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# Exploratory characterization of the milk bacterial microbiome in subclinical mastitis at CMT scores 2 and 3 in smallholder dairy farms using full-length nanopore 16S rRNA sequencing

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**Abstract**

This exploratory study characterized the taxonomic profiles of bacterial microbiota associated with CMT scores 2 and 3 in Holstein-Friesian dairy cows from smallholder farms in Batu City, East Java, Indonesia. A total of 126 cows from 29 smallholder farms were screened using the California Mastitis Test (CMT), and 27 cows had CMT scores of 2 and 3. One representative milk sample was selected for each condition: SP3 for CMT score 2 and SP4 for CMT score 3. Full-length 16S rRNA gene sequencing was performed using Oxford Nanopore Technology (ONT), and taxonomic classification was conducted using Centrifuge against the NCBI 16S RefSeq database. The SP3 sample yielded 99,600 high-quality reads and showed a *Streptococcus*-dominated taxonomic profile, with *Bacillota* as the predominant phylum and the *S. dysgalactiae group* as the dominant species-level taxonomic assignment. *Corynebacterium* and *Clostridium* were also detected. The SP4 sample yielded 98,300 high-quality reads and exhibited a highly skewed taxonomic profile, with *S. agalactiae* as the predominant species-level taxonomic assignment. These observations indicate distinct taxonomic profiles between the two representative samples, but they should be interpreted descriptively because only one sample was analyzed for each CMT condition. The findings provide an exploratory basis for further investigation of milk microbiome patterns associated with subclinical mastitis severity using full-length Nanopore 16S rRNA sequencing. Larger studies with adequate biological replication and independent species confirmation are required to validate these taxonomic patterns and assess their potential diagnostic relevance.



## 1. Introduction

Mastitis in dairy cows leads to major financial loss for farmers around the world. The significant economic impact results from declined milk production, altered milk composition, inflated treatment costs, and loss of productive cows by early culling (Rasmussen et al. 2024; Borș et al. 2024). Subclinical mastitis is the most commonly encountered form of mastitis, which is generally not detected easily because of lack of obvious swelling or visible changes in the milk (Nuraini et al. 2023). However, it is consistently associated with increased somatic cell count (SCC), reduced milk quality, and shifts in the bacterial composition living in the udder, which ultimately threaten animal health and the safety of dairy products (Winther et al. 2023).

Laboratory-based bacterial culture for conventional diagnosis of mastitis-associated pathogens holds significant historical value in identifying primary pathogens, such as *Streptococcus agalactiae* or *Staphylococcus aureus* (Langhorne et al. 2024). However, it does not account for the dynamics and complex interplay of the microbial communities inhabiting the mammary gland. Recent studies have shown mastitis as a disturbance of the ecological balance of the entire milk microbiota rather than as an outcome of a specific pathogen (Zhao et al. 2025; Surjowardojo et al. 2025b). This paradigm shift has led to looking at mastitis as a microbial dysbiosis where the community of bacteria changes in structure and how diverse it is in the milk (Guo et al. 2023).

The rise of next generation sequencing, particularly the high-throughput 16S rRNA gene sequencing technology, has offered new perspectives on identifying the bovine milk microbial community (Steinberg et al. 2022; Rifa'i et al. 2025b). Recent findings have shown that the healthy udder microbial community is relatively rich with dominant bacteria within the phyla like Bacillota (Firmicutes), Actinomycetota, Bacteroidota and Proteobacteria (Jiang et al. 2023). Yet, as the mastitis progresses, the succession pattern of the microbial community may alter, typically characterized by the expansion of opportunistic pathogens and a decrease in overall microbial diversity (Yu et al. 2025). Several metagenomic studies stated that bacteria like *Streptococcus*, *Staphylococcus*, and *Corynebacterium* become dominant during mastitis progression (Salman et al. 2023), indicating that microbiota imbalance plays a pivotal role in the pathogenesis of mastitis.

Nevertheless, most of the studies of mastitis microbiome to date have relied on a short-read sequencing platform that only targets partial regions of 16S rRNA gene, typically the V3-V4 region. It enables taxonomic identification only to genus level and does not properly resolve to the species level due to limitations in read length (Buetas et al. 2024). This limitation is particularly critical in mastitis research, given that closely related species such as *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* exhibit significant differences in virulence, epidemiology, and response to treatment (Kabelitz et al. 2021; Maeda et al. 2024). Therefore, developing a deep understanding of the dynamics of the mastitis-associated microbiota remains an ongoing challenge.

Long-read sequencing technology, such as that developed by Oxford Nanopore Technologies (ONT), enables full-length sequencing

of the 16S rRNA gene to provide greater taxonomic resolution for species-level identification and real-time profiling of the microbial community (Hu et al. 2021; Tigrero-Vaca et al. 2025) that helps in discriminating bacterial species in a complex microbial community and understanding their dynamics during disease progression (Aja-Macaya et al. 2025). However, studies specifically applying full-length ONT sequencing to characterize the milk microbiome at various stages of subclinical mastitis severity remain very limited (Johnson et al. 2019; Urrutia-Angulo et al. 2024), particularly in smallholder dairy systems in tropical regions.

Smallholder dairy farms, which dominate milk production in many developing countries, including Indonesia (Saputra et al. 2025), present unique epidemiological conditions in the context of mastitis (Rifa'i et al. 2024). Limited biosecurity practices, variability in hygiene during milking, and uncontrolled use of antimicrobials can influence the structure and evolution of microbial communities in the mammary gland (Hayashi et al. 2023; Surjowardojo et al. 2025a). Previous research from our group revealed that in fresh milk and milk with subclinical mastitis (CMT score 1), there is a shift in dominance from *Streptococcus parauberis* toward *Ralstonia pickettii*, indicating early ecological changes during the very early stages of infection (Rifa'i et al. 2025a). However, how these changes progress as mastitis severity increases, particularly at CMT scores 2 and 3, remains poorly understood at the species resolution level.

Understanding these changes is vital, because CMT scores 2 and 3 indicate differences in reaction intensity and potentially reflect the differences in intramammary inflammatory status. CMT score 2 generally reflects a moderate intramammary infection where the microbial community retains some of its diversity, while CMT score 3 indicates a more advanced infection with the potential for single-pathogen dominance and the collapse of microbiome balance (Tan et al. 2025; Zhu et al. 2024). Understanding the ecological transition between these two conditions can provide valuable insights into disease progression mechanisms and open opportunities for the identification of microbial biomarkers that can be used in early detection strategies.

Therefore, this study aims to characterize the milk bacterial microbiota associated with subclinical mastitis at CMT severity scores of 2 and 3 using full-length 16S rRNA sequencing based on Oxford Nanopore Technologies. By comparing the microbial community structure between these two conditions in samples from smallholder farms, this study seeks to descriptively characterize and compare the taxonomic profiles of representative CMT score 2 and CMT score 3 milk samples. The resulting findings are expected to contribute to a deeper understanding of mastitis pathogenesis and support the development of microbiome-based strategies for early detection and sustainable management of mastitis in smallholder dairy production systems.

## 2. Materials and Methods

### 2.1 Sample collection

This study was part of a broader investigation of the milk microbiota of Holstein-Friesian dairy cows with different levels of subclinical mastitis severity in smallholder farms. A total of 126 Holstein-Friesian dairy cows managed by 29 smallholder farmers in Toyomerto Hamlet, Pesangrahan Village, Batu Subdistrict, Batu City, East Java, Indonesia, were initially screened using the California Mastitis Test (CMT). Based on the screening results, 27 cows were identified with CMT scores of 2 or 3. From these cows, one representative milk sample was selected for each CMT condition for exploratory microbiome profiling. Sample SP3 represented CMT score 2, whereas sample SP4 represented CMT score 3. Selection of the representative samples considered consistency of the CMT results, the general health condition of the cows, and the quality of DNA obtained during the extraction process (Urrutia-Angulo et al. 2024; Couvillion et al. 2023). Milk samples were collected aseptically into sterile 250 mL bottles. Before sampling, the teats were cleaned and the first milk stream was discarded. Samples were transported to the laboratory within 24 h in a refrigerated container. Aliquots were stored at 4 °C until DNA extraction was performed (Usui et al. 2023; Faust et al. 2024).

### 2.2 California mastitis test (CMT)

The California Mastitis Test (CMT) was used as a field-based semi-quantitative screening method for subclinical mastitis and for categorizing cows according to CMT reaction scores. Approximately 2-3 mL of milk from each quarter was placed into a CMT paddle and mixed with an equal volume of CMT reagent (Rifa'i et al. 2024; Surjowardojo et al. 2025a). The mixture was gently swirled for 10–15 seconds, and the resulting viscosity was visually assessed using a 0–3 scoring scale. In this study, samples representing CMT score 2 showed clear gel formation with a convex surface, whereas samples representing CMT score 3 showed a strong and stable gel formation that did not flow back upon swirling. These reactions were used to classify the representative samples as CMT score 2 (SP3) and CMT score 3 (SP4), respectively (Rust et al. 2023; Ashraf and Imran 2018).

### 2.3 DNA extraction and quality assessment

Genomic DNA was extracted from 200 $\mu$L of each milk sample using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, catalogue no. D4300), according to the manufacturer's instructions. Following DNA extraction, DNA concentration and purity were initially assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific) based on the absorbance ratios at A260/280 and A260/230. The DNA concentration varied among samples, with the highest concentration recorded by NanoDrop being 209.2 ng/$\mu$L. DNA concentration was subsequently quantified using the Qubit dsDNA HS Assay Kit (Thermo Scientific) to provide fluorescence-based quantification of double-stranded DNA. The extracted DNA samples were subjected to quality assessment before amplification of the full-length 16S rRNA gene.

### 2.4 PCR amplification and amplicon quality assessment

The universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used to amplify the full-length 16S rRNA gene with Phusion™ Plus PCR Master Mix (Thermo Fisher Scientific, F631L). Amplification was done according a standardized laboratory protocol, with pre-denaturation at 95 °C for 5 min, followed by denaturation at 95 °C for 30 s, annealing at 60 °C for 45 s, and extension at 72 °C for 60 s; and a post-extension step at 72 °C for 5 min. PCR products were analysed by 1% TBE agarose gel electrophoresis using a DNA ladder as a molecular size marker. The expected amplicon size was approximately 1,500 bp. A negative template control (NTC) was included in the PCR assessment.

### 2.5 Full-length 16S rRNA gene sequencing

The bacterial community profiles of the selected milk samples were characterized by full-length 16S rRNA gene sequencing, using Oxford Nanopore Technologies (ONT). The SP3 and SP4 samples were subjected to exploratory microbiome profiling after PCR amplification

and assessment of amplicon quality. Following the manufacturer's instructions, ONT sequencing kit was used for library preparation. Multiplex sequencing of multiple samples in the single sequencing run was done by use of barcodes. The libraries formed were loaded onto a MinION sequencing device using MinKNOW software (version 24.02.16). Raw sequencing data were base-called using Dorado software (version 7.3.11) with a high-accuracy model. Read quality was assessed using NanoPlot, and sequence filtering was performed using NanoFilt to retain reads suitable for downstream taxonomic classification. Following quality processing, 99,600 high-quality reads were obtained from SP3 (CMT score 2), whereas 98,300 high-quality reads were obtained from SP4 (CMT score 3). The reads had an average fragment length of approximately 1,500 bp, consistent with the expected size of the full-length 16S rRNA gene amplicon.

**2.6 Taxonomic classification and bioinformatics analysis**

Taxonomic classification of the quality-filtered 16S rRNA gene reads was performed using Centrifuge (Kim et al. 2016) with the NCBI 16S RefSeq database as the reference database. Reference indexes for Bacteria and Archaea were constructed from the NCBI 16S RefSeq sequences. Taxonomic assignments were obtained across multiple taxonomic ranks, including phylum, family, genus, and species, where supported by the reference database and sequence classification results. The resulting taxonomic classification outputs were further explored and visualized using Pavian. Sankey diagrams were generated to visualize the distribution of classified reads across taxonomic ranks from phylum to species level. The taxonomic profiles of SP3 (CMT score 2) and SP4 (CMT score 3) were subsequently compared descriptively based on the relative distribution of classified reads. Species-level assignments were interpreted as sequence-based taxonomic classifications and were not independently confirmed using culture-based or isolate-level identification methods.

**2.7 Microbial composition and descriptive comparative analysis**

The relative abundance of each bacterial taxon was calculated based on the proportion of classified reads assigned at each taxon at the phylum, family, genus, and species levels relative to the total classified reads for each sample. Because one representative sample was analyzed for each CMT condition (SP3 for CMT score 2 and SP4 for CMT score 3), the microbiota profiles were evaluated descriptively. No inferential statistical comparisons or sample-level alpha- or beta-diversity analyses were performed between the two CMT conditions. Taxonomic composition was visualized using Sankey diagrams to illustrate the distribution of classified reads across taxonomic ranks from phylum to species level. Comparative bar charts were used to display the relative proportions of dominant species-level taxonomic assignments in SP3 and SP4. Differences between the two sample profiles were interpreted descriptively based on the relative distribution and dominance of the detected taxa. Given the use of one representative sample per CMT condition, these observations were considered exploratory and hypothesis-generating and were not interpreted as statistically generalizable differences between CMT severity groups.

**2.8 Methodological limitations**

This study used one representative sample per CMT condition as an exploratory pilot approach to characterize the milk microbiome using full-length Nanopore 16S rRNA sequencing in a tropical smallholder dairy setting. The use of a single representative sample for SP3 (CMT score 2) and SP4 (CMT score 3) limits statistical inference, biological replication, and generalizability of the observed taxonomic patterns. Therefore, the differences observed between the two samples should be interpreted as descriptive and hypothesis-generating rather than as evidence of statistically validated changes in microbial diversity or progressive microbiome restructuring. Species-level taxonomic assignments obtained from 16S rRNA sequencing were not independently confirmed using culture-based isolation or biochemical characterization. Accordingly, the identified taxa should be interpreted as sequence-based taxonomic assignments rather than culture confirmed etiological agents. Further studies involving larger sample sizes, adequate biological replication culture based confirmation, and complementary metagenomic or multi-omics approaches are warranted to validate the observed microbial community patterns and determine their potential relevance to subclinical mastitis severity.

## 3. Results

**3.1 PCR amplification and amplicon quality assessment**

Prior to sequencing, PCR amplification of the 16S rRNA gene was performed to assess the quality of the extracted DNA. PCR amplification of the full-length 16S rRNA gene was evaluated by 1% agarose gel electrophoresis in TBE buffer. Visible amplicon bands at approximately 1,500 bp were observed for SP3 (CMT score 2) and SP4 (CMT score 3), consistent with the expected full-length 16S rRNA gene product (Fig. 1). No visible amplification band was observed in the negative template control (NTC).

**3.2 Milk microbiota profile of CMT 2 subclinical mastitis cows**

Nanopore sequencing of the milk samples from subclinical mastitis cows (CMT score 2) successfully identified bacteria as the dominant

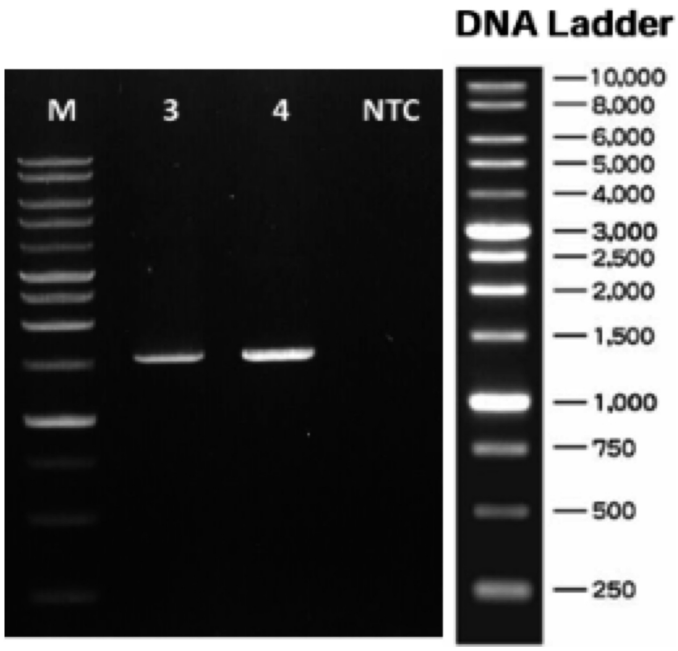


***Fig. 1.*** *Agarose gel electrophoresis of full-length 16S rRNA gene PCR products. Lane M: DNA ladder; Lane 3: SP3 (CMT score 2); Lane 4: SP4 (CMT score 3); NTC: negative template control. SP3 and SP4 showed amplicon bands at approximately 1,500 bp, whereas no visible amplification band was observed in the NTC. PCR products were subsequently quantified using the Qubit dsDNA HS Assay Kit before downstream library preparation and Nanopore sequencing*

component of the microbiota, resulting in a total of 99,600 high-quality sequences. Taxonomic assignments were detected across multiple taxonomic ranks, from phylum to species level of taxonomic classification; the entire taxonomic distribution within the microbiota from these samples can be seen below in Fig. 2.

### *3.2.1 Phylum Level*

The analysis results show bacterial diversity across all the taxonomic classifications levels. Bacillota was the predominant phylum in almost every read (98,000 sequences, 98.4%) and other phyla of far less abundance included Actinomycetota (484 sequences, 0.5%), Pseudomonadota (503 sequences, 0.5%), Bacteroidota (456 sequences, 0.5%), Campylobacterota (5 sequences), Fusobacteriota (12 sequences), Lentisphaerota (9 sequences), Mycoplasmatota (10 sequences), Spirochaetota (11 sequences), and Verrucomicrobiota (11 sequences). At the phylum level, Bacillota was predominant in the CMT-2 sample, accounting for 98.4% of the high-quality reads, indicating a highly skewed taxonomic composition.

### *3.2.2 Family Level*

The family-level taxonomic profile was dominated by Streptococcaceae, which had majority of sequences (around 92,500 reads) of all those detected in the milk microbiome. Among the families included Peptostreptococcaceae (2,290 reads), Aerococcaceae (1,390 reads), and Carnobacteriaceae (365 reads) along with several other families detected in small proportions, such as Corynebacteriaceae (266 reads), Clostridiaceae (223 reads), Lachnospiraceae (154 reads), Oscillospiraceae (142 reads), Turicibacteraceae (121 reads), and Rikenellaceae (130 reads). Overall, the taxonomic levels were heavily dominated by one pathogenic group of bacteria and were supplemented by a few other types.

### *3.2.3 Genus Level*

At the genus level, most predominant taxonomic assignment was *Streptococcus* with approximately 92,500 reads, which indicates this genus as a major contributor of the overall microbial community structure. The other bacterial genera found in the milk sample included *Corynebacterium* (266 reads), *Fundicoccus* (450 reads), *Suicoccus* (652 reads), *Atopostipes* (185 reads), *Jeotgalibaca* (137 reads), *Clostridium* (209 reads), *Faecalimicrobium* (415 reads), *Paraclostridium* (812 reads), and *Romboutsia* (886 reads). This shows a clear dominance of a single pathogenic genus and minor percentages from the remaining environmental or commensal groups.

### *3.2.4 Species Level*

At the species level, the *S. dysgalactiae* group was the predominant species-level taxonomic assignment, with 8,820 classified reads. Other assignments included *S.* pyogenes (3,110 reads), *Paraclostridium tenue* (708 reads), *Romboutsia timonensis* (667 reads), *Suicoccus acidiformans* (652 reads), *Faecalimicrobium dakarense* (415 reads), and other taxa at

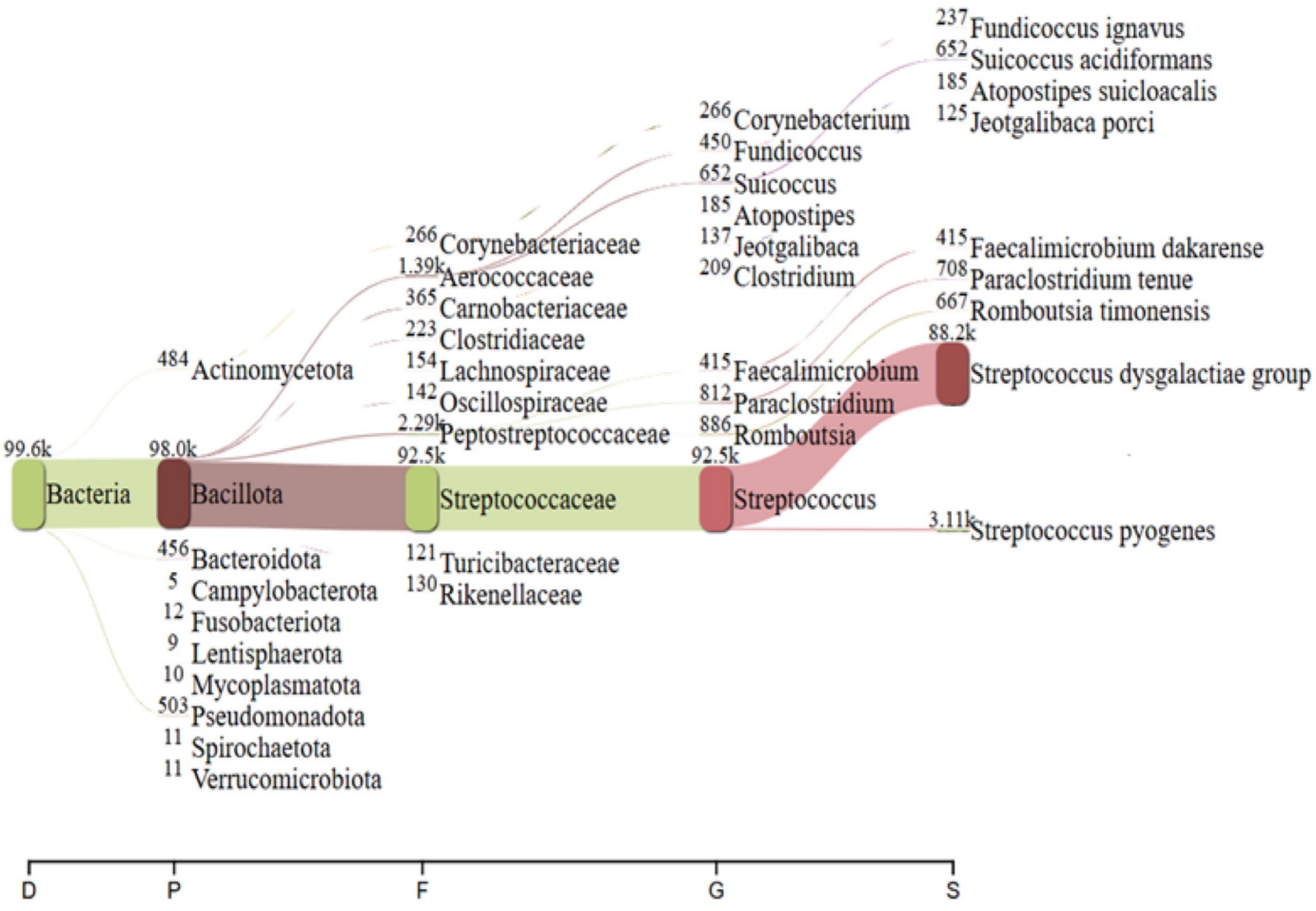


***Fig. 2.*** *Sankey diagram illustrating the taxonomic composition of the milk microbiota in the CMT score 2 representative sample based on full-length 16S rRNA Sequencing*

lower relative abundances.

Overall, the results showed that the milk microbiota from samples of subclinical mastitis (CMT score 2) was dominated by the *Streptococcus* genus, more specifically the *S. Dysgalactiae* group. *Corynebacterium* and *Clostridium* were also detected, indicating the presence of several minor bacterial taxa the *streptococcus*-dominated CMT score 2 community. Fig. 2 presents a Sankey diagram illustrating the taxonomic flow of the milk microbiome in subclinical mastitis with a CMT score of 2, with the dominant pathway Bacillota → Streptococcaceae → *Streptococcus* → *the Streptococcus dysgalactiae group* indicated by a thick flow representing relatively high abundance.

**3.3 Milk microbiota profile of CMT 3 subclinical mastitis cows**

Nanopore sequencing analysis of milk samples from cows with subclinical mastitis (CMT score 3) revealed a far more dramatic shift in microbial community structure compared to the CMT score 2 samples. The CMT score 3 sample exhibited a highly skewed taxonomic profile dominated by *Streptococcus*. The complete taxonomic distribution of the microbial community in these samples is presented in Fig. 3.

***3.3.1 Phylum Level***

The bacterial community at CMT score 3 was extremely taxonomically skewed. The Bacillota phylum was by far the most abundant phylum at around 98,200 reads (>99% of total reads), and all other phyla were detected at very low read counts, such as Actinomycetota (6 reads), Bacteroidota (21 reads), Fusobacteriota (11 reads), Pseudomonadota (34 reads), Planctomycetota (3 reads), Spirochaetota (2 reads), Thermodesulfobacteriota (2 reads), Bdellovibrionota (1 read), and Campylobacterota (1 read). It is clear that this represented an extremely skewed community, where the remaining non-dominant taxa were merely remnants in the milk.

***3.3.2 Family Level***

At the family level, the microbial community was almost entirely dominated by Streptococcaceae with around 98,100 reads. This high dominance at the family level is expected, considering the prominent association between Streptococcaceae and mastitis, particularly at increasingly severe levels of udder inflammation. Other families detected were extremely low in number, including Aristaellaceae (6 reads), Bacillaceae (41 reads), Oscillospiraceae (5 reads), Prevotellaceae (7 reads), Leptotrichiaceae (9 reads), Burkholderiaceae (4 reads), Neisseriaceae (12 reads), Pasteurellaceae (8 reads), and Succinivibrionaceae (4 reads). This distribution indicates that the CMT score 3 representative sample exhibited a highly skewed taxonomic profile at the family level, with Streptococcaceae accounting for most classified reads and several other families detected at much lower read counts.

***3.3.3 Genus Level***

A highly skewed taxonomic profile was observed in the CMT score 3 sample, in which *Streptococcus* constituted approximately 98,100 reads. Other genera detected only in minimal numbers included *Bacillus* (41 reads), *Aristaella* (6 reads), *Lactococcus* (4 reads), and *Selenomonas* (3 reads). *Leptotrichia* (9 reads), *Ralstonia* (4 reads), *Neisseria* (7 reads), *Aggregatibacter* (4 reads), and *Ruminobacter* (4 reads). These taxa are generally associated with environmental microbiota or opportunistic organisms, and their minimal presence underscores that the remaining classified reads were distributed among a small number of low-abundance taxa.

***3.3.4 Species Level***

The dominant bacterial species, with around 97,800 reads, was *Streptococcus agalactiae*. All others species, such as *Streptococcus*

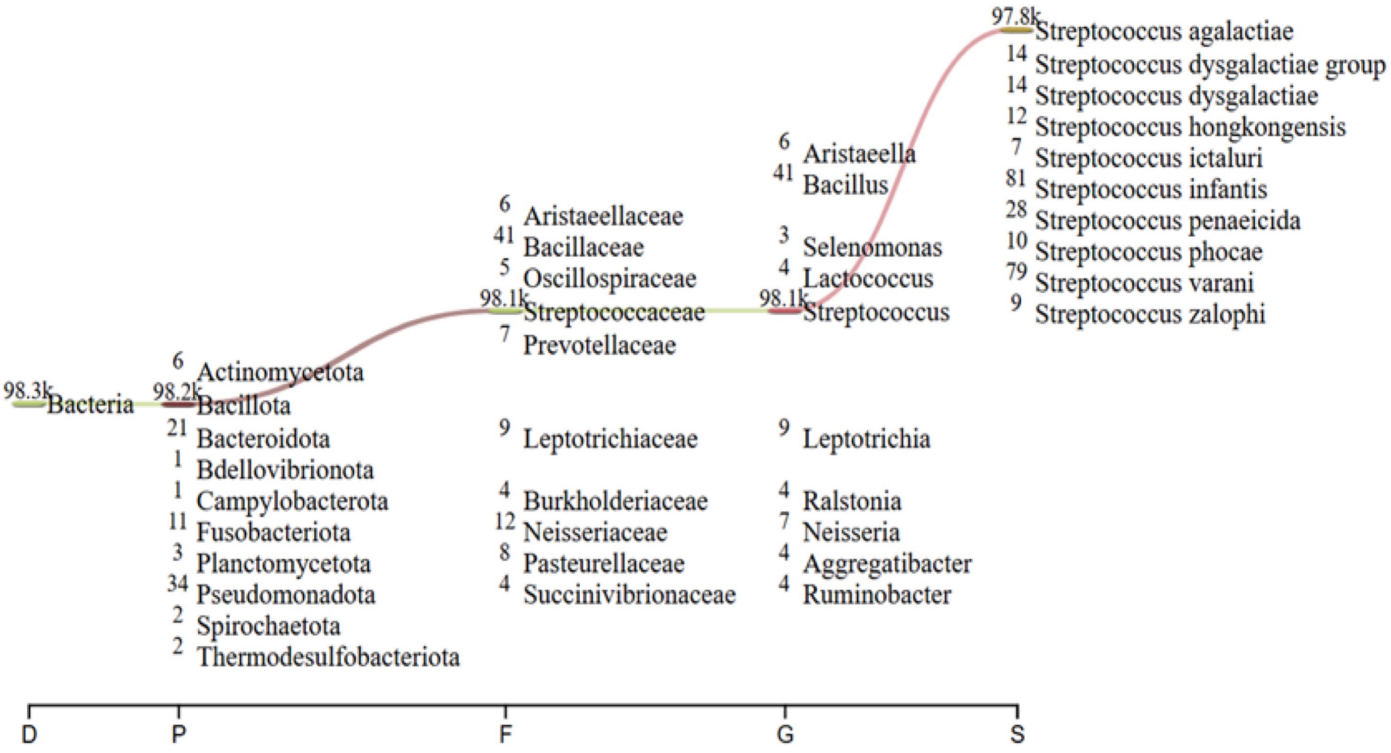


***Fig. 3.*** *Sankey diagram illustrating the milk bacterial community structure from CMT-3 Subclinical mastitis cows using full-length 16S rRNA sequencing*

*dysgalactiae*, *Streptococcus hongkongensis*, *Streptococcus ictalurid*, and *Streptococcus infantis* were far low in number. The dominance suggests a later stage of intramammary infection where the milk microbiota shifts towards the pathogens and the loss of normal protective commensal bacteria such as *Moraxella* and *Corynebacterium*, which can still be identified in CMT 2 score milk. A highly skewed taxonomic profile of the CMT score 3 sample revealed the dominance *S. agalactiae* because of its known strong adhesion properties for mammary epithelial cells and biofilm-forming capability that contribute to resistance to host immunity and antibiotic treatment. Fig. 3 presents a Sankey diagram illustrating the composition of the milk microbiota in CMT score 3 subclinical mastitis, with the dominant taxonomic pathway: Bacillota → Streptococcaceae → *Streptococcus* → *S. agalactiae*, indicated by a visibly thicker flow corresponding to its higher relative representation.

### 3.4 Comparison of microbiota composition between CMT score 2 and CMT score 3

A descriptive comparison of the two representative samples showed distinct taxonomic profiles, including a difference in the dominant species-level taxonomic assignment between SP3 (CMT score 2) and SP4 (CMT score 3). The main differences identified are summarized in Table 1. The two representative samples showed distinct taxonomic profiles, with different dominant species-level taxonomic assignments: *S. dysgalactiae group* at CMT score 2 to *S. agalactiae* at CMT score 3. The two representative samples differed in their detectable taxonomic composition. *Corynebacterium* and *Clostridium* were detected in CMT score 2 but were not detected among the classified reads from CMT score 3. Because only one representative sample was analyzed for each CMT condition, these observations are descriptive and cannot be used to infer population-level differences or progressive changes in microbial diversity. From a functional perspective, this shift indicates that changes in the milk microbiome may serve as a biological indicator of the severity of subclinical mastitis.

## 4. Discussion

Full-length Nanopore 16S rRNA profiling of representative milk samples from CMT scores 2 and 3 revealed distinct taxonomic patterns. The CMT score 2 sample was characterized by *Streptococcus* dominance with several minor taxa still detectable, whereas the CMT score 3 sample showed a highly skewed profile dominated by *S. agalactiae*. Because only one representative sample was analyzed for each CMT condition, these observations are descriptive and should not be interpreted as evidence of statistically significant changes in microbial diversity or progressive ecological disruption (Zhao et al. 2025; Guo et al. 2023).

In the representative milk sample with CMT score 2, several bacterial taxa remained detectable across multiple taxonomic levels, resulting in a less skewed taxonomic profile than that observed in the CMT score 3 representative sample. At the phylum level, Bacillota was dominant at approximately 98.4% of the total identified reads. The dominance of Bacillota at the early to middle stage of mastitis has been observed and reported consistently among a number of recent metagenomic studies. At phylum level, Bacillota together with Bacteroidota, Actinomycetota, and Proteobacteria constitute the dominant bacterial phyla in bovine milk microbiota (Reuben and Torres 2025). The predominance of Bacillota in the CMT score 2 sample indicates a strongly Bacillota dominated taxonomic profile, consistent with patterns reported in previous studies of bovine milk microbiota. This aligns with the report by Zhang et al. (2024), which reported that the abundance of Bacillota in healthy cows and subclinical mastitis is higher than in clinical mastitis cases, suggesting that certain degree of stability may be retained in the mammary gland microbial ecosystem.

At the family level, the dominance of Streptococcaceae and the presence of Corynebacteriaceae, Aerococcaceae, and Carnobacteriaceae indicate an initial stage of opportunistic pathogen invasion in the community, where relative biodiversity has not yet been substantially lost. It has been recently reported that increased abundance of Streptococcaceae shows strong correlation with increased CMT scores and mammary gland inflammatory response, and there are still no clinical symptoms visible (Salman et al. 2023). *Streptococcus* has several adaptive advantages that facilitate colonization of the mammary gland. They can efficiently utilize nutrients from milk, withstand the inflammatory environment, and outcompete other microbes in the mammary gland (Kabelitz et al. 2021).

At the species level, *Streptococcus dysgalactiae* was the dominant species in the samples with CMT score of 2. It is known as an etiological agent of both clinical and subclinical mastitis of cows. *Streptococcus dysgalactiae* isolates from mastitis cases were reported to harbor a number of virulence genes related to adherence to the epithelium, invasion of the gland tissue and immune avoidance (Crippa et al. 2023). Furthermore, antibiotic resistance genes were identified in *Streptococcus dysgalactiae* strains isolated from mastitis cases, which is a major challenge in mastitis treatment and control (Shen et al. 2021). The presence of this pathogen at a relatively high relative abundance in CMT score 2 implies that dysbiosis is already underway, although it may not have reached severe clinical symptoms. The detection of *Corynebacterium* and *Clostridium* in the CMT score 2 sample indicates that several minor bacterial taxa remained detectable within the *Streptococcus* dominated community, suggesting a less pronounced

**Table 1. Comparison of microbiota composition between CMT score 2 and CMT score 3**

| Characteristics | CMT score 2 | CMT score 3 |
|---|---|---|
| Total reads | 99,600 | 98,300 |
| Dominant phylum | Bacillota (98.4%) | Bacillota (>99%) |
| Dominant family | Streptococcaceae (92,500) | Streptococcaceae (98,100) |
| Dominant genus | *Streptococcus* (92,500) | *Streptococcus* (98,100) |
| Dominant species | *S. dysgalactiae* group (8,820) | *S. agalactiae* (97,800) |
| Commensal/ environmental taxa | *Corynebacterium*, *Clostridium* (detected) | Not detected |
| Taxonomic Profile | *Streptococcus*-dominated, with several minor taxa detected | highly skewed profile dominated by *S. agalactiae* |

taxonomic alteration than that observed at CMT score 3.

In striking contrast to the CMT score 2 sample, the microbial community structure in CMT score 3 sample exhibited an extremely simplified community and taxon dominance. At the phylum level, Bacillota accounted for greater than 99% of the classified reads in the CMT score 3 representative sample, whereas the other detected phyla were represented by substantially fewer reads. The change in predominant phyla at this high level has been observed consistently in severe mastitis cases. Inflammation and the alteration of milk physicochemical properties in the severe mastitis environment may impose severe selective pressures that favor certain bacterial taxa over others (Kizil et al. 2024; Tan et al. 2025). Bacteroidota and Proteobacteria were represented by fewer classified reads in the CMT score 3 representative sample is believed to represent the ecological filtering processes occurring in the inflammatory mammary gland niche, as inflammation causes shifts in milk pH, immune response activation and changes in nutrient availability that are detrimental to commensal and environmental microbes (Zhu et al. 2024). This phenomenon of ecological filtering becomes clearer at the family and genus levels where Streptococcaceae and *Streptococcus* showed dominant occupation. Recent studies have shown that severity of mastitis with increased somatic cell count has a high association with an increase of the *Streptococcus* and *Staphylococcus* genera abundance (Angelopoulou et al. 2024). Furthermore, these two opportunistic pathogens will not only affect the structure of milk microbiome but also worsen the milk quality.

At the species level, *Streptococcus agalactiae* was the predominant species-level taxonomic assignment in the CMT score 3 sample (>97% of total identified reads). This pathogen is known as a primary cause of contagious mastitis that can efficiently adhere to mammary epithelial cells and provoke a strong localized inflammatory immune response leading to gland tissue damage and increased SCC (Liu et al. 2024). In fact, the highly specialized, pathogen-driven microbial community formation in advanced stage of mastitis is correlated with the elimination of the protective microbes and the loss of microbial homeostasis in the mammary gland (Surjowardojo et al. 2025). The absence of these taxa in the CMT score 3 sample, together with the overwhelming dominance of *S. agalactiae,* indicates a highly skewed detectable microbial community profile. This may attributed to the formation of biofilm on mammary gland epithelial cells, which allows this species to evade the host's immune response while eliminating microbial competitors within the mammary gland environment (Bochniarz et al. 2024).

Comparison of the two representative samples revealed distinct dominant taxonomic profiles. The CMT score 2 representative sample was characterized by the *S. dysgalactiae group* as the dominant species-level taxonomic assignment, whereas the CMT score 3 representative sample was dominated by *S. agalactiae*. These observations indicate distinct detectable taxonomic compositions between the two representative samples. Because only one representative sample was analyzed for each CMT condition, these observations should be interpreted descriptively and cannot be used to infer population level differences or progressive changes in microbial diversity (Zhao et al. 2025). From the perspective of food safety, alterations in the milk microbial community during mastitis dysbiosis leads to changes in the quality and safety of processed dairy products. An increase in somatic cell count due to mastitis infection can lead to a reduction in protein, lactose, and non-fat solids content in milk, while also altering the physicochemical properties that are critical for milk processing (Maçi et al. 2025). These changes can reduce the suitability of milk for products such as cheese and yogurt, and increase the risk of microbial contamination during processing if hygiene management is suboptimal (Zalewska et al. 2025). Therefore, continuous monitoring of the milk microbiome using nanopore sequencing technology has the potential to be a valuable tool in food safety surveillance systems on smallholder farms.

The finding that shifts in the milk microbiome reflect the severity of subclinical mastitis provides opportunities for the development of microbiome-based diagnostic approaches. The distinct taxonomic profiles observed in the two representative samples suggest that *S. dysgalactiae* and *S. agalactiae* may represent candidate microbial signatures associated with different CMT severity states. These candidate signatures require validation in larger cohorts with adequate biological replication before their diagnostic utility can be established. 16S rRNA sequencing using nanopore technology has emerged as a promising method for portable microbiome monitoring in smallholder dairy farms due to its real-time analytical capabilities and species-level identification accuracy (Urrutia-Angulo et al. 2024; Aja-Macaya et al. 2025). Future diagnostic tools developed based on this method are expected to support more appropriate mastitis management, reduce unnecessary antibiotic use, and sustainably improve udder health and milk quality in smallholder dairy farming systems in the tropics.

Although this study offers valuable insights into microbial diversity and ecological dynamics in subclinical mastitis milk with CMT scores of 2 and 3, several limitations need to be acknowledged. The limited sample size, with only one representative sample per condition, restricts the ability to make statistical inferences and generalize the findings. Microbial diversity and function may vary due to natural biological variability among individual animals, farms, and different management systems. Species confirmation via culture-based methods and biochemical characterization was not performed in this study, although nanopore-based full-length 16S rRNA sequencing has been shown to provide reliable species-level resolution for complex microbial communities in milk (Urrutia-Angulo et al. 2024). Further research with larger sample sizes, longitudinal sampling designs, adequate biological replication, and a combination of metagenomic and culture-based approaches is highly recommended to validate and expand upon the findings of this pilot study.

## 5. Conclusions

This exploratory study characterized distinct taxonomic profiles of the milk microbiome in representative samples from CMT scores 2 and 3 in Holstein-Friesian dairy cows raised on smallholder farms in the tropics. Using full-length 16S rRNA sequencing with Oxford Nanopore Technologies, the CMT score 2 sample was dominated by *Streptococcus* dysgalactiae, while several minor bacterial taxa, including *Corynebacterium* and *Clostridium*, remained detectable. In contrast, the CMT score 3 sample exhibited a highly skewed taxonomic profile, with *Streptococcus agalactiae* accounting for more than 97% of the total classified reads and only a small proportion of other taxa detected. The distinct dominant taxonomic profiles observed in the two representative samples, with *S. dysgalactiae group* in the CMT score 2 sample and *S. agalactiae* in the CMT score 3 sample, indicate differences in detectable microbial community composition between these samples.

These observations underscore the potential of full-length Nanopore sequencing in providing high resolution characterization of microbial community patterns associated with different CMT score conditions in smallholder dairy systems. However, because each CMT condition was represented by a single sample in this study, the observed patterns should be considered descriptive and hypothesis-generating rather than evidence of a progressive microbiome collapse or a statistically validated microbial biomarker. Therefore, future studies with larger sample sizes, adequate biological replication, and complementary multi-omics approaches are warranted to validate these microbial signatures and determine their potential utility for assessment of mastitis severity and evidence-based disease management in smallholder dairy production.

## Declarations

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**Data Availability:** The data supporting this study are available upon request from the corresponding author

**Ethics approval:** Milk samples used in this study were obtained during routine milking without any additional handling or invasive procedures. Therefore, no ethical approval was required. All procedures were conducted in accordance with standard animal welfare guidelines and farm management practices

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