables long-read sequencing of the full-length 16S rRNA gene encompassing the V1–V9 regions, thereby providing improved taxonomic resolution (Bahram *et al.*, 2019). All procedures, including DNA extraction, library preparation, and sequencing, were conducted at PT Genetika Science Indonesia. Library preparation followed the standard ONT protocol using official ONT kits (PT Genetika Science Indonesia, 2023). Sequencing runs were carried out on a MinION device operated with MinKNOW software version 23.04.5, while basecalling was performed using Guppy software version 6.5.7 with the high-accuracy model (Wick *et al.*, 2019). Prior to taxonomic classification, quality control and data preprocessing were conducted. The resulting FASTQ files were first examined using NanoPlot version 1.41.0 to evaluate read length distribution and sequence quality metrics. Low-quality reads were subsequently filtered using NanoFilt version 2.8.0, applying a minimum Q-score threshold of ≥7 and a minimum read length of ≥500 bp, ensuring that only high-quality reads were retained for downstream analysis (De Coster *et al.*, 2018; Nygaard *et al.*, 2020).

Taxonomic assignment was carried out using Centrifuge software (Kim *et al.*, 2016). The reference database included bacterial and archaeal sequences obtained from the NCBI 16S RefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/). The use of full-length 16S rRNA gene sequences allowed improved taxonomic resolution, enabling putative identification of microbial taxa at the species level.

Analysis and Statistics

Relative abundances of microbial taxa were derived from the 16S rRNA sequencing data and used to generate community profiles at the phylum, genus, and species levels. To ensure biological relevance and minimize analytical noise, only taxa with a relative abundance of ≥1% were considered. Within this threshold, analyses were further restricted to the ten most abundant taxa (Supamri *et al.*, 2025).

Alpha diversity metrics, including the Shannon, Simpson, and Inverse Simpson indices, were calculated to characterize patterns of microbial richness and evenness among treatments. For each treatment, one representative sample was selected for sequencing and analyzed twice during the sequencing workflow to confirm technical consistency. Descriptive statistics (mean ± standard deviation) were calculated from these duplicate measurements. Because the dataset was derived from repeated technical measurements of the same sample rather than from independent samples, the alpha diversity results were interpreted descriptively to identify trends in microbial diversity among treatments. Sankey diagrams were constructed from the relative abundance matrix to visualize microbial community dynamics across treatments, with branch width representing the number of operational taxonomic units (OTUs). All analyses and data visualizations, including relative abundance calculations, stacked bar plots, alpha diversity boxplots, Sankey diagrams, and principal component analysis (PCA), were performed using OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA).

RESULTS

Relative Abundance of Dominant Microorganisms Microbiota Composition Analysis

The analysis of microbial composition revealed that ten phyla were detected with relative abundances exceeding 1%, whereas the remaining taxa occurred below this threshold. Among these groups, Bacillota represented the predominant phylum across all treatments (Figure 1a). Its relative abundance was particularly high in the SKC (98%) and SKD (95%) samples, indicating a consistent dominance of the core microbial community. In contrast, in SKT, the abundance of *Bacillota* drastically decreased to 60%, accompanied by an increase in the proportion of *Cyanobacteriota* (31%), *Pseudomonadota* (5%), and *Planctomycetota* (2%). In addition, other taxa such as *Actinomycetota* (1.3%) and *Bacteroidota* (1.2%) were also noticeably present in SKT.

At the genus level, five taxa were identified with a relative abundance above 1%, while the other genera were below that threshold (Figure 1b). SKC and SKD showed very strong dominance by *Lactobacillus*, at 96% and 86%, respectively. In contrast, SKT displayed a more even and complex microbial community distribution, with various dominant genera such as *Lysinibacillus* (20%), *Bacillus* (19%), and *Brevibacillus* (16%). Other genera with proportions above 1% in SKT included *Thalassoporum* (12%) and *Paenibacillus* (4%).

Seven taxa at the species level had a relative abundance above 1%, while other species were detected in lower amounts (Figure 1c). SKC and SKD showed relatively similar dominant compositions, marked by the presence of *Lactobacillus helveticus* (SKC: 32%; SKD: 30%), *L. gallinarum* (SKC: 39%; SKD: 40%), and *L. crispatus* (9% in both samples). In contrast, the SKT samples exhibited a shift in microbial community composition, characterized by the predominance of *Brevibacillus parabrevis* (26%), *Thalassoporum komareki* (12%), and *Lysinibacillus boronitolerans* (10%). Lower but still notable abundances were also observed for members of the *Bacillus cereus* group (4%) and *L. helveticus* (4%).

These observations suggest that the form of horse milk and the drying process substantially influence the structure and diversity of the microbial community. This interpretation is supported by the microbial community profiles shown in Figure 1, which illustrate clear differences among the treatments.

Alpha Diversity Analysis of Microbial Communities

Because the dataset was derived from technical replicates, the statistical analyses presented here should be interpreted as exploratory. Alpha diversity indices calculated from the 16S rRNA gene sequencing data are summarized in Table 1 and illustrated in Figure 2. The number of observed species (Figure 2a) increased markedly across treatments, from 190 in SKC to 963 in SKD and 1185 in SKT. This pattern indicates that powdered horse milk samples were associated with a higher detected taxonomic richness under the sequencing and analytical conditions applied in this study compared with the liquid milk sample (SKC). Overall, these findings suggest that processing horse milk into powdered form is linked to a broader detection of microbial taxa within the analytical framework used in this research.

The Shannon diversity index (Figure 2b) likewise increased across treatments, rising from 2.02 ± 0.06 in SKC to 2.40 ± 0.99 in SKD and reaching 3.43 ± 0.23 in SKT. This trend suggests a gradual increase in microbial diversity and evenness from pasteurized liquid milk to powdered milk without dextrin under the applied processing conditions. In contrast, the Simpson index (Figure 2c) displayed the opposite trend, decreasing from 0.18 ± 0.03 in SKC and 0.17 ± 0.01 in SKD to 0.07 ± 0.00 in SKT. Because the Simpson index is inversely related to diversity, the lower value observed in SKT indicates a more even and diverse microbial community compared with SKC and SKD.

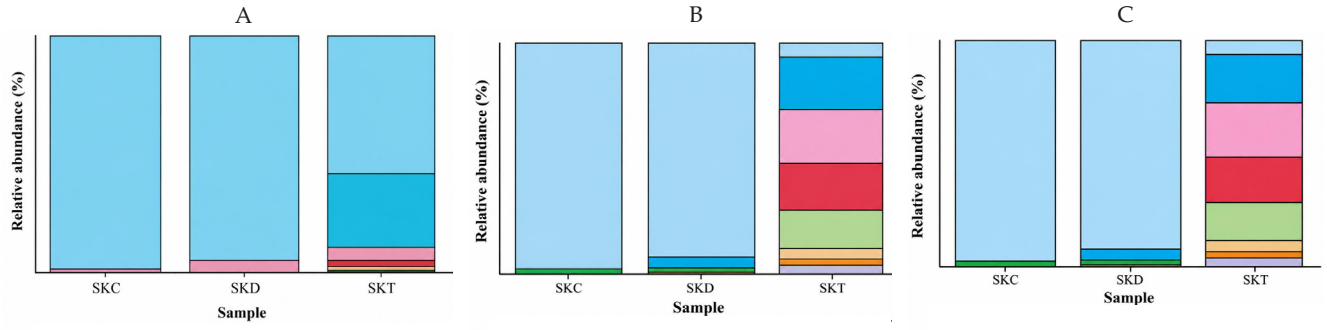

Figure 1. Relative abundance of bacterial communities in fermented horse milk products at different taxonomic levels based on 16S rRNA gene sequencing: (a) phylum, (b) genus, and (c) species. Bacillota was the predominant phylum across all samples, with particularly high abundance in SKC and SKD. In contrast, SKT exhibited greater microbial diversity at the genus and species levels, including notable representation of Lactobacillus, Lactococcus, and several other taxa. SKC: liquid fermented horse milk; SKD: powdered horse milk + 15% dextrin; SKT: powdered horse milk. Note: A : Cyanobacteriota, Actinomycetota, Nitrospirota, Pseudomonadota, Synergistota, Fusobacteriota, Planctomycetota, Bacteroidota, Spirochactota. B,C : Lactobacillus, Brevibacillus, Paenibacillus, Bacillus, Thalassoporum, Acinetobacter, Lysinibacillus, Lacticaseibacillus, Planctopirus, Others

Table 1. Alpha diversity metrics (Observed species, Shannon, Simpson, and Inverse Simpson indices) of horse milk products (SKC, SKD, and SKT) derived from 16S rRNA gene sequencing analysis.

Sample	Observed	Shannon (Mean ± SD)	Simpson (Mean ± SD)	Inv. Simpson (Mean ± SD)
SKC	190	2.02 ± 0.06	0.18 ± 0.03	5.61 ± 0.99
SKD	963	2.40 ± 0.99	0.17 ± 0.01	5.93 ± 0.50
SKT	1185	3.43 ± 0.23	0.07 ± 0.00	14.26 ± 0.57

Note: Values are expressed as mean ± standard deviation (SD). The statistical results are exploratory in nature and are based on technical replicates, representing analytical patterns associated with processing conditions rather than biological or population-level variation. SKC: pasteurized liquid milk; SKD: powdered milk supplemented with 15% dextrin; SKT: powdered milk without dextrin.

A similar trend was observed for the Inverse Simpson index (Figure 2d), which increased substantially from 5.61 ± 0.99 in SKC and 5.93 ± 0.50 in SKD to 14.26 ± 0.57 in SKT. This pattern suggests a more complex microbial composition in SKT under the applied experimental conditions. The consistency among the alpha diversity metrics indicates that the different milk treatments, particularly powder production without dextrin, were associated with distinct patterns of microbial richness and evenness. Overall, these results suggest that the drying process and dextrin supplementation may influence the detectable microbial diversity of horse milk.

Taxonomic Distribution of Microbial Communities Based on Phylogenetic Relationships

The Sankey diagram depicts the hierarchical distribution of microbial taxa from the phylum to the species level across the different treatments (Figure 3). To facilitate meaningful comparison among samples, only taxa with a relative abundance of ≥1% were considered in the visualization. In SKC (Figure 3a), the microbial community was largely dominated by members of the family *Lactobacillaceae*, particularly the genera *Lactobacillus*, *Lactococcus*, and *Enterococcus*. The SKD sample (Figure 3b) exhibited an intermediate pattern, sharing characteristics with both SKC and SKT. By contrast, SKT (Figure 3c) showed a more diverse taxonomic composition, including representatives of the families *Bacillaceae* and *Paenibacillaceae*. Dominant genera detected in this treatment included *Bacillus*, *Paenibacillus*, *Lysinibacillus*, *Brevibacillus*, *Thalassoporum*, and *Gloeothece* under the sequencing and processing conditions applied in this study.

To complement the descriptive visualization, alpha diversity metrics, including Observed OTUs, Shannon, Simpson, and Inverse Simpson indices, were also calculated. The results indicated that SKT had the highest number of observed OTUs (1185), followed by SKD (963) and SKC (190). This pattern aligns with the broader taxonomic distribution illustrated in the Sankey diagrams. These findings suggest that powder production without dextrin was associated with a broader range of detectable taxa than either pasteurized liquid milk or powdered milk supplemented with dextrin under the applied sequencing conditions.

In the Sankey diagrams, the width of each connecting band reflects the relative abundance of the corresponding taxa, providing a visual representation of how different microbial groups contribute to the overall community structure. The SKT treatment displayed both broader and more numerous connections across taxonomic levels, suggesting a more complex distribution of detected taxa compared with SKC and SKD. These visual patterns correspond with the alpha diversity trends; however, they should be interpreted as descriptive representations of microbial profiles associated with the different milk treatments and the drying process rather than as evidence from inferential statistical comparisons.

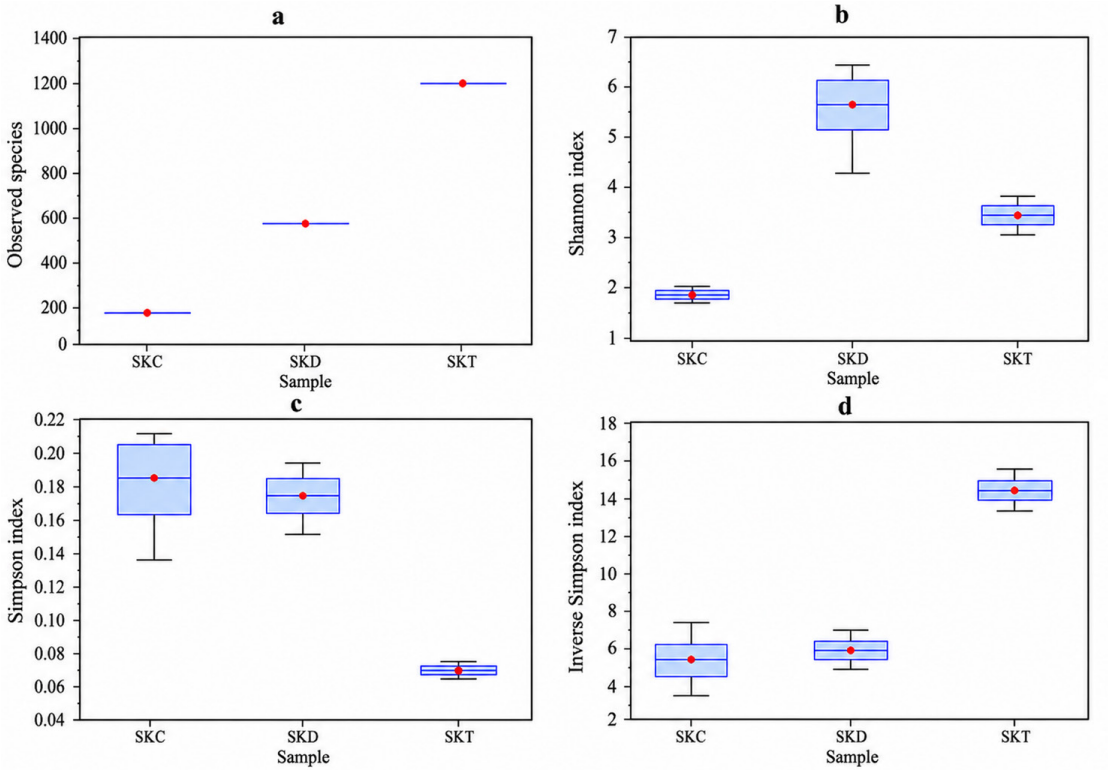

Figure 2. Alpha diversity metrics of horse milk products (SKC: pasteurized liquid milk; SKD: powdered milk supplemented with 15% dextrin; SKT: powdered milk without dextrin) derived from 16S rRNA gene sequencing analysis: (a) observed species, (b) Shannon index, (c) Simpson index, and (d) Inverse Simpson index. Values are presented as mean ± standard deviation (SD) from duplicate technical measurements and are intended to descriptively illustrate trends in microbial diversity among treatments.

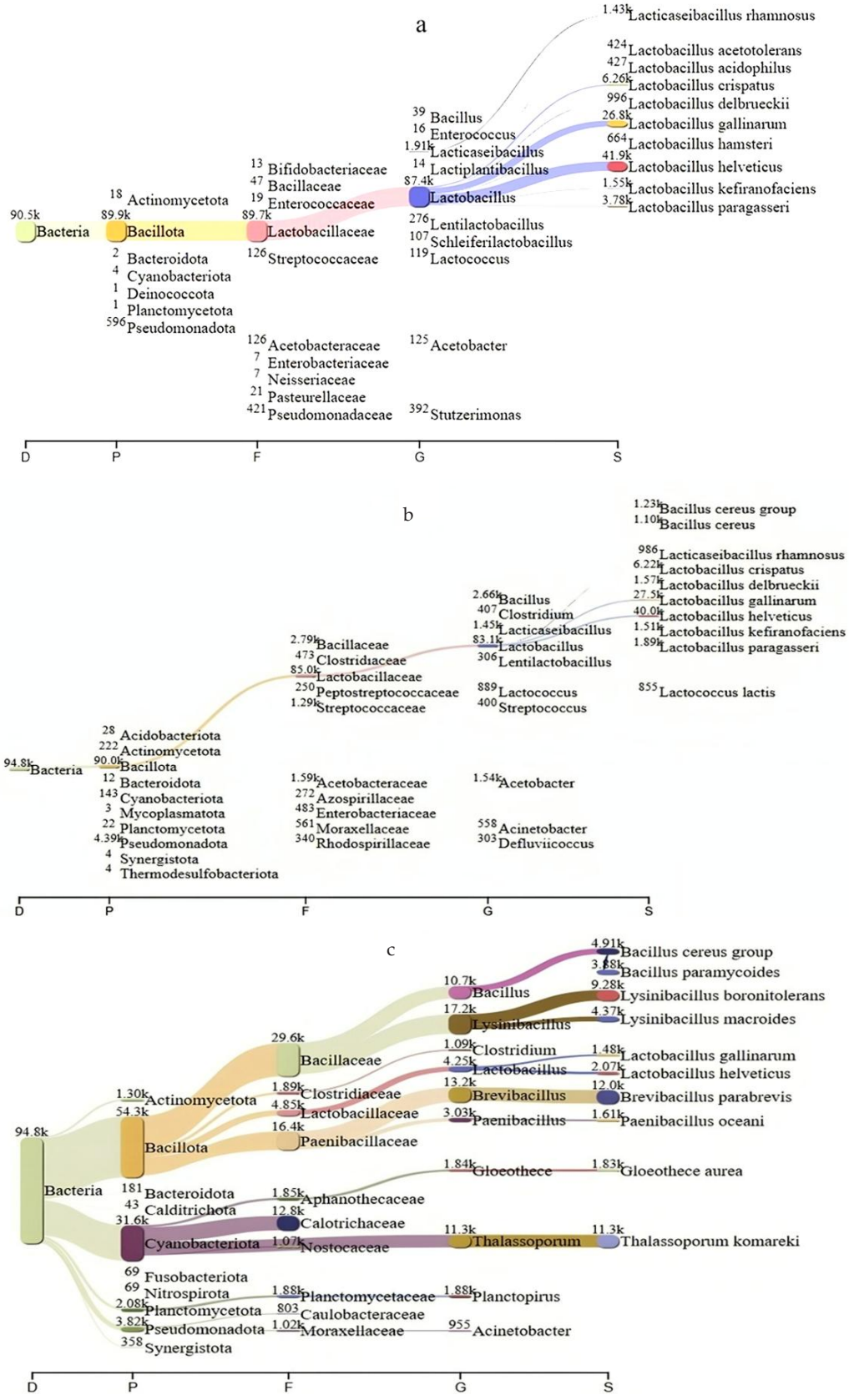

Figure 3. Sankey diagrams showing the hierarchical distribution of dominant microbial taxa (≥1% relative abundance) from the domain to species levels in SKC (a), SKD (b), and SKT (c). Taxonomic ranks (D–P–F–G–S) serve as positional references, since Sankey diagrams do not employ conventional axes. Legends and labeling were standardized across all panels to enable direct comparison among treatments. Numerical values indicate relative abundances obtained from full-length 16S rRNA gene sequencing, representing processing-associated compositional patterns based on technical replicates, with taxonomic assignments resolved to the species level whenever sequence resolution allowed. SKC: pasteurized liquid milk; SKD: powdered milk supplemented with 15% dextrin; SKT: powdered milk without dextrin.

Overlapping Microbial Communities

The Venn diagram (Figure 4) presents the distribution of operational taxonomic units (OTUs) detected in SKC, SKD, and SKT, highlighting both shared and unique microbial members among the treatments. Only OTUs that passed the quality-filtering step were considered in the analysis. Among the samples, SKT showed the highest OTU richness (944 OTUs), followed by SKD (310 OTUs) and SKC (72 OTUs), suggesting a greater number of detectable taxa in the powdered horse milk samples under the sequencing and processing conditions applied in this study. A total of 74 OTUs were common to all three treatments, representing the core microbial community based on presence–absence within this dataset. In addition, 151 OTUs were shared between SKT and SKD, 28 OTUs between SKC and SKD, and 16 OTUs between SKC and SKT.

These quantitative patterns align with the alpha diversity results presented in Table 1, where SKT showed the highest observed OTU richness, followed by SKD and SKC. Although several dominant lactic acid bacteria—including *Lactobacillus gallinarum*, *L. helveticus*, and *L. crispatus*—were identified in all treatments, their relative abundances varied noticeably among the different processing conditions. Samples from SKC and SKD were characterized by a greater relative contribution of lactic acid bacteria, whereas SKT showed increased representation of spore-forming genera such as *Bacillus* and *Brevibacillus*. This pattern suggests a shift in the detected microbial composition associated with the drying process without dextrin.

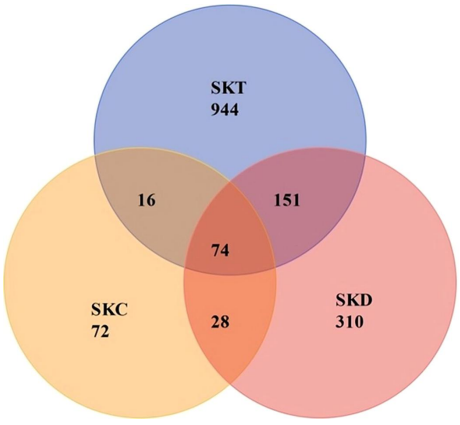

Figura 4. Venn diagram showing the unique and shared operational taxonomic units (OTUs) among the treatment groups SKC (yellow), SKD (red), and SKT (blue). Each circle represents one treatment, and overlapping regions indicate OTUs shared between groups based on presence–absence data. Numerical values denote the total number of OTUs detected within each treatment or shared combination. OTUs were assigned to the species level when sequence resolution permitted, based on full-length 16S rRNA gene sequencing, and represent processing-associated detection patterns derived from technical replicates rather than independent biological replication. SKC: pasteurized liquid milk; SKD: powdered milk supplemented with 15% dextrin; SKT: powdered milk without dextrin.

Separation of Microbiota Community Structure

Principal Component Analysis (PCA) revealed a separation pattern of microbial community structures among treatments based on relative abundance profiles derived from technical replicates. Two principal components, PC1 (34.01%) and PC2 (33.61%), explained 67.62% of the total variation in the data. SKT samples were predominantly distributed in the positive quadrants of both PC1 and PC2, indicating a distinct ordination pattern under the applied processing and analytical conditions. SKC was positioned in the positive PC1 and negative PC2 quadrant, while SKD was located in the negative quadrants of both components. This distribution reflects processing-associated differences in detected microbial composition, with minimal overlap observed between the samples. within this exploratory ordination space. This pattern is clearly illustrated in Figure 5.

DISCUSSION

Substrate form and the incorporation of functional carbohydrates such as dextrin have been reported to influence microbial community structures in fermented dairy products (Kaur *et al*. 2022; Ge *et al*. 2025). In the present study, clear differences in microbial diversity were observed among the treatment groups. These variations reflect patterns associated with the different milk treatments. Powdered horse milk without dextrin (SKT) showed the highest microbial diversity, characterized by a relatively balanced distribution at

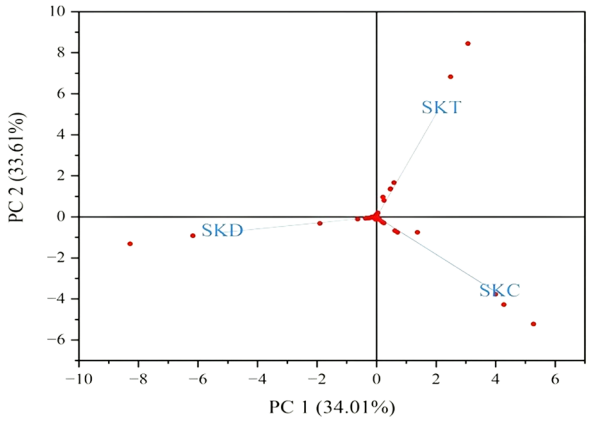

Figure 5. Principal component analysis (PCA) was used to visualize differences in microbial community composition based on full-length 16S rRNA gene sequencing data. The first two principal components (PC1 and PC2) together accounted for 67.62% of the total variance in the dataset. Within the ordination plot, SKT formed a distinct cluster compared with SKC and SKD under the analytical conditions applied in this study. This separation indicates differences in microbial composition associated with the processing treatments, as observed from technical replicates, rather than reflecting population-level biological variation. Each treatment, therefore, displayed a characteristic distribution within the PCA ordination space. SKC: pasteurized liquid milk; SKD: powdered milk supplemented with 15% dextrin; SKT: powdered milk without dextrin.

the genus level and the presence of less commonly reported genera, including *Lysinibacillus*, *Thalassoporum*, and *Brevibacillus*, within the dataset. By contrast, both pasteurized liquid milk (SKC) and powdered horse milk supplemented with dextrin (SKD) were strongly dominated by *Lactobacillus*, which comprised more than 85% of the detected community. The greater microbial diversity observed in SKT may be related to the absence of dextrin during the drying process. Without a protective carbohydrate matrix, the relative dominance of heat-sensitive lactic acid bacteria may have decreased, allowing stress-tolerant and spore-forming taxa to become more detectable under the applied sequencing conditions (Selmi *et al*., 2026). In addition, the low water activity typically associated with drying may contribute to the persistence of genera such as *Bacillus*, *Brevibacillus*, and *Lysinibacillus*, which are commonly reported as tolerant to harsh environmental conditions (Saha *et al*., 2025).

This pattern of dominance aligns with findings reported in probiotic goat milk and fermented camel milk, where *Lactobacillus* species often prevail due to their tolerance to acidic conditions and competitive advantage during fermentation (Monareng *et al*., 2025; Shori, 2024). In the present dataset, SKC largely maintained microbial characteristics typically associated with fresh milk. The SKD samples exhibited a slightly more heterogeneous community structure, whereas SKT showed a greater presence of spore-forming and stress-tolerant taxa under the drying conditions applied in this study. These observations are in agreement with previous reports suggesting that thermal processing and storage conditions can affect microbial survival and resilience (García *et al*., 2023; Hebishy *et al*., 2023). The compositional patterns observed were further reflected in the alpha diversity metrics (Observed, Shannon, Simpson, and Inverse Simpson), which showed higher microbial richness and evenness in SKT within the analyzed dataset.

These observations suggest that powder production without dextrin was associated with the detection of a broader range of microorganisms within the analytical scope of this study. From an ecological perspective, the higher diversity observed in SKT indicates a more complex microbial community within the dataset, where spore-forming and stress-tolerant taxa occur alongside lactic acid bacteria under the experimental conditions applied (Sibanda *et al*., 2024). Such community patterns may reflect potential ecological resilience rather than demonstrating confirmed functional advantages (Tan *et al*., 2022).

The differences observed in microbial community profiles may also be influenced by physicochemical factors associated with the drying process. Dextrin functions as a carbohydrate carrier capable of forming a semi-crystalline matrix around microbial cells, which may help reduce thermal and osmotic stress during dehydration (Bamidele & Emmambux, 2021). In the SKD samples, this matrix may have contributed to the persistence of lactic acid bacteria, particularly *Lactobacillus* spp., thereby supporting a moderate level of diversity within the analyzed sample. By contrast, the absence of dextrin in SKT likely exposed microorganisms to greater stress conditions, which may have favored the detection of stress-tolerant or spore-forming taxa such as *Bacillus* and *Brevibacillus*. Although water activity (a_w) was not directly measured in this study, the drying process was conducted at 70 °C and produced powders with low final moisture contents. Under such low-moisture conditions, bacterial cellular proteins and membrane integrity may be affected, whereas spore-forming microorganisms generally remain more resistant (Tong *et al*., 2025; Shymialevich *et al*., 2024). Therefore, the observed microbial differences may be associated with the drying conditions applied, although this interpretation remains tentative because a_w was not determined experimentally. These differences in microbial composition may also influence the quality characteristics of the final product. The greater persistence of lactic acid bacteria in SKD may be relevant to maintaining microbial characteristics commonly associated with fermented milk products commonly associated with fermented milk, including a more stable microbial profile and possible contributions to flavor development. In contrast, the higher representation of spore-forming taxa in SKT may be relevant to product stability during storage, as some species of *Bacillus* and *Brevibacillus* are known to survive harsh processing conditions and may contribute to spoilage in dried dairy products (Medjahdi *et al*., 2025). Consequently, the microbial structure observed in the powdered horse milk samples likely reflects interactions between the presence of dextrin and the drying conditions applied in this experiment.

From a microbial ecology standpoint, the higher alpha diversity detected in SKT may indicate a broader metabolic potential within the microbial community identified in this dataset; however, such interpretations remain descriptive and exploratory. The simultaneous presence of both spore-forming and non-spore-forming taxa under the applied processing conditions suggests a pattern consistent with survival under environmental stress rather than evidence of confirmed ecological adaptation (Manyi-Loh & Lues, 2025; Toor *et al*., 2024). Additionally, the identification of microbial taxa uniquely detected in SKT, including *Lysinibacillus boronitolerans* and *Thalassoporum komareki*, represents analytical findings within the current dataset, and their functional roles were not experimentally verified in this study (Tuesta-Popolizio *et al*., 2021).

Taxonomic shifts were also reflected in the dominance of particular taxa across the treatments. Although several core microbial groups were detected in all samples, their relative abundances varied within the limits of the technical replicate dataset. Samples from SKC and SKD were primarily characterized by the presence of species such as *L. helveticus* and *L. gallinarum*. In contrast, SKT showed relatively higher detection of *Brevibacillus parabrevis* and *Bacillus* spp., which may be associated with the processing conditions applied. These descriptive observations are consistent with the alpha diversity patterns reported earlier, although they should not be interpreted as statistical evidence of population-level differences. Previous

research has also shown that the use of additives can influence patterns of microbial dominance in processed food systems, providing contextual support for these observations (Xiong *et al*., 2022). Taken together, the compositional differences observed among treatments indicate that each sample type exhibited a distinct microbial profile under the applied experimental conditions, with SKT displaying the broadest taxonomic representation. These results highlight processing-related trends rather than confirmed biological effects and suggest that food processing practices, such as those applied to horse milk in this study, can influence the detected structure and relative abundance of microbial communities (Blanco-Doval *et al*., 2024; García *et al*., 2023).

Several limitations of the present study should be considered. First, the microbial analyses were based on technical replicates derived from a limited number of processed samples; therefore, the patterns observed should be interpreted as analytical trends associated with processing conditions rather than as indicators of population-level biological variability. Second, key physicochemical parameters—including water activity (a_w), drying kinetics, and temperature gradients—were not measured directly, which restricts the ability to relate the observed microbial shifts to specific processing factors quantitatively. Furthermore, microbial profiling was performed using full-length 16S rRNA gene sequencing, which provides detailed taxonomic information but does not permit direct assessment of microbial functionality, viability, or metabolic activity. As a result, the ecological and biotechnological interpretations presented here remain exploratory and would benefit from further validation through studies that include biological replication, functional characterization, and genome-resolved analytical approaches. Despite these limitations, the present findings provide preliminary descriptive information relevant to the milk powder industry, particularly in the selection of drying conditions and carrier additives to help maintain desirable microbial groups and improve the microbiological stability of horse milk powder products.

CONCLUSION

The results of this study indicate that different milk treatments were associated with distinct microbial profiles in horse milk. Pasteurized liquid milk (SKC) and powdered milk supplemented with 15% dextrin (SKD) were dominated by lactic acid bacteria, particularly *Lactobacillus gallinarum* and *Lactobacillus helveticus*. In contrast, powdered milk without dextrin (SKT) showed higher microbial diversity and greater representation of stress-tolerant and spore-forming taxa, including *Brevibacillus parabrevis*, *Lysinibacillus boronitolerans*, and members of the *Bacillus cereus* group. These findings indicate that dextrin supplementation during drying was associated with maintaining lactic acid bacteria in powdered horse milk.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this study.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this manuscript, the authors used generative AI tool to assist with language editing, grammar correction, and improvement of sentence clarity. All sections of the manuscript were subsequently reviewed and revised by the authors, who take full responsibility for the final content of the publication.

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