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Microbiome Dynamic: Diversity in Healthy and Mastitis Milk Herd

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ABSTRACT

Bovine mastitis is the inflammation of udder due to physical injury or microbial infections. The milk from different mastitis statuses present different microbial profiles that can impact the mechanisms and pathophysiology of mastitis. An increasing number of studies provided evidence indicating the occurrence of dysbiosis in the microbiota during clinical mastitis. Our study aimed to investigate the shifts in mastitis milk microbiota over a three-week period within a Jersey Friesian mastitis herd in a local farm (n=20). The milk samples were collected from healthy animals (HT), clinical mastitis milk at different time frames throughout three weeks (W1, W2, and W3) (n=5). Microbial genomic DNA from milk samples was extracted and then submitted for 16S amplicon sequencing. The 16S amplicon sequencing analysis revealed that the predominant phyla in the core microbiota were *Firmicutes, Actinobacteriota, Proteobacteria,* and *Bacteroidota*. Alpha diversity indicated the presence of lower bacterial diversity in the clinical mastitis group across the weeks (W1, W2, W3) in comparison to the healthy (HT) group. Among the four dominant phyla, *Firmicutes* exhibited the highest percentage of abundancy (HT=35.40%; W1=63.10%; W2=89.32%; W3=90.86%), followed by *Actinobacteriota* (HT=34.08%; W1=7.87%; W2=1.01%; W3=6.95%), *Proteobacteria* (HT=11.17%; W1=18.69%; W2=7.50%; W3=1.14%), and *Bacteroidota* (HT=14.77%; W1=1.86%; W2=1.01%; W3=0.88%). The diversity indices exhibited a decreasing trend from W1 to W3 (Chao1 index: HT=323, W1=297, W2=69, W3=35; Shannon index: HT=3.41, W1=3.87, W2=1.50, W3=0.92). Beta diversity displayed a scattered pattern of sample clustering in PCA plots among different groups. In conclusion, the dominance of *Firmicutes* persisted throughout the weeks, while other populations decreased over the specified time frame. The healthy (HT) group maintained a more diverse distribution of phyla.

Keywords: amplicon sequencing; dysbiosis; mastitis; milk microbiota; next generation sequencing

INTRODUCTION

Bovine mastitis is the inflammation of udder due to physical injury or microbial infections. The milk from different mastitis statuses present a different microbial profile that can impact the mechanisms and pathophysiology of mastitis. Various studies were done in Malaysia using culturable methods to reveal the causative microorganisms in order to get an insight into the bacteria involved in bovine mastitis (Ariffin *et al.,* 2019; Marimuthu *et al.,* 2014). However, studies examining the intramammary bacteria population of dairy cattle through conventional culture methods have been restricted to limited selective media, which isolate a limited spectrum of bacteria. Such studies are not conclusive in representing the total bacterial diversity existing in different clinical statuses of mastitis.

Numerous studies reported that 10 % to 40 % of clinical mastitis cases with "no growth", which is defined as no bacterial growth was observed, when subjected to conventional culture methods (Kuehn *et al.,* 2013), and evidence suggests that such cases are increasing (Kuehn *et al.,* 2013). There is no scientific evidence on the real reason behind this phenomenon.

However, the failure to culture bacteria might be due to their concentrations being below detectable levels, or the mastitis infection may be caused by non-bacterial agents. (Kuehn *et al.,* 2013).

High-throughput next-generation sequencing (NGS) technology involving 16S amplicon analysis and bioinformatic tools is a way to overcome the limitations of culture-based approaches (Kennedy *et al.,* 2016). This method has proven it to be possible to identify more than 90% of the majority of bacterial or pathogen communities at the genus level (Hoque *et al.,* 2019).

Mastitis is thought to be linked to alterations of microbiota composition in the udder, which can trigger an inflammatory response (Derakhshani *et al.*, 2018). Mastitis may not only be caused by pathogenic microorganisms but also by microbial imbalance in the milk, a condition referred to as dysbiosis (Derakhshani *et al.*, 2018; Kuehn *et al.*, 2013). To date, the microbial profiles of healthy versus mastitic mammary glands remain insufficiently characterized (Derakhshani *et al.*, 2018). In Malaysia, studies on mastitis milk microbiota are limited. One study conducted in Malaysia has proven that mastitis related to dysbiosis and the balance of microbiota is a condition that provides a protective role against mastitis (Tan *et al.,* 2023). However, there is a lack of reported evidence demonstrating the shift of the microbiota profile over time. Therefore, our study aimed to investigate the microbiome in healthy bovine and clinical mastitis milk, and the shifts in mastitis milk microbiota over a three-week period, which allows a comprehensive and practical time frame, within a Jersey Friesian mastitis herd in a local farm. This duration is ideal for studying the invasion pattern and the host-pathogen population interactions.

Most of the studies in Malaysia relied on conventional culture methods with limited bacterial detection capability; this research provides a comprehensive and culture-independent analysis of microbial dynamics, capturing shifts in bacterial community composition. Upon completion of this study, we have a more comprehensive and better understanding of the dynamics of diversity and population of the microbiota profile of healthy and animals with clinical mastitis throughout three weeks. The knowledge gained from this study offers new insights into the role of dysbiosis in mastitis development and highlights the potential for microbiome-based approaches as a strategy for mitigating dysbiosis-related diseases in improving udder health.

MATERIALS AND METHODS

Ethic Statement

The study was conducted in a commercial dairy farm in Pahang, Malaysia. The research underwent approval by the Animal Ethics Committee (AEC) at the Malaysian Agricultural Research and Development Institute (MARDI), with the protocol number 20190215/R/MAEC00054. All methods were conducted according to the approved guidelines.

Experiment Animals and Milk Samples Collection

The study was carried out on Jersey Friesian cows, cows consisting of 50% Jersey and 50% Friesian blood, located on a local farm (Pahang, Malaysia). The dairy cattle population consisted of cows in <300 days of lactation and were managed under an intensive production system. At sampling time, approximately 50 ml of milk samples from each cow were collected. The milk samples from healthy animals that are not infected with mastitis (HT), as a control, and clinical mastitis milk at different time frames throughout three weeks (W1, W2, and W3), with 5 animals in each group (n=5), to investigate the bacterial population shift at different time frames. Animals with normal milk appearance and somatic cell counts (SCC) below 200,000 cells/mL were considered free from mastitis infection (Lam *et al.*, 2009) and were included in the HT group. However, animals that produce milk with SCC >200,000 cells/mL with observed milk color changes and clotting were considered infected and were grouped in the CM groups. All samples were collected and kept at -80 °C prior to DNA extraction.

Isolation of DNA

Genomic DNA from milk samples was extracted using the DNeasy® PowerFood® Microbial Kit (Qiagen, Germany), following the manufacturer's protocol with slight modifications. The milk samples were first homogenized, and 1.8 mL of each was transferred into a 2 mL collection tube. The tubes were then centrifuged to remove any residual solids. The resulting pellets were collected and subjected to cell lysis at 70 °C for 10 minutes, followed by a 15-minute bead beating step, as recommended by the manufacturer. The tubes were centrifuged to remove the remaining contaminating non-DNA organic and inorganic materials. DNA from each sample was bound to a silica membrane and subsequently washed to eliminate salts and other impurities. In the final elution step, 50 µL of purified DNA was collected from each tube. The concentration and purity of the extracted DNA were assessed using a Nanodrop™ 1000 spectrophotometer at a wavelength of 260 nm. A 260/280 nm absorbance ratio of approximately 1.8 is considered indicative of high-purity DNA, in accordance with the Nanodrop TM1000 Spectrophotometer's protocol. The milk DNA samples were then submitted for 16S amplicon sequencing to Apical Scientific Sdn. Bhd.

16S rRNA Gene Amplicon Sequencing

The 16S rRNA gene of the V1-V2 hypervariable region was amplified using specific primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 338R (5'-TGCTGCCTCCCGTAGGAG-3') (Hamady *et al.*, 2008), with sample-specific barcodes. The pooled DNA products were used to construct an Illumina Pair-End library, following the Illumina genomic DNA library preparation procedure. Sequencing was conducted on an Illumina MiSeq platform according to the standard

protocols. The amplicon library was subjected to next-generation sequencing using the MiSeq platform 300PE.

Bioinformatic Sequencing

The 16S rRNA sequences were generated using the PE Illumina MiSeq platform, producing raw reads approximately 300 bp in length. The forward and reverse reads were merged using QIIME2, a software tool that removes sequence adapters and eliminates low-quality reads from the raw data (Caporaso *et al.*, 2010). The Divisive Amplicon Denoising Algorithm 2 (DADA2) pipeline version 1.14 (Callahan *et al.*, 2016) was employed for denoising, aiming to correct inaccurate reads, low-quality regions, and chimeric errors, resulting in amplicon sequence variant (ASV) data. These ASV data were then utilized for taxonomic classification, aligning them with the SILVA version 132 database (Quast *et al.*, 2013) to assign individual taxonomies. The SILVA database was used to assess sequence similarity within ASV reads, following recommended parameters at a 97% similarity threshold. Statistical analyses were performed to evaluate alpha and beta diversities, including diversity, Principal Component Analysis (PCA), and relative percentage comparison.

RESULTS

A total of 20 samples were grouped (n=5) in four groups, which consist of HT (HT[a], HT[b], HT[c], HT[d], HT[e]), W1 (W1[a], W1[b], W1[c], W1[d], W1[e]), W2 (W2[f], W2[g], W2[h], W2[i], W2[j]), and W3 (W3[k], W3[l], W3[m], W3[n], W3[o]). The clinical status is categorized into healthy non-mastitis (HT), and clinical mastitis throughout three weeks (W1, W2, W3), with 5 animals in each category representing different stages of severity of the condition to investigate the microbiota dynamic shift across three weeks in the same herd. Microbial DNA was isolated from each milk sample. The V1-V2 hypervariable regions were amplified (Table 1). Sequencing of 20 milk samples produced 8,000,852 sequences with sizes ranging from 35 to 301 bp. A total of 2,481,342 sequences were eventually used for analysis after trimming and chimeric sequence exclusion.

The median and distribution of the Chao and Shannon index demonstrated that Clinical groups across the three weeks decreased (Figure 1). The diversity of

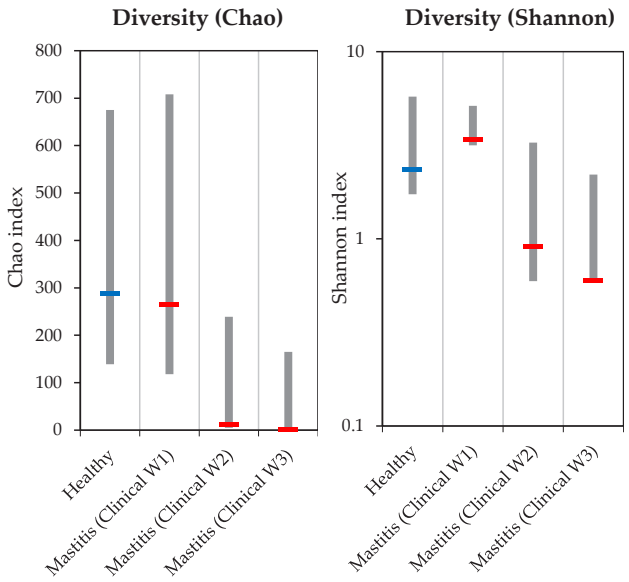

Figure 1. Median and distribution of Chao and Shannon diversity index in Clinical Week 1 (W1), Clinical Week 2 (W2), and Clinical Week 3 (W3) of the mastitis samples.

Table 1. Individual data of cow's parity, days in milk, somatic cell count, and DNA concentration from individual samples in healthy (HT), and different time frame clinical mastitis (W1, W2, W3) groups

Clinical status	Samples	Parameters		
		Days in milk	Somatic cell count (cells/mL)	DNA concentration (mean ng/µL ± SD)
Non mastitis (HT)	HT (a)	18	89,000	14.2 ± 0.14
	HT (b)	60	16,000	28.15 ± 0.63
	HT (c)	22	24,000	36.4 ± 0.42
	HT (d)	29	30,000	21.9 ± 0.14
	HT (e)	43	17,000	11.05 ± 0.49
Clinical Mastitis Week 1 (W1)	W1 (a)	262	>2,000,000	186.03 ± 2.51
	W1 (b)	28	>2,000,000	967.30 ± 24.81
	W1 (c)	57	>2,000,000	36.25 ± 0.07
	W1 (d)	258	>2,000,000	351.93 ± 3.69
	W1 (e)	59	>2,000,000	848.36 ± 17.33
Clinical Mastitis Week 2 (W2)	W2 (f)	200	>2,000,000	479.06 ± 2.61
	W2 (g)	82	>2,000,000	1016.06 ± 12.71
	W2 (h)	11	>2,000,000	885.36 ± 20.96
	W2 (i)	114	>2,000,000	759.96 ± 9.86
	W2 (j)	254	>2,000,000	290.83 ± 1.68
Clinical Mastitis Week 3 (W3)	W3 (k)	281	>2,000,000	911.00 ± 19.17
	W3 (l)	258	>2,000,000	646.36 ± 58.24
	W3 (m)	180	>2,000,000	1787.53 ± 92.23
	W3 (n)	22	>2,000,000	833.43 ± 7.29
	W3 (o)	224	>2,000,000	1021.56 ± 18.94

Note: SD – Standard deviation.

W2 and W3 is significantly lower compared to HT and W1. The microbial diversity, as well as species richness and evenness, were shifted in the period of 3 weeks of study. Mostly similar genera of pathogens were domineering the herd across the time frame, as shown in the diversity indexes. The PCA analysis illustrated varying bacterial sequence compositions in the HT group in contrast to the W1, W2, and W3 groups. The HT group exhibited a unique distribution pattern, whereas the W1, W2, and W3 groups showed overlapping patterns. Despite this overlap, distinct differences in bacterial distributions were observed, indicating clear disparities among these groups (Figure 2).

The HT group displays a more balanced distribution of phyla compared to W1, W2, and W3 (Figure 3). Over the specified time frame, there was a noticeable increase in *Firmicutes* within the population (HT=35.40%; W1=63.10%; W2=89.32%; W3=90.86%). Conversely, most other phyla, including *Actinobacteriota* (HT=34.08%; W1=7.87%; W2=1.01%; W3=6.95%), *Proteobacteria* (HT=11.17%; W1=18.69%; W2=7.50%; W3=1.14%), and *Bacteroidota* (HT=14.77%; W1=1.86%; W2=1.01%; W3=0.88%) showed a decreasing trend from W1 to W3 (Figure 4). This study supports the idea of dysbiosis in mastitis samples. The prevalence of the dominant bacteria in clinical mastitis samples indicates their potential as causative mastitis pathogens on the respective farm.

The data indicated that *Mycoplasma* spp. dominated the clinical mastitis cases during the study period (Figure 5). The W1, W2, and W3 exhibited a rapid increase in the percentage of *Mycoplasma* spp., reaching as high as 86.9% in W3, whereas this percentage was only 0.35% in the HT group. Conversely, the HT group showed a high prevalence of *Rhodococcus* spp. (27.81%) and *Streptococcus* sp. (11.76%), while other genera accounted for approximately 5% or less (Table 2).

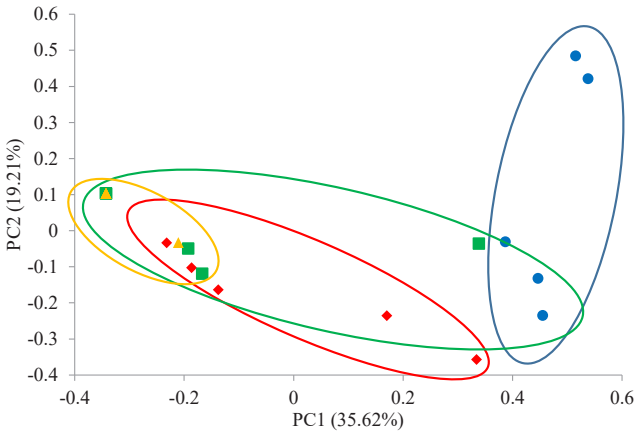

Figure 2. Principle component analysis (PCA) in healthy (●), HT (HT[a], HT[b], HT[c], HT[d], HT[e]); clinical week 1 (♦), W1 (W1[a], W1[b], W1[c], W1[d], W1[e]); clinical week 2 (■), W2 (W2[f], W2[g], W2[h], W2[i], W2[j]); and clinical week 3 (▲), W3 (W3[k], W3[l], W3[m], W3[m], W3[o]) of the mastitis samples. Each dot represents an individual, and colours indicate the populations in four metagenomes.

DISCUSSION

The data from 16S amplicon sequencing was collected over three weeks of milk sampling from the same cow herd. The microbial population went through a dynamic change in its composition (Figure 5). From the HT milk microbial population, these changes in the microbial population dynamics range from the dominance of mutual symbionts to opportunistic pathogens while entering clinical mastitis and eventually towards the dominance of a pathogenic species during extreme conditions, as represented by microbial dysbiosis (Porcella *et al.*, 2020).

In the overall analysis, *Firmicutes* clearly dominated the farm during the sampling period, leaving no doubt that this bacterial group was the phyla of the causative agent of mastitis at the sampled point (Figure 3). The data provides a fairly unbiased snapshot of the phyla and genera present in the milk microbial community. The data might be a good representation of the insight into cow mastitis intramammary of untreated cows across the timeline of infection, as a mastitis treatment will take approximately 5-21 days. The escalating population of *Firmicutes* coincided with a decrease

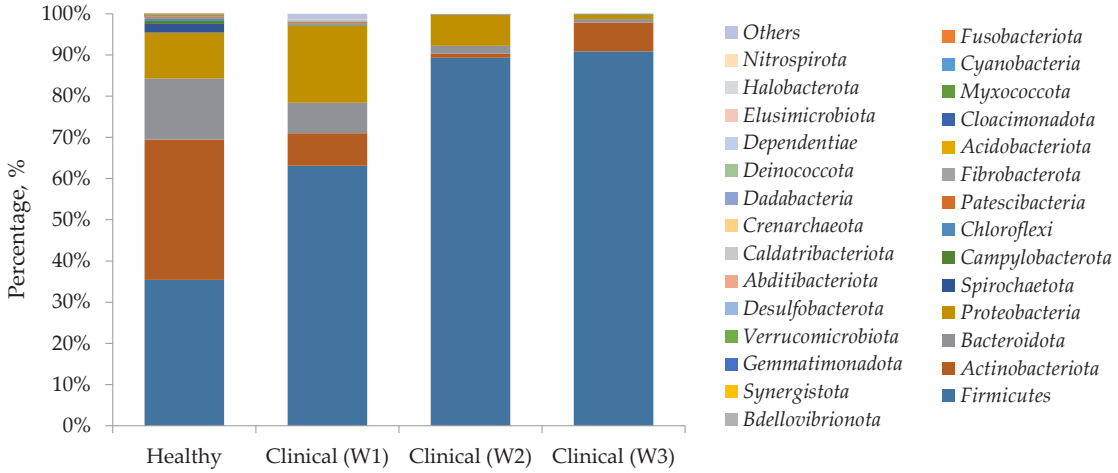

Figure 3. The proportions of abundant microbial phyla in milk from clinical mastitis of Jersey Friesian cows collected throughout week 1 (W1), week 2 (W2), and week 3 (W3) of mastitis infection.

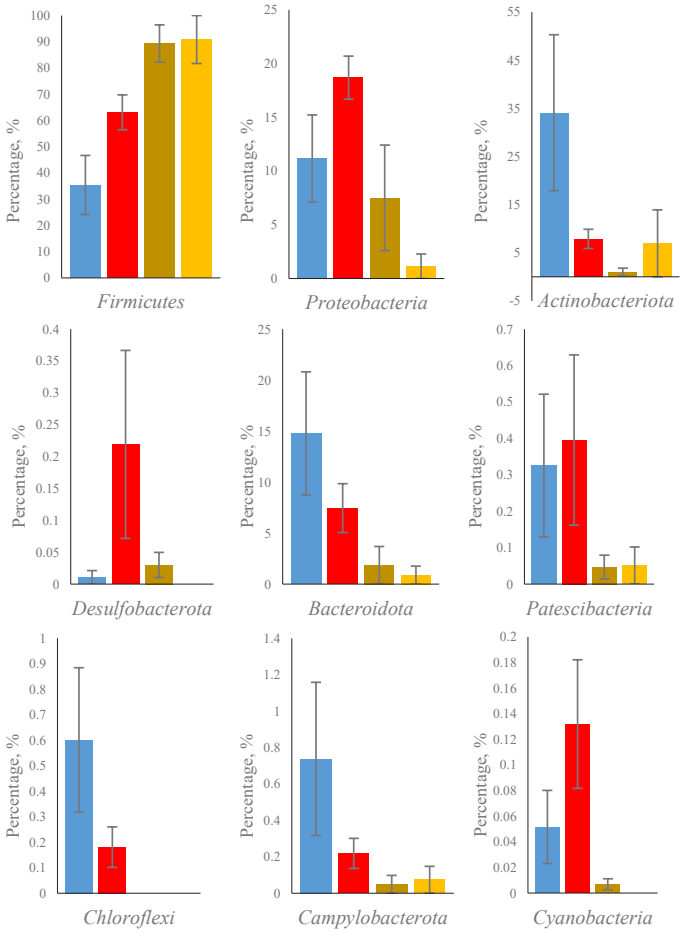

Figure 4. The microbial phyla changes in milk from clinical mastitis of Jersey Friesian cows collected throughout week 1 (W1), week 2 (W2), and week 3 (W3) of mastitis infection. Healthy (■), Clinical (W1, ■), Clinical (W2, ■), and Clinical (W3, ■).

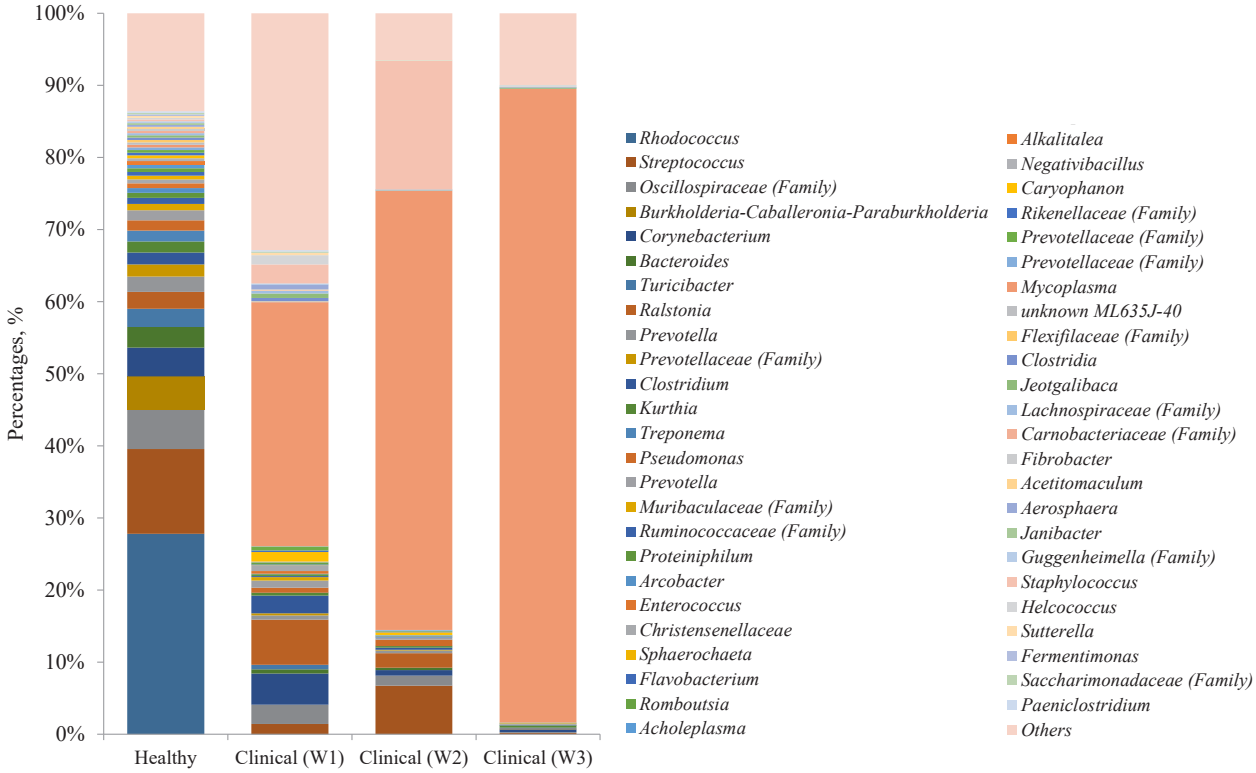

Figure 5. The proportions of abundant microbial genera in milk from clinical mastitis of Jersey Friesian cows collected throughout week 1 (W1), week 2 (W2), and week 3 (W3) of mastitis infection

Table 2. The most abundant microbial genera (> 0.5%) in clinical mastitis week1 (W1), week2 (W2), and week3 (W3) samples

Genus / Family	Week of clinical mastitis sampling			
	HT (%)	W1 (%)	W2 (%)	W3 (%)
Mycoplasma	0.35	33.85	60.91	89.66
Ralstonia	2.37	6.27	2.03	0.00
Rhodococcus	27.81	0.00	0.00	0.00
Burkholderia-Caballeronia-Paraburkholderia	4.65	0.00	0.00	0.00
Corynebacterium	4.00	4.31	0.75	0.11
Oscillospiraceae (Family)	5.39	2.68	1.40	0.17
Staphylococcus	0.24	2.63	17.80	0.00
Clostridium sensu stricto 1	1.64	2.45	0.25	0.14
Methylobacterium	0.02	1.82	0.23	0.02
Undibacterium	0.18	1.77	0.08	0.02
Streptococcus	11.76	1.40	6.73	0.05
Caryophanon	0.39	1.32	0.37	0.04
Helcococcus	0.24	1.29	0.03	0.17
Alloprevotella	0.14	1.06	0.18	0.00
Chryseobacterium	0.01	0.99	0.05	0.14
Prevotella_9	1.39	0.97	0.25	0.01
Christensenellaceae R-7 group	0.54	0.79	0.08	0.08
Rhodocyclaceae (Family)	0.02	0.73	0.00	0.39
Serratia	0.00	0.70	2.38	0.00
Pseudomonas	1.41	0.70	0.86	0.00
Aerococcus	0.17	0.69	0.04	0.06
Comamonas	0.08	0.68	0.00	0.03
Trueperella	0.13	0.62	0.00	0.00
Turicibacter	2.51	0.61	0.00	0.00
Jeotgalibaca	0.32	0.61	0.04	0.02
Aerosphaera	0.27	0.60	0.02	0.00
Bacteroides	2.84	0.60	0.31	0.03
Prevotella	2.10	0.57	0.35	0.06
Prevotellaceae (Family)	0.36	0.56	0.09	0.00
Cutibacterium	0.06	0.53	0.00	0.00
Blautia	0.12	0.51	0.03	0.00
Globicatella	0.11	0.51	0.01	0.00
Muribaculaceae (Family)	0.88	0.46	0.00	0.03
Prevotella	2.10	0.57	0.35	0.06
Kurthia	1.54	0.40	0.23	0.00
Treponema	1.52	0.01	0.86	0.02
Proteiniphilum	0.68	0.25	0.00	0.04
Acrobacter	0.67	0.05	0.04	0.07
Erwiniaceae (Family)	0.00	0.37	0.73	0.03
Enterococcus	0.64	0.34	0.00	0.01
Sphaerochaeta	0.52	0.04	0.00	0.00
Flavobacterium	0.51	0.10	0.01	0.01
Romboutsia	0.51	0.29	0.09	0.03
Romboutsia	0.51	0.29	0.09	0.03
Prevotellaceae (Family)	1.68	0.29	0.13	0.04

Note: The most abundant percentage within the groups was in bold. HT – Healthy; W1 – Week 1; W2 – Week 2; W3 – Week 3.

in other phyla, such as *Proteobacteria*, *Actinobacteria*, *Desulfobacterota*, *Bacteroidota*, *Patescribacteria*, *Campylobacterota*, *Chloroflexi*, and *Cyanobacteria*, indicating that *Firmicutes* dominated other phyla (Figure 4).

At the genus level, *Mycoplasma*, known as one of the main pathogens in bovine mastitis, exhibited a noticeable increase, reaching 89.66% in W3, becoming the predominant member within the *Firmicutes* phylum. *Mycoplasma* species are significant contributors to bovine mastitis, causing both clinical and subclinical infections in dairy cows (Fox 2012). *Mycoplasma* tends to spread rapidly within a herd, as evidenced by our investigation of clinical mastitis infection over a three-week sampling period (Figure 5). *Mycoplasma* infection led to alterations in milk SCC, as well as changes in milk composition and quality (Al-Farha *et al*., 2017). Our findings suggest that

Mycoplasma infections in cows' mastitis can be rapidly transmitted, leading to a significant prevalence of the bacteria in affected animals.

Comparatively, *Mycoplasma* was found at 0.35% in the HT group, indicating its role as one of the balanced microbes within a diverse microbial population, similar to other mastitis pathogens such as *Streptococcus* (11.76 %), *Staphylococcus* (0.24 %), *Clostridium* (1.64 %), *Pseudomonas* (1.41 %), and *Enterococcus* (0.64 %) (Table 2). *Mycoplasma* mastitis is usually excluded from standard mastitis screening tests due to its unique growth needs and the time delay involved (Okella *et al.*, 2023). Traditional culturing of *Mycoplasma* from milk samples used to be a slow process, often taking up to two weeks and frequently resulting in non-growth due to these bacteria's specific culture requirements (Parker *et al*., 2018). Recent research has shown that some *Mycoplasma*-infected samples yield negative cultures but positive PCR results (Al-Farha *et al.,* 2017). However, metagenomic technology provides comprehensive information about the causative agents in respective farms.

Rhodococcus in the phyla of *Actinobacteria* is a genus of aerobic, gram-positive bacteria. It has a relatively fast growth rate and a simple development cycle, and exists in a high percentage in the HT group (Figure 3). *Rhodococcus* can be pathogenic, but most of the species are benign and largely found in environments such as water and soil (Patek *et al.,* 2021). Livestock, according to research by Vechi *et al.* (2018) and Żychska *et al*. (2021), can serve as hosts for *Rhodococcus*. However, in our study, *Rhodococcus* was identified as one of the mutual microbes in the HT milk samples, as it does not affect the milk quality despite the high population in the HT milk (27.81 %).

Besides, *Streptococcus* did not emerge as a pathogenic bacteria during this specific sampling period, presenting a contrast to previous findings. In earlier samplings, *Streptococcus* exhibited a significantly high prevalence in the CM milk group and is known as the causative agent of mastitis (Table 2). Conversely, *Streptococcus* was identified as one of the common microbes in the HT group during other sampling instead (Table 2). It highlights that different pathogens might cause distinct clinical mastitis cases, contingent on the prevailing causative agents in each situation. Intriguingly, pathogenic bacteria were also detected in the Healthy group's milk, indicating their existence as mutual symbionts within a balanced microbial population. This further supports the concept of dysbiosis in mastitis disease within our local farm.

Ralstonia was associated with the contamination of water, as well as water purifying systems, potentially as a source of contamination in milking that relies heavily on water (Ryan *et al.*, 2011). Therefore, the genera may exist in higher amounts in the farm, as the local farm is highly reliant on underground water. As for other genus, decreasing trends were observed except for *Mycoplasma*. This finding provides valuable information for developing targeted treatment strategies for dairy herds. Therefore, vigilant monitoring and management strategies are crucial to prevent the spread of *Mycoplasma*-induced mastitis and maintain the overall health of the dairy herd on the farm.

Analysis of microbiota dynamics over the three weeks reinforced the association between the incidence of microbiota dysbiosis and mastitis. The decreasing Chao and Shannon diversity index during this time (Figure 1) suggest a shift in microbial community composition. This imbalance, characterized by the overrepresentation of certain pathogens, likely contributes to mastitis development. While the precise mechanisms by which specific microbial taxa influence this process remain unclear, our findings further support the link between microbiota dysbiosis and clinical mastitis. The balance of microbiota provides a certain degree of protection against infectious diseases, like mastitis. The investigation reported in Malaysia to characterize shifts in milk microbiota over time using 16S rRNA amplicon sequencing, offering new insights into microbial dynamics associated with mastitis, and the importance of incorporating molecular tools for detection of mastitis-related pathogens and targeted treatment strategies for the dairy herds.

CONCLUSION

The dominance of *Firmicutes* persisted throughout the weeks, particularly *Mycoplasma* in the clinical mastitis (CM) group, while other populations decreased over the specified time frame. The healthy (HT) group maintained a more diverse distribution of phyla. This study supports the idea of dysbiosis in mastitis samples. The prevalence of specific bacteria in clinical mastitis samples indicates their potential as causative mastitis pathogens on the respective farm. This finding provides valuable information in developing targeted treatment strategies for the dairy herds.

CONFLICT OF INTEREST

We certify that there is no conflict 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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