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Profiling of Dominant Bacteria in Traditional Buffalo Milk Cheese "Dangke" Based on 16S rRNA Sequencing

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ABSTRACT

Metagenomic approaches are highly valuable in food microbiology, particularly for the investigation of traditional fermented products such as dangke buffalo milk cheese from South Sulawesi, Indonesia. Buffalo milk, a primary raw material, contains diverse biochemical and bioactive components that are produced by microbial activity during fermentation. Unlike conventional culture-based techniques, metagenomics enables the comprehensive characterization of microbial communities directly from food matrices. This study aimed to identify the key microbial taxa that contribute to the dangke quality of fermentation and to understand the factors influencing it. Bacterial 16S rRNA genes were amplified using primers 27F and 1492R under optimized polymerase chain reaction conditions. DNA concentrations were measured with NanoDrop and Qubit instruments, and sequencing was conducted using Oxford Nanopore Technology with MinKNOW software (v23.04.5). Metagenomic analysis revealed *Enterococcus faecium* as the dominant lactic acid bacterium across all dangke samples (P1–P3), emphasizing its crucial role in fermentation and probiotic potential. These findings suggest that *E. faecium* can enhance the quality and functional properties of dangke. Moreover, metagenomic tools can support the development of standardized fermentation practices and microbial safety assessments for traditional dairy products.

Keywords: 16S rRNA; bacteria; buffalo; dangke; milk; sequencing

INTRODUCTION

Metagenomics is a powerful tool for identifying microbial communities in environmental samples without requiring prior cultivation. Traditional techniques often fail to detect microorganisms that do not grow well under laboratory conditions. In contrast, metagenomic profiling enables the comprehensive analysis of microbial communities by identifying both culturable and non-culturable species, thereby providing a more complete representation of bacterial diversity. A traditional approach that requires culture can only detect approximately 1% of microorganisms, leaving the remaining 99% of bacteria undetected (Torsvik & Øvreås, 2002). Advances in next-generation sequencing have provided insights into microbiota composition and function in complex samples by enabling the direct sequencing of DNA and RNA (Gilbert *et al*., 2014). The integration of metagenomic sequencing and bioinformatics allows for the comprehensive analysis of microbial diversity, metagenomic potential, and the characteristics of various samples (Niccum *et al*., 2020; Walsh *et al*., 2020).

Comprehensive microbial profiling provides a detailed microbial community profile, including dominant, subdominant, and rare bacteria. This helps us understand how microbial interactions influence the flavor, texture, fermentation process, and safety of dangke products. 16S rRNA gene sequencing offers objective microbial classification compared to other methods, especially bacterial identification and classification. Microbial communities are often grouped into taxonomic units (e.g., species, genera, families, and phyla) to assess species richness, diversity, composition, and community structure (Kim *et al*., 2011).

In addition, 16S rRNA gene sequencing facilitates the early identification of potential pathogens and spoilage microorganisms, thereby strengthening food safety monitoring. This technique supports quality assurance without disrupting the fermentation methods established for traditionally fermented cheeses. Additionally, metagenomic profiling enables the characterization of distinct microbial characteristics associated with high-quality dangkes, offering valuable insights for developing targeted starter cultures. This contributes to the standardization of flavors, textures, and safety in industrial-scale production. Metagenomic approaches are particularly valuable in food microbiology, especially for investigating the potential

hazards associated with traditional buffalo milk cheese from South Sulawesi, Indonesia.

Buffalo milk, the primary component of dangke, contains diverse biological components that are influenced by microbial activity during fermentation. The addition of papaya latex during production plays a pivotal role in modifying texture and flavor. Papaya latex contains proteolytic enzymes, notably papain, which breaks down proteins into peptides and amino acids. This enzymatic activity can alter the fermentation environment (pH and nutrient availability), potentially inhibiting the spoilage of pathogenic microbes while promoting the growth of specific lactic acid bacteria (LAB) or other beneficial fermentative microbes. Thus, characterizing the microbiota in buffalo milk using metagenomics is essential for preserving traditional food products. The microbial composition of cheese is similar to that of raw milk (Martins *et al*., 2018). This composition is shaped by various factors, including the farm's microbial environment, as well as soil, air, and processing conditions (Lopez *et al.,* 2018). Results in a protein content that meets or exceeds Indonesian national standards because of the fermentation process and the addition of papain, which helps coagulate milk and concentrate protein (Mutmainna *et al*., 2025).

Another important aspect of this study was the discovery of novel probiotics, which may reveal unique or beneficial bacterial strains not typically found in other fermented dairy products. These strains may be used for functional food development and have potential health applications. This method helps to identify LAB involved in the fermentation of dangke, which contributes to its preservative and probiotic properties. Therefore, this study aimed to elucidate the microbial interactions in buffalo milk and their influence on milk quality. Deep metagenomic sequencing has emerged as a robust methodology for characterizing fermented food microbiota, offering taxonomic resolution at the species and strain levels (Niccum *et al*., 2020; Walsh *et al*., 2020).

Studying the genomes of dangke microorganisms is essential to understanding the microbial composition, functionality, and safety of traditional fermented products. This genomic information may help authenticate conventional production methods, enhance quality control, and identify probiotic strains that contribute to health benefits. Furthermore, genome-based analyses support the development of starter cultures and the optimization of fermentation conditions, ensuring consistency, safety, and scalability in dangke production conditions, while preserving the unique cultural and nutritional value of the product. Moreover, the epigenome comprises different mechanisms, such as DNA methylation, remodeling, histone tail modifications, chromatin microRNAs, and long non-coding RNAs, that interact with environmental factors, such as nutrition, pathogens, and climate, to influence the expression profile of genes and the emergence of specific phenotypes (Barazandeh *et al*., 2016). Multilevel interactions among genomic, epigenomic, and environmental factors may occur (Amiri Roudbar *et al.,* 2020). Eukaryotic gene expression is temporarily and multidimensionally controlled. Only a relatively small portion of the entire genome is expressed in each tissue type, and gene expression depends on the developmental stage (Heidarpour *et al*., 2011; Khabiri *et al*., 2023). Therefore, gene expression in eukaryotes is tissue-specific (Safaei *et al*., 2023). In addition, the number of gene products produced in the same tissue and other tissues that make up the product regulates gene expression. One of the basic activities of genomic research is the study of genes and proteins related to traits and their study at the cellular or chromosomal levels (Bordbar *et al*., 2022). Furthermore, numerous lines of evidence suggest that epigenomic variation influences health, reproduction, and production (Alavi *et al.,* 2022). Metagenomic profiling has been widely applied to various dairy products (yogurt, kefir, and cheese). However, there is a lack of scientific literature on dangke, a traditional cheese made from buffalo milk in Indonesia. This study fills this gap by documenting the microbiota of dangke for the first time using a molecular approach. This contributes to a broader understanding of the microbial diversity in traditional foods globally. Thus, this study aimed to identify potential microbes that enhance dangke quality and elucidate the factors influencing the fermentation process. These findings provide valuable insights for optimizing traditional Indonesian fermented food production, with potential applications in food safety and public health.

MATERIALS AND METHODS

Sample Handling Protocol

The following samples of buffalo milk and dangke were collected from three villages in Enrekang, South Sulawesi, Indonesia: P1 – Rogo Village, P2 – Rogo2 Village, and P3 – Sumbang Village. Raw buffalo milk was obtained from a fourth site (P4) in the same region. Micro-, small-, and medium-sized enterprises (UMKM) processed approximately 4 liters of fresh buffalo milk, yielding a total of 12 liters used in this study. Dangke preparation followed the traditional method used by the Enrekang community. Fresh buffalo milk was heated to 85 °C, and 12 mL of diluted papaya (*Carica papaya*) latex was added as a natural proteolytic enzyme to induce milk coagulation. Salt was added, and the mixture was gently stirred until visible curds were formed. The curds were then separated from the whey and molded using coconut shell-shaped containers until they solidified.

Both buffalo milk and dangke samples were collected directly from local producers who rear buffaloes and prepare dangke onsite. All samples were transported under cooled conditions (4 °C) to the laboratory for further DNA extraction and metagenomic analysis. This traditional method is known to yield white-colored dangke with predominant microbial activity from LAB, reflecting the rich microbial diversity typically found in fermented Indonesian food.

Determination of DNA Concentration

DNA was extracted using a modification of the method reported by Purwantiningsih *et al.* (2025).

The DNA concentration was quantified using dual measurement methods: spectrophotometric analysis using a NanoDrop instrument and fluorometric assessment using a Qubit system (Thermo Fisher Scientific, Waltham, MA, USA). Quality assessment included electrophoretic separation on an agarose gel with subsequent visualization and documentation using a Gel-Doc EZ imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Samples (10 mL) were centrifuged at 2,500 rpm for 10 min, and the obtained pellet was washed with saline and rinsed with sterile distilled water. Genomic DNA was extracted from the pellet using a ZymoBIOMICS DNA Miniprep Kit D4300 (Zymo Research, Cambridge, UK). DNA quality was assessed using agarose gel electrophoresis and subsequent visualization using Gel-Doc EZ imaging (Bio-Rad Laboratories).

Amplification of *16S rRNA*

The 16S rRNA gene for identifying bacterial species was amplified using the 27F and 1492R primers following the method described by Kim *et al*. (2011). The 27F primer (5'- AGAGTTTGATCMTGGCTCAG-3') and 1492R primer (5'- GGTTACCTTGTTACGACTT-3') produced an amplification of approximately 1,500 bp. DNA amplification was conducted in a total volume of 25 μL consisting of 0.3 μL of bacterial DNA samples, 11.9 μL of NFW, 0.15 μL of forward primer, 0.15 μL of reverse primer, and 12.5 μL of KOD Multi & Epi (KME-101). PCR analysis was set as follows: initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s. Nanopore sequencing was performed using MinKNOW software version 23.04.5. Base calling was performed using Guppy version 6.5.7, with a high-accuracy model (Wick *et al*., 2019). The quality of the FASTQ files was visualized using NanoPlot and the quality of filtering was performed using NanoFilt (De Coster *et al*., 2018).

Bioinformatics Analysis

The filtered reads were classified using a previously described centrifuge classifier (Kim *et al*., 2016). Bacterial and archaeal indices were constructed using the NCBI 16SRefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/). Downstream analyses and visualizations were performed using Pavian (https://github.com/fbreitwieser/pavian), Krona Tools (https://github.com/marbl/Krona), and R Studio using R version 4.2.3 (https://www.R-project.org/).

RESULTS

DNA Amplification

The electrophoresis results (Figure 1) demonstrated successful amplification of the 16S rRNA gene across all tested samples (P1–P3 and buffalo milk), as indicated by clear and distinct bands at approximately 1,500 bp. The DNA molecular marker in the leftmost lane served as a size reference, confirming that the amplified fragments consistently corresponded to the correct gene region across all samples.

Although minor DNA degradation was observed in some dangke samples, the amplicon integrity was sufficient for sequencing. All PCR products met the quality control standards required for high-throughput sequencing, confirming their suitability for downstream metagenomic analyses using the Oxford Nanopore system. The uniform band size across all samples indicated specific and consistent amplification of the 16S rRNA region.

Taxonomic Profiling of Dangke

Figure 2 shows that milk processing significantly influenced microbial diversity. P1 exhibited a less complex microbial ecosystem because of standardized or controlled fermentation using pasteurized milk or starter cultures. In contrast, P2 showed slightly higher microbial diversity, suggesting exposure to ambient microbiota and the use of raw milk. P3 demonstrated the highest microbial richness, including Clostridia, Actinomycetia, and Chitinophagia, indicating spontaneous fermentation in traditional dangke. The presence of *Clostridia* microorganisms plays a role in proteolysis and aroma formation, while the potential bioactivity of products and *Chitinophaga* (a class of the phylum Bacteroidetes) microorganisms plays a less-controlled sanitation, raw materials, or equipment surfaces. Buffalo milk (P4) exhibited the broadest taxonomic diversity, harboring classes such as Flavobacteria, Sphingobacteria, and Bacteroidia.

Amplicon sequence variant (ASV) analysis revealed the following key insights into microbial overlap. Three hundred and thirty-eight ASVs were shared among all four samples, indicating a core microbiome (Figure 3A). P3 had the highest number of unique ASVs (913), reflecting its microbial complexity. Five hundred and forty-seven ASVs were common to P1, P2, and P3 (Figure 3B); however, only 67 ASVs were shared exclusively between P1 and P2, suggesting a weak microbial overlap in the absence of P3. Three hundred and fifty-seven ASVs were shared between buffalo milk (P4), P1, and P2, with P4 containing 909 total

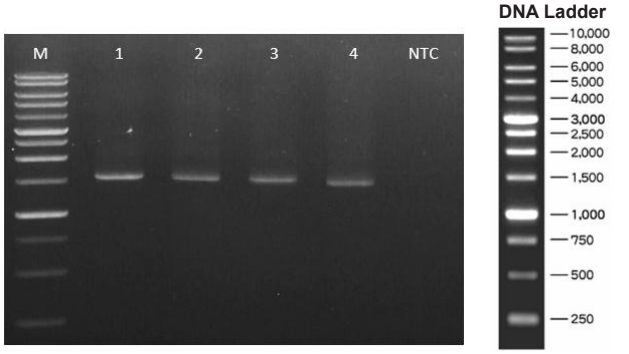

Figure 1. Agarose gel electrophoresis (1% gel, run time 40 min at 100 V) of metagenomic DNA from dangke samples lane 1(P1), lane 2 (P2), lane 3 (P3), lane 4 (raw buffalo milk), and control (NTC). DNA Ladder 1500 bp equals 1.5 kb.

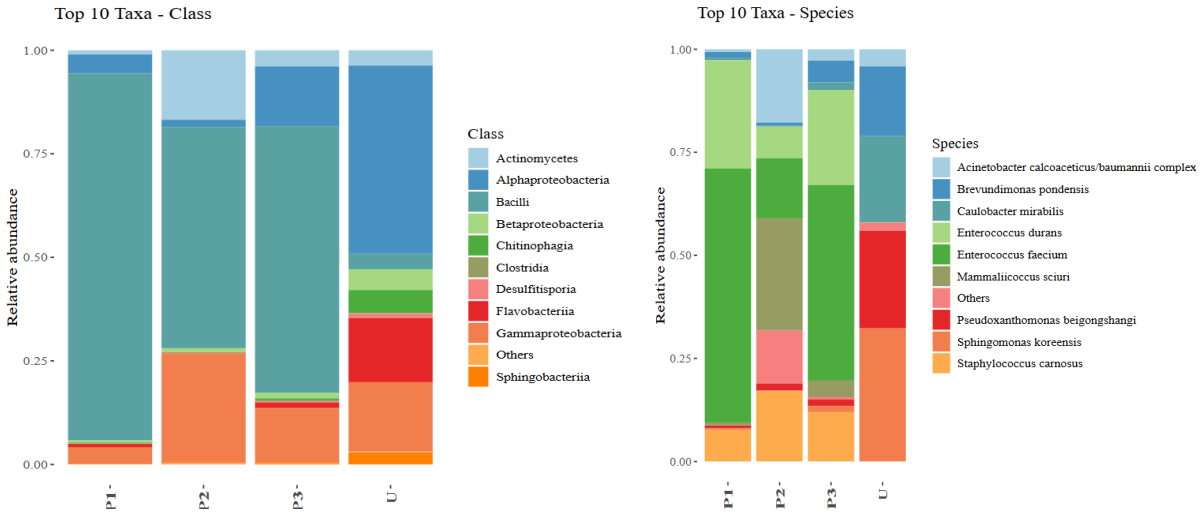

Figure 2. Taxonomic profiling of samples of dangke in group P1, group P2, group P3, and buffalo milk in group U.

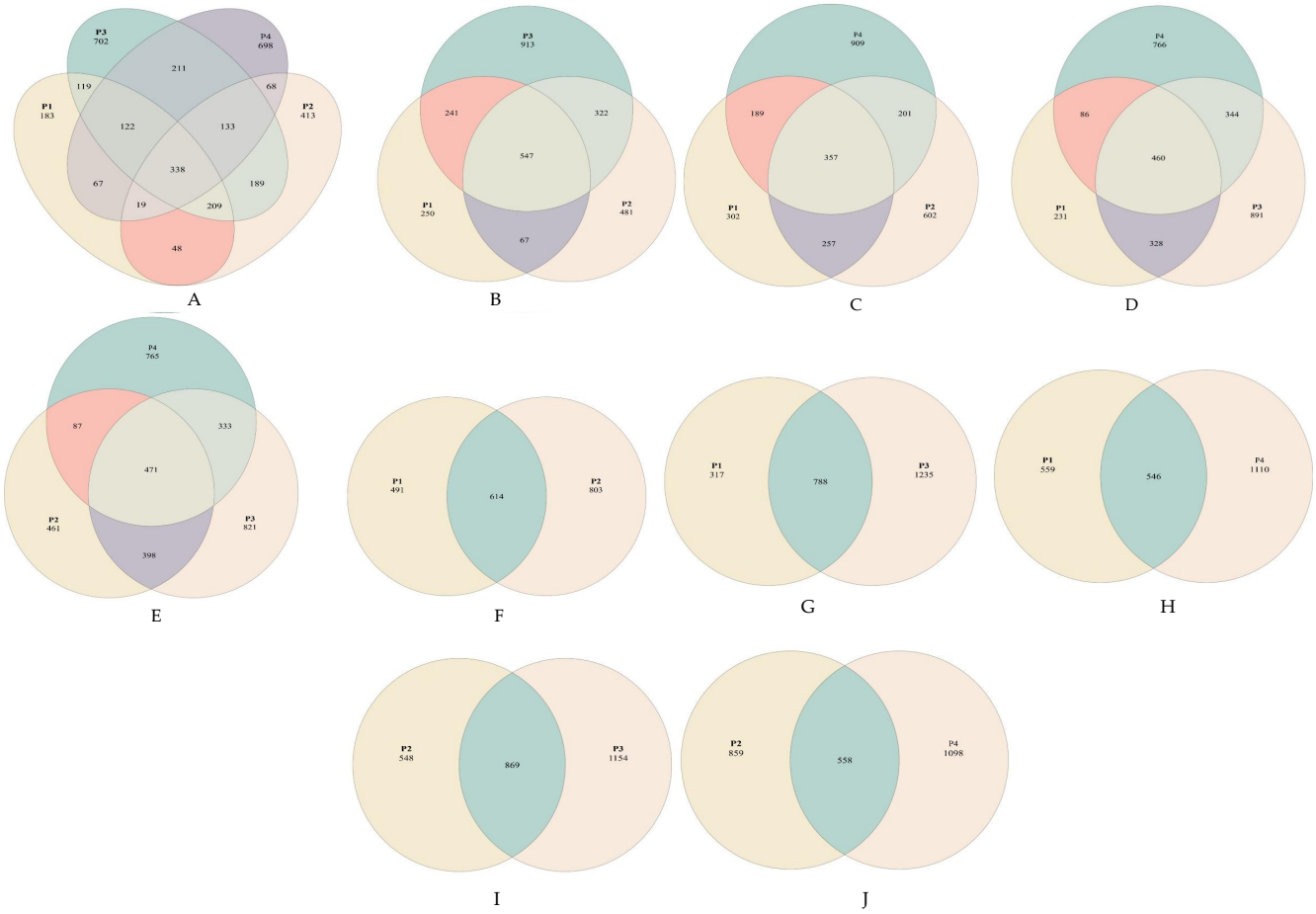

Figure 3. A Venn diagram showing the position of the dangke and buffalo milk sample groups. Venn diagram illustrating the distribution of the microbiome in: A) P1 (Rogo Village), P2 (Rogo Village), P3 (Sumbang Village), and P4 (Buffalo milk); B) P1, P2, and P3; C) P1, P2, and P4; D) P1, P3, and P4; E) P2, P3, and P4; F) P1 and P2; G) P1 and P3; H) P1 and P4; I) P2 and P3; and J) P2 and P4.

ASVs, supporting its role as a microbial source (Figure 3C). Figure 3D–F presents the shared and unique ASV counts between groups. For instance, P2 had 803 unique ASVs compared to 491 for P1, indicating a greater environmental or compositional influence by P2.

The overlap between P3 and P4 was particularly high (e.g., 344 and 333 ASVs were shared), suggesting microbial transfer from raw milk into spontaneously fermented milk (Figure 3G–J). Figures 3H and 3I show the highest ASV richness (869 and 788 ASVs, respectively), confirming P3 as the most microbiologically rich sample (Figure 3H–I).

The implications of high-throughput sequencing and the high resolution generated by next-generation

sequencing allow the identification of novel microbial taxa and environmental adaptation patterns. This highlights the microbial responses to different processing environments and reveals the role of raw milk microbiota in shaping fermented dairy ecosystems. These findings are vital for future culture development, probiotic strain isolation, and the optimization of traditional fermentation practices for commercial applications.

Taxonomic Composition of Dangke

Taxonomic profiling revealed that LAB were the predominant microbial group in all analyzed samples. The phylum Bacillota (formerly Firmicutes) was identified as the most abundant taxonomic group, with *Enterococcus faecium* (45%) and *Enterococcus durans* (19%) showing particular dominance in the samples presented in Figure 4B and Figure 4D. Although Bacillota was also dominant (Figure 4C), its relative abundance was significantly lower (Figure 4A), suggesting sample-specific variation in microbial composition.

Notably, samples with the highest viable cell counts, particularly those shown in Figures 4B and 4D, contained heat-resistant LAB strains, such as *E. faecium* and *E. durans*. These bacteria are known to withstand thermal treatment and are well-adapted to dairy fermentation environments. Their presence is crucial not only for flavor and texture development but also for microbial safety and preservation.

Understanding the factors that influence shifts in the microbiome is essential because certain microbes contribute positively to sensory attributes, whereas others may generate spoilage metabolites. Given that microbial activity drives the biochemical transformation of fermented dairy products, knowledge of the underlying microbiota is critical for product quality and safety, especially for products intended for prolonged storage.

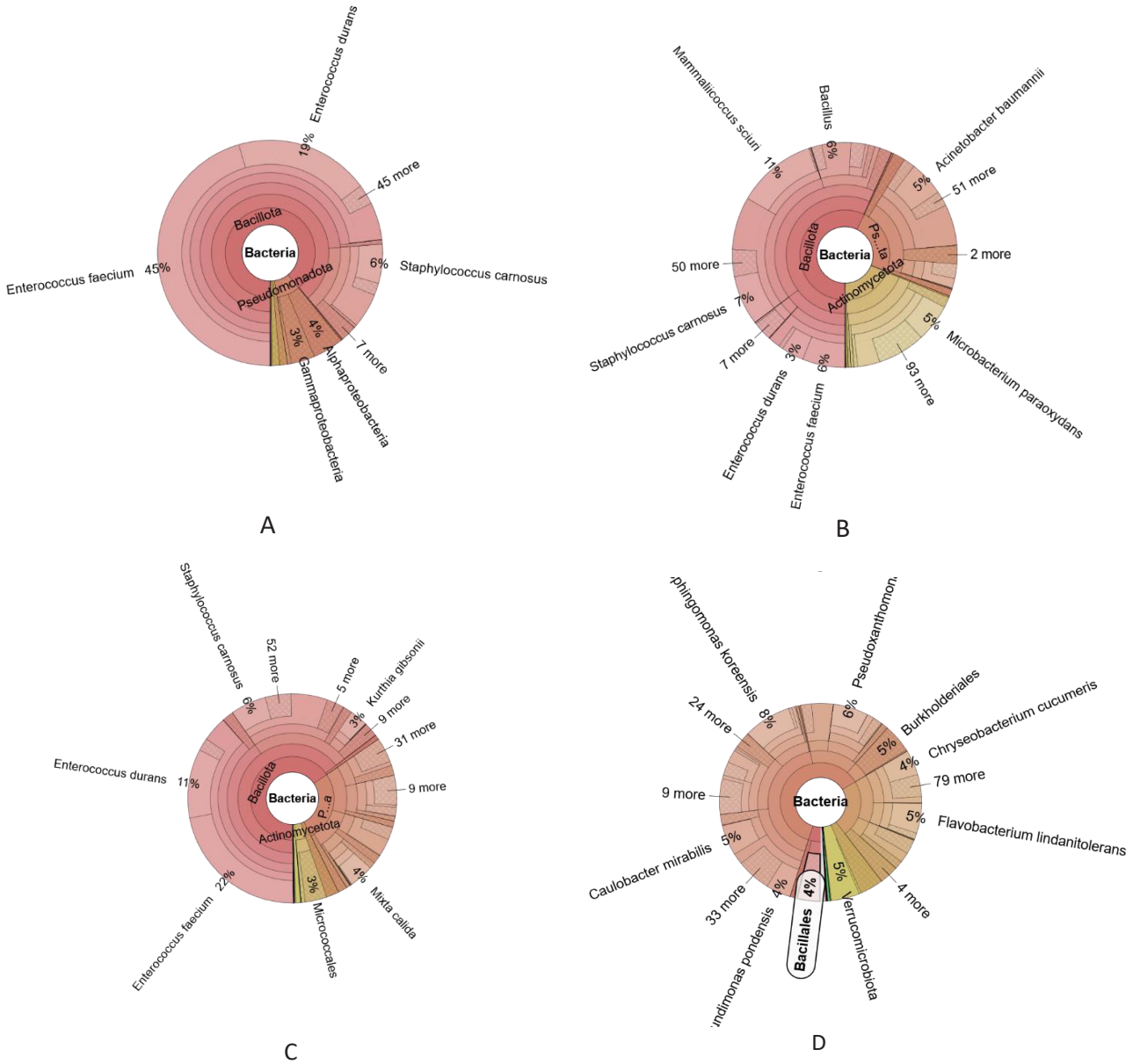

Figure 4. The Krona species composition map in group P1 Rogo Village (A), group P2 Rogo2 Village (B), group P3 Sumbang Village (C), and group P4 buffalo milk (D).

Phylogenetic Tree of the Microbiota in Dangke

Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity revealed notable differences in microbial community composition among the samples (Figure 6). In the left panel, the first two principal coordinates (PCoA1 and PCoA2) accounted for 78.37%, 56.12 %, and 22.25% of the total variation, respectively. In the right panel, PCoA1 and PCoA3 explained 70.49% of the total variation.

The PCoA plot exhibited distinct clustering patterns, where the spatial distances between points reflected the degree of microbial dissimilarity. Notably, the buffalo milk sample (P4) formed a separate cluster from all dangke samples (P1–P3), indicating a significantly different microbial profile in raw milk than in the fermented products. In contrast, the dangke samples clustered tightly together, suggesting relatively similar microbial community structures regardless of the production site. This similarity likely reflects shared fermentation processes or dominant microbial taxa.

The gray vectors shown in the plot represent the contribution of specific microbial features (e.g., bacterial genera or species) to the differentiation among samples. The length of each vector is proportional to its influence on community separation, with longer vectors indicating higher discriminatory power. These findings demonstrate that while fermentation tends to standardize microbial communities, the microbiota of raw buffalo milk remains distinct and more variable.

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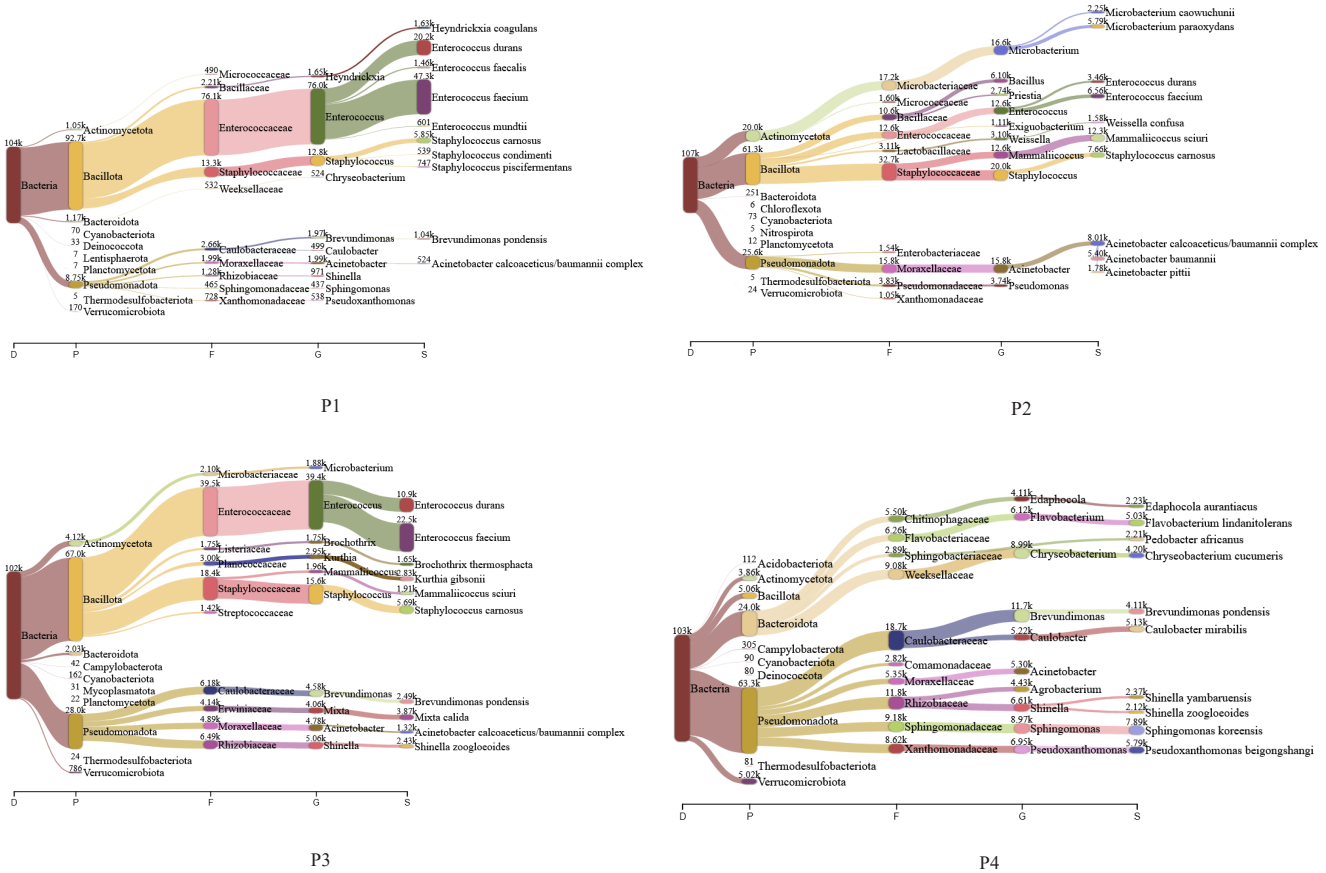

Figure 5. Phylogenetic relationships of 16S rRNA gene sequences from different dangke samples based on production location P1 (Rogo Village), P2 (Rogo2 Village), P3 (Sumbang Village), and P4 (Buffalo milk).

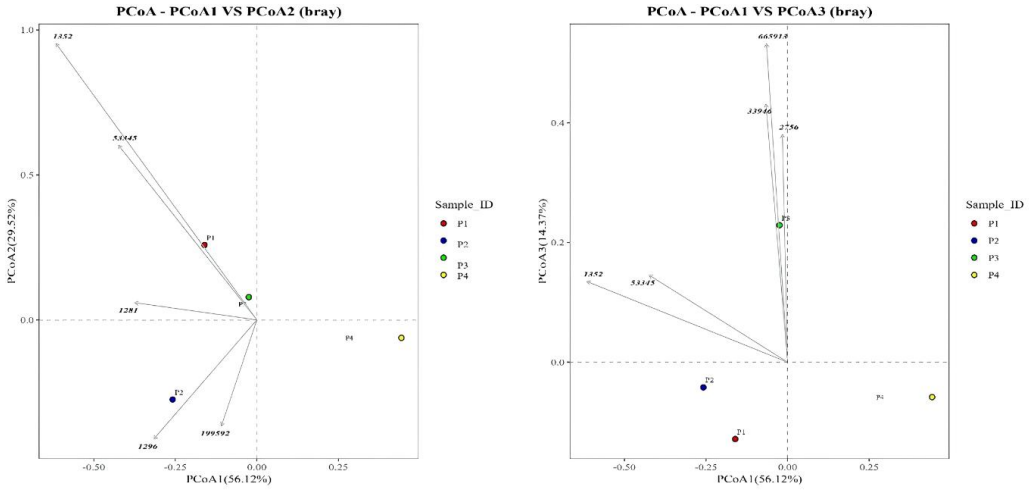

Figure 6. Principal coordinates analysis (PCoA) of different dangke samples, P1 (Rogo Village), P2 (Rogo2 Village), P3 (Sumbang Village), P4 (Buffalo milk).

DISCUSSION

DNA Amplification

All three samples exhibited similar electrophoretic band characteristics, that is thick and well-defined bands. As previously reported by Haris *et al*. (2003), DNA concentration significantly affects fragment visualization. Excessive concentrations produce thick bands, whereas insufficient quantities yield faint or undetectable bands (Table 1). Electrophoresis separates DNA fragments by molecular weight and charge, with migration rates dependent on the DNA size and the applied electric current (Novitasari *et al*., 2014).

Total DNA samples are composites of various bacterial genomes. Although universal 16S rRNA primers can "attach" to different bacterial DNA templates, they preferentially amplify dominant bacterial populations. This banding pattern reflects the microbial diversity in the dangke. This is in agreement with the findings of Randazzo *et al*. (2010) that traditional cheeses have greater microbial diversity than industrial varieties. This difference stems from the use of raw milk and cross-contamination with potential pathogens (Delcenserie *et al*., 2014).

Gel electrophoresis is a fundamental technique in molecular biology that separates charged biomolecules, including nucleic acids and proteins, using a stationary colloidal matrix under an electric field (Rudge & Monnig, 2000). Figure 1 shows the presence of distinct bands on the agarose gel, confirming successful DNA amplification. The leftmost lane displays the DNA molecular weight marker (1 kb ladder) as a size reference. All sample lanes consistently exhibited amplified products of approximately 1,500 bp, corresponding to the expected size of the 16S rRNA amplicons.

Taxonomic Profiling of Dangke

Milk processing methods (such as pasteurization or raw, unpasteurized milk) significantly influence microbial richness and diversity, affect fermentation outcomes, and result in more diverse microbial taxa (Figure 2). P1 exhibited a less diverse or simpler microbial ecosystem, likely because of standardized or controlled fermentation using a starter culture or pasteurized milk. The taxonomic abundance was quantified by comparing the number of sequence reads classified into specific taxa. Data were visualized at the genus level to highlight the predominant microbial groups in each sample. Notably, high-depth metagenomic sequencing enables precise identification of novel species in traditional fermented dairy products. This capability provides critical data for developing culture biology, strain screening for optimized fermentation, and the industrial-scale production of fermented dairy products. Microbiome sequencing, using the 16S rRNA gene, is a powerful tool for assessing and quantifying the structure and diversity of microbial communities in ecosystems (Choi *et al*., 2020). 16S rRNA gene sequencing is widely used to characterize microbial taxonomic composition and phylogenetic diversity (Aßhauer *et al.,* 2015).

The results for P2 suggest a slightly more diverse fermentation environment than for P1. The factors influencing diversity included the use of raw milk and exposure to ambient microbiota. The difference in bacterial diversity between dangke products may result from the use of different raw buffalo milk depending on where the dangke is produced. The raw materials differ markedly because their quality depends on the milking process and cross-contamination during production (McHugh *et al*., 2021). In addition, as previously explained by Kamimura *et al*. (2019), variations in microbial profiles depend on processing technologies, storage conditions, seasonal fluctuations, and climatic factors.

Table 1. The quality of extracted metagenomic DNA from dangke samples

Sample code	Volume (µL)	Concentration (ng/µL)	Remarks
N967-1	10	51.04.00	PASS QC
N967-2	10	28.08.00	PASS QC
N967-3	10	40.06.00	PASS QC
N967-4	10	28.04.00	PASS QC

P3 showed greater microbial diversity, including a significant presence of *Clostridia, Actinomycetia*, and *Chitinophagia*. This indicated that natural or fermentation processes and factors influence diversity, including the use of unpasteurized. The deacidification of cheese is supported by the genus *Clostridia* (*Clostridium tyrobutyricum*) during ripening determines the intensification of the metabolic activity of *C. sporogenes* and *C. beijerinckii* and, consequently, an increase in butyric acid fermentation (Le Bourhis *et al*., 2005).

The presence of *Chitinophagia* also suggests anaerobic or less stringent sanitation conditions during production. Specific bacterial populations remain viable throughout the fresh milk transportation and milking processes (Vithanage *et al*., 2017). These findings highlight the urgent need for improved hygiene practices, proper handling, and better storage conditions in traditional dangke production to reduce contamination and ensure consumer safety (Syah *et al*., 2025). Thermophilic bacteria are resilient and produce heat-stable enzymes that maintain their activity under high-temperature conditions. Several microbial species generate extracellular peptidases capable of withstanding pasteurization temperatures (Glück *et al*., 2016).

Buffalo milk (P4) exhibited the most diverse microbial profile, including *Flavobacteria, Sphingobacteria*, and *Bacteroides.* Raw milk naturally harbors a wide range of environmental- and host-associated microbes. Dairy-associated microbiota frequently exhibit proteolytic and lipolytic activities that contribute to the characteristics of the final product. According to a previous study by Rizvi *et al*. (2021), psychrophilic microorganisms exhibit distinct thermal adaptation profiles, characterized by a minimum growth temperature of 0 °C, an optimal growth temperature of ≤15 °C, and a maximum threshold around 20 °C. These cold-adapted microbes produce enzymes with flexible molecular architectures and reduced structural rigidity (Jin *et al*., 2022). Certain psychrotrophic strains exhibit heat tolerance, and psychrotrophic microorganisms are important in the low-temperature preservation of foods (Erkmen & Bozoglu, 2016).

Venn diagram analysis (Figures 3A–J) revealed important patterns in microbial diversity and similarity among samples (P1, P2, P3, and buffalo milk [P4]). A range of 338–547 ASVs were shared across multiple samples, suggesting that common microbial communities are likely involved in basic functions, such as metabolism or fermentation. P3 and P3 consistently showed the highest microbial richness (913 ASV), with many similarities among the samples (547 ASV). This is because of the BAL content in dangke. LAB plays a pivotal role in product maturation through substrate metabolism, using residual lactose and other carbohydrates, citrate, peptides, and amino acids, ultimately generating volatile aromatic compounds. In addition, bacteria have bioprotective effects because of the production of bacteriocins and other antimicrobial metabolites (Ristagno *et al*., 2012). A previous study corroborated these findings (Marino *et al*., 2019), reporting a high prevalence of LAB species (particularly *Lactococcus* spp. and *Weissella*) in the traditionally produced buffalo milk product, Mozzarella, from local markets. The LAB *Lactobacillus plantarum* IIA-2C12 and *Lactobacillus acidophilus* IIA-2B4 show extraordinary proteolytic activity against casein and sarcoplasmic proteins (Afiyah *et al*., 2015). Microorganisms present in cheese influence its flavor profile by producing volatile compounds (Percival & Percival, 2017). The bacterial community structure in samples is influenced by several factors, such as raw milk quality, fermentation conditions, and the addition of inoculation cultures (Yang & Yu, 2019).

Taxonomic Composition of Dangke

Microbiota diversity in dairy products originates from multiple sources, including teat surfaces, milking equipment, barn hygiene, and manure contamination. However, despite high-temperature treatment, a small population of microorganisms may remain in the final product (Quigley *et al*., 2013). The microbiome of cheese plays a vital role in determining its organoleptic and physicochemical properties, which affect its quality and safety (Yeluri Jonnala *et al*., 2018). Therefore, it is essential to implement additional control measures to reduce the transmission of pathogenic bacteria in dairy products. Metagenomic analysis was used to investigate diversity (Qu *et al*., 2024).

LAB constitutes a natural microbial community in milk, with significant populations of *Lactobacillus, Streptococcus,* and *Enterococcus* consistently reported in bovine milk (Yuan *et al*., 2022). The present study identified heat-resistant LAB strains, including *E. faecium,* which exemplifies a broad spectrum of thermotolerant LAB. These findings suggest that pasteurized milk is a valuable source for isolating heat-resistant LAB strains, indicating that in the presence of LAB, antimicrobial activity is also increased by antimicrobial peptides produced during fermentation (Hanifah *et al*., 2016). Proteomic analysis of two bacteriocin-producing *E. faecium* strains isolated from milk revealed significant antibacterial activity against *Staphylococcus aureus* and *Listeria monocytogenes* (Aspri *et al*., 2017). The bacteria were reported to exert bacteriocinogenic properties (Bagde & Vigneshwaran, 2019), making their antimicrobial metabolites valuable for the natural preservation of livestock-derived products (e.g., milk and meat). LAB, widely used to increase the functionality of various foods, is limited by its short lifespan. In addition, LAB are categorized as probiotics and can extend the shelf life and maintain the quality of products (Sulaiman *et al*., 2016). LAB isolated from Indonesian beef has been recommended as a preservative agent (Sihombing *et al*., 2015). There is a relatively high abundance of *Lactobacillaceae* in dry areas of cheddar cheese (Choi *et al*., 2020). The production process of traditional fermented milk products is relatively open and poorly controlled; microorganisms present in the production environment can easily enter containers and participate in the fermentation process (de Melo Pereira *et al*., 2022). LAB isolates from dangke produced pH values, titratable acidity, and viscosity that were relatively good compared to commercial cultures and even tended to be better, specifically regarding the total number of LAB from fermented milk (Syah *et al*., 2024).

Phylogenetic Tree of the Microbiota in Dangke

The microbiome of P1 reflected a controlled fermentation process using pasteurized milk, characterized by low microbial diversity, but a strong dominance of LAB. These conditions favor the growth of beneficial fermentative bacteria, such as *Enterococcus faecium* and *Staphylococcus condimenti*, while minimizing the presence of environmental or spoilage microbes, indicating a technological intervention to ensure product safety and consistency. The resulting phylogenetic tree exhibited limited stability, as indicated by low bootstrap values at specific nodes. Bootstrap values are displayed numerically on the phylogenetic branches to represent the confidence level. Higher bootstrap values indicate greater consistency in the clade arrangement (Simbolon & Aji, 2021). However, as Chatrou *et al*. (2012) explained, despite their low bootstrap scores, phylogenetic trees provide more robust results than those of morphology-based clustering.

Phylogenetic trees fundamentally depict ancestral relationships among species, genes, and organisms (Baum, 2008), and serve as critical tools for reconstructing evolutionary events, refining taxonomic classifications, and enhancing crucial insights into biodiversity. Branch length correlates with evolutionary divergence, with longer branches signifying greater genetic changes and more distant relationships between clades. Environmental factors affect the growth of microorganisms and, therefore, change the composition of the microbiota. Correlative analysis of the relationship between the metagenomic composition and environmental gradients can help elucidate the main ecological factors and principles of microbial community formation (Satoh *et al*., 2023).

The microbial profile of P2 revealed controlled fermentation using raw or minimally processed milk. LAB and environmental microbes reflect a less selective fermentation environment, possibly because of traditional processing methods, limited hygiene practices, or fermentation. Compared to P1, P2 exhibited greater microbial complexity. A phylogenetic tree of the microbiota was constructed using evolutionary-distance-based methods, specifically the neighbor-joining algorithm (Saitou & Nei, 1987). This method optimizes branch length estimation by selecting sequence joins that best reflect the actual evolutionary distances between sequences (Dharmayanti, 2011). *Bacillus* is a genus of gram-positive, rod-shaped bacteria belonging to the phylum Firmicutes (Setiaji *et al*., 2023). In P2, *Bacillota* clustered with *Lactobacillaceae* and *Weissella* and the facultative anaerobic *Weissella confusa,* which belongs to the family *Leuconostocaceae* (Salazar *et al*., 2018).

P3 is a traditionally fermented dangke product made from raw milk with minimal processing. It exhibited higher microbial diversity than P1 and P2 and was characterized by beneficial LAB and various environmental microorganisms. The detection of genera such as *Shinella, Acinetobacter, Brevundimonas*, and *Mixta,* many of which are environmentally derived, suggests limited hygiene control or exposure to environmental microbiota during processing (e.g., unclean equipment, ambient air, or manual handling). These findings highlight how the microbial community structure varies between samples, reflecting phylogenetic constraints and functional adaptations during dairy product processing. Non-random microbial assembly patterns that result in parallelism between host phylogeny and microbial similarity have been described as phylosymbiosis (Tang *et al*., 2021).

Sample P4 represented a microbiome shaped by raw buffalo milk. Its microbial diversity reflected this condition, with the dominance of Pseudomonadota and Bacteroidetes and the presence of families such as *Caulobacteraceae, Xanthomonadaceae, Sphingobacteriaceae, Flavobacteriaceae,* and *Weeksellaceae*, indicating a highly diverse and environmentally influenced community. Genera such as *Shinella, Brevundimonas, Sphingomonas,* and *Pseudoxanthomonas,* which are typically found in soil, water, and plant environments, suggest extensive environmental exposure during handling or storage. P4 (buffalo milk) was dominated by *Pseudomonadota* (formerly *Proteobacteria*), with *Caulobacteraceae* showing a strong phylogenetic affinity. These gram-negative bacteria display diverse morphologies (rods, spirals, and cocci) and metabolic strategies (phototrophic, heterotrophic, and chemolithotrophic) with varying oxygen requirements (Wangka *et al*., 2020).

Principal Coordinates Analysis (PCoA)

The principal axis (PCoA1) explained 56.12% of the variation, while PCoA2 and PCoA3 explained 23.59% and 14.72% of the total variation, respectively. Samples P1 and P2 tended to cluster, indicating relatively similar microbiota compositions, while P3, P4, and buffalo milk separated, indicating differences in their microbial communities. Dominant ASV vectors (e.g. ASVs 199592, 53345, and 1352) were strongly associated with sample divergence along the PCoA axes, indicating that specific taxa significantly differentiated the microbiota profiles between samples. These results confirmed that the source and fermentation substrate (buffalo milk) significantly influenced the final product microbiome ($p<0.05$).

Based on Quantitative Insights into Microbial Ecology, clusters P1–P2 were associated with ASV vectors 199592 and ASV1296, which are annotated as Lactococcus (e.g. *L. lactis*), as well as ASVs 53345/ASV1352 (*Streptococcus*/*Enterococcus*), reflecting the dominance of homolactic LAB in the final product. Sample P3 extended along PCoA2 toward ASV 1281 (*Weissella*), consistent with the contribution of heterofermentative LAB to the formation of volatile compounds (e.g. acetoin/diacetyl). In contrast, P4 and buffalo milk samples were driven by ASVs 65913/33946/2756 (*Staphylococcus*/*Bacillus*/*Pseudomonas*/*Acinetobacter*), indicating the influence of environmental flora and aerobic microbes from raw materials/equipment. Overall, LAB taxa, particularly *Lactococcus* and *Streptococcus*/*Enterococcus*, were the primary drivers of microbiota composition convergence in dangke, while environmental taxa separated buffalo milk samples and their products. LAB dominance benefits human health by serving as a probiotic that supports digestive health. The antimicrobial properties of LAB in cheese whey are beneficial (Mutmainna *et al.,* 2021).

In addition, kefir contains lactic acid bacteria (LAB) made from milk, colostrum, and a milk-colostrum mixture that has antimicrobial properties against *S. aureus*, *S. typhimurium*, *E. coli*, and *P. aeruginosa* (Setyawardani *et al*., 2020). These lactic acid bacteria are expected to inhibit the growth of pathogenic microbes and maintain the stability of the intestinal microflora of Indonesians (Astawan *et al*., 2012). These findings underscore how processing variables, particularly milk treatment, sanitation, and fermentation management, play critical roles in shaping the microbial community structure during dangke production. This highlights the potential of microbiome profiling as a tool for authenticating traditional food processes and optimising microbial composition to improve product quality and safety. Temperature, humidity, storage time, and contamination encourage bacterial growth (Mutmainna *et al*., 2023). These findings demonstrate that buffalo milk maintains a substantially distinct microbiota compared to dangke fermented dairy products. However, further research is required to elucidate the specific mechanisms governing bacterial activity.

CONCLUSION

Metagenomic analysis revealed rich and diverse microbial communities in milk and dangke samples, highlighting the complexity of the microbiota. Robust PCR amplification (approximately 1,500 bp) confirmed the presence of the target microbial DNA. Notably, *Enterococcus faecium* consistently dominated the lactic acid bacterial population in all dangke variants (P1–P3), showing a significantly higher abundance than that in raw milk. These results confirm that *E. faecium* plays a central role in fermentation and is the predominant LAB species involved. This study successfully demonstrated the utility of metagenomics in characterizing microbial communities in traditional dairy products and provided foundational data for understanding microbial succession and dominance during dangke fermentation.

CONFLICT OF INTEREST

I. I. Arief and C. Budiman serve as editors of the Tropical Animal Science Journal but have no role in the decision to publish this article. We certify that there are no conflicts of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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