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FULL PAPER

Genomic mapping milk microbiota from healthy, sub-clinical and clinical mastitis of Jersey Friesian cattle in a Malaysian farm

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Abstract

Bovine mastitis is the persistent, inflammatory reaction of the udder due to physical trauma or microorganism infection. The milk from healthy and mastitis cows presents an ecosystem of microbial communities, which can influence the mechanisms and pathophysiology of mastitis. Hence, there is a possible shift in microbiome composition in healthy, subclinical and clinical mastitis. This study reported the composition of microbiota in the udder of Jersey Friesian cows in one of the local farms located in Pahang, Malaysia. From the 16s amplicon sequencing analysis, the core microbiota was dominated by phyla of *Firmicutes*, *Proteobacteria*, *Actinobacteriota*, and *Bacteroidota*. Most of the predominant genera from healthy groups were mainly *Ralstonia*, *Staphylococcus*, *Corynebacterium*, *Turicibacter*, *Acinetobacter*, *Escherichia*, *Aerococcus*, and *Streptococcus*. Furthermore, the majority of subclinical mastitis milk was populated by genera of *Ralstonia*, *Escherichia*, *Aerococcus*, and *Corynebacterium,* whereas, *Streptococcus*, *Ralstonia* and *Escherichia* were predominant in clinical mastitis samples. The alpha and beta diversity analysis indicated that microbiota from healthy and subclinical were more diverse compared to clinical mastitis microbiota. Therefore, predominant genera from clinical mastitis samples might be the potential of causative mastitis pathogens in the respective farm. The use of culture-independent analysis presented here revealed a wide bacterial diversity and variation between different clinical statuses.

1. Introduction

Bovine mastitis is a major production disease with high incidence and prevalence that has caused considerable loss to the dairy industry. These losses are attributed to reduced milk production, poor milk quality, pharmacotherapy and labour cost. In Malaysia, increasing dairy cattle production is one of the important projects undertaken by the Malaysian government to increase the milk sufficiency level (Malay Mail 2020).

Bovine mastitis is caused by bacterial infection, following the inflammation of the udders. An inflammation response in the mammary gland is initiated when there is an intrusion of bacteria, after which leukocytes are attracted into the milk in large numbers. The interaction between the bacteria and the mammary cells in milk stimulates the production of numerous mediators of inflammation that leads to the pathogenesis of the disease (Zacconi and Smith, 2000). This resulted in an increase in milk somatic cell count (SCC), as part of the natural defence mechanism (Harmon, 2001). The SCC is an indicator of the quality of milk, of which the

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somatic cells in milk samples are stained with a fluorescence dye prior to counting under a fluorescence microscope. A threshold value below 200,000 cells/mL is normally used as a cut-off for an indicator of subclinical mastitis (Lam *et al*., 2009).

Mastitis is caused by either contagious or commensal microorganisms. In Malaysia, reported mastitis causing pathogens include *Staphylococcus aureus* (Othman and Bahaman, 2005; Ariffin *et al*., 2019; Ali *et al.,* 2020), non-aureus Staphylococci (Othman and Bahaman, 2005; Marimuthu *et al*., 2014; Ali *et al*., 2020), *Streptococcus agalactiae* (Ariffin *et al*., 2019; Ali *et al*., 2020), *Escherichia coli* (Othman and Bahaman, 2005; Marimuthu *et al*., 2014; Ariffin *et al*., 2019), *Klebsiella pneumoniae* (Marimuthu *et al*., 2014; Ali *et al*., 2020), *Streptococcus uberis* (Othman and Bahaman, 2005; Ali *et al*., 2020), *Bacillus* (Othman and Bahaman, 2005; Marimuthu *et al.*, 2014), *Corynebacterium* sp. (Othman and Bahaman, 2005; Marimuthu *et al*., 2014), *Yersinia* (Othman and Bahaman, 2005; Marimuthu *et al*., 2014), *Neisseria* (Marimuthu *et al*., 2014), *Acinetobacter* (Othman and Bahaman, 2005; Marimuthu *et al*., 2014), *Pseudomonas* (Othman *et al*., 2005; Marimuthu *et al*., 2014) and *Micrococcus* (Othman and Bahaman, 2005).

Conventionally, studies on the bacterial composition of the mammary gland from dairy cattle were restricted to the use of a few selective media suited for the isolation of a narrow spectrum of Gram-positive and -negative bacteria. Therefore, the results of such studies cannot be considered illustrative of total bacterial diversity existing in healthy and mastitis milk, since the presence of other Gram-positive bacteria or Gram-negative bacteria was possibly overlooked. Furthermore, approximately 10-40% of clinical mastitis cases yield "no growth" in clinical culture assays, as reported by Kuehn *et al*. (2013); Taponen *et al*. (2009); Makoccc and Ruegg (2003). On the other hand, the 16S rRNA gene can identify almost the entire bacterial community, both commensal and pathogenic, since it can overcome the limitations of the culture-based bacterial detection method (Kennedy *et al*., 2016).

Recent metagenomic studies suggested that mammary secretion from a healthy udder consists of a diverse bacterial group, which has not been associated with mastitis (Derakhshani *et al*., 2018). Mastitis is believed to occur with changes in the composition of udder microbiota, resulting in the activation of an inflammatory response (Derakhshani *et al*., 2018). In addition, a recent study suggested that mastitis may be associated not only with mastitis pathogens but also with an imbalance of the milk microbiota (Kuehn *et al*., 2013). This gives the idea of microbiota dybiosis is the consequence of infectious mastitis (Derakhshani *et al*., 2018). However, the profile of microbiota in the healthy and mastitis mammary glands has not been fully investigated (Derakhshani *et al*., 2018). The understanding of microbiota profile and host-pathogen mechanisms will develop an effective therapeutic treatment of bovine mastitis.

Limited studies have been carried out to understand the diversity of microbiota in healthy and mastitis milk samples (Kuehn *et al*., 2013; Oikonomou *et al*., 2014; Oultram *et al*., 2017). The objective of the present study was to understand the diversity and dominant bacteria existing in bovine milk of healthy, subclinical and clinical mastitis Jersey-Friesian cows from a local dairy farm using the 16S rRNA amplified gene approach.

2. Material and methods

2.1 Ethic statement

The study was conducted in one of the local commercial dairy farms situated in Pahang, Malaysia. The research protocol was reviewed and approved by the Animal Ethics Committee (AEC) in the Malaysian Agricultural Research and Development Institute (MARDI) with the protocol number 20190215/R/MAEC00054. The methods were carried out in accordance with the approved guidelines.

2.2 Sample collection

The dairy cattle population for the present study consisted of Jersey Friesian cows at middle to late lactation in a local farm (Pahang, Malaysia). The animals were managed under an intensive production system. Average days in milk (DIM) and milk yield (MY) were recorded for each cow. Prior to sample collection, the udder was washed with water, dried, and swabbed with 75% of alcohol. Subsequently, the first 5 mL of milk was discarded and a sample of 50 mL was collected in a sterile tube. After collection, the tubes were brought to the laboratory in an ice box and were processed immediately.

2.3 Screening of mastitis

Screening of healthy, subclinical and clinical mastitis was done based on clinical examination of the udder physically via visual and palpitation to detect redness, swelling, pain, warmth, hardness of udder tissue, and abnormal milk appearance. Milk abnormality can be detected via the California mastitis test (CMT), and somatic cell count (SCC). The CMT was carried out by mixing an equal volume of CMT reagent and milk by swirling it in the specific paddle and the results were recorded. The results were further confirmed by SCC,

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which was quantified using Chemometex NucleoCounter® SP- 100TM (Chemometex, Denmark). Milk from healthy cows with negative CMT and SCC <200,000 cells/mL was classified as healthy (HT) cow's milk. While, milk from a cow with positive CMT and SCC >200,000 cells/mL, without any sign of clinical mastitis, was classified as subclinical (SC) cow's milk, and subsequently milk from a cow with positive CMT, and SCC >200,000 cells/mL, with a visible abnormality of milk or udder were classified as clinical mastitis (CM) cow's milk. To determine the causative agents, cows that were having a history of mastitis infections will be selected in the clinical mastitis group.

2.4 DNA extraction

The DNA from milk samples were extracted using DNeasy® PowerFood® Microbial Kit (Qiagen, Germany) and in accordance with the manufacturer's protocol with minor modifications. Milk samples were homogenized. An amount of 1.8 mL milk was added to a 2 mL collection tube. Tubes were centrifuged to remove the food residues. The pallet from each tube was collected and subjected to cell lysis at 70ºC for 10 mins, followed by a bead beating process for 15 mins, as suggested by the manufacturer. The tubes were centrifuged to remove the remaining contaminating non-DNA organic and inorganic materials. The DNA from each tube was bound to silica and washed to remove salt and other contaminants. A final volume of 50 µL of the total DNA from each tube was eluted and was collected in the final elution step. The purity and concentration of extracted DNA were analysed using Nanodrop TM1000 spectrophotometer at 260 nm wavelength. The ratio of the absorbance at 260/280 nm between 1.8 - 2.2 is considered optimum in DNA purity. Samples with good purity but low concentration will be amplified with Genomiphi V2. The DNA samples from HT (n = 5), SC (n = 5), and CM (n = 5) were sent to Shanghai Biozeron Biological Technology Co. Ltd (http:// www.biozeron.com) for 16s amplicon sequencing analysis.

2.5 PCR amplification

The V3-V4 marker region of the bacteria was amplified by PCR (95°C for 2 mins, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 mins using primers sequences: 5' - barcode 1 - (forward primer 515F) - 3' and 5'- barcode 2 - (Reverse primer 997R) - 3', where a barcode is an eight-base sequence unique to each sample. PCR reactions were performed in a triplicate 20 µL mixture containing 4 µL of 5 × FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.).

2.6 Sequences library construction and sequencing

The sequences library was constructed in accordance with the protocol adopted from the Shanghai Biozeron Biological Technology Co. Ltd. The purified PCR products were quantified, and every 24 amplicons with different barcodes were mixed equally. 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 pair-end sequenced (2 × 300) on the platform.

2.7 Processing of sequencing data

Raw fastq files were demultiplexed, and quality-filtered using QIIME (version 1.9.1). Based on the overlapping relationship, paired-reads were merged into a single read. The merged reads were used to Operational Taxonomic Units (OTUs) clustering, taxonomy classifying and community diversassessmentsing. The 300 bp reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50 bp. Only exact barcode matching will be considered, and 2 nucleotide mismatches in primer matching, and reading containing ambiguous characters were removed. Also, only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. The microbial community could be used to compare similarities or dissimilarities between different sample groups, analyses of the relationship between microbial community and environmental factors, phylogenetic analysis, and other statistical analyses.

2.8 Alpha- and Beta- diversity analysis

Based on the results of OTUs cluster analysis, the Alpha-diversity of all samples was estimated. Sequences from each OTUs ranged from large to small according to the OTUs richness, and the Rank abundance curves were drawn with the relative abundances of each OTUs ranked against the OTUs ranks. Principal coordinate analysis (PCoA) was performed with QIIME program to examine dissimilarities in the community composition profile of the samples by plotting all samples in different dimensional spaces. The samples were grouped based on unweighted and weighted UniFrac distance metrics.

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2.10 Statistical analysis

The data of each group were compared among each genotype. Analysis of the associations between the group was conducted using SAS software SAS/STAT® 9.4 (SAS Institute Inc. 2011). The *P-value* of less than 0.05 is regarded as statistically significant.

2.11 Data access

All raw sequences are deposited to Sequence Read Archive (SRA) in National Centre for Biotechnology Information (NCBI) with the accession number PRJNA775085. https://www.ncbi.nlm.nih.gov/sra/PRJNA775085

3. Results

3.1 Cow data, milk analysis and DNA extraction

To identify suitable samples for analysis, milk was collected from the udder of five healthy (HT), five subclinical mastitis (SC), and five clinical mastitis (CM) Jersey Friesian dairy cows. The parity, days in milk (DIM), somatic cell count (SCC), and histories of milking and mastitis disease are shown in Table 1. Samples with an SCC reading <200,000 cells/mL are categorized as HT milk, while samples with SCC reading >200,000 cells/mL without any sign of clinical mastitis were classified as SC milk, and subsequently milk with SCC reading >200,000 cells/mL, with a visible abnormality of milk or udder were classified as clinical mastitis (CM) cow's milk samples (Table 1).

The extraction of DNA from HT, SC and CM milk samples using DNeasy® PowerFood® Microbial Kit is optimized. However, low DNA yield was obtained from HT milk samples, with a DNA concentration of 1.42 – 21.73 ng/µL. Minor modifications were made according to the manufacturer's protocol by treating all HT milk samples with Genomiphi V2 Whole Genomic DNA Amplification System in order to obtain sufficient amounts of DNA for downstream use. The concentration of extracted DNA is shown in Table 1.

3.2 Sequencing results

The DNA was successfully sequenced from all samples. However, sequencing data of sample N16 from HT group was lost during data transfer by the service provider, thus this sample was excluded from the study. The 16s amplicon sequencing of all remaining milk samples generated a total of 1693046 reads, with an average of 417 bp in length. Due to the trimming and quality control, the number of effective reads per sample ranged from 100025 to 148170. The milk samples were grouped into HT, SC and CM in the experiment as well as analysis. The effective read per sample in HT, SC and CM groups were approximately 123124±15329, with an average length of 417.5±7.1 bp, 119267±17930, with an average length of 414.1±13.6 bp, and 120842±18464, with average length 421.3±3.6 bp, respectively. No significant difference (p>0.05) was observed among the number of effective reads of different groups, indicating that the data can be used for further analysis.

The number of OTUs in Venn diagram for different groups and rare fraction graph is shown in Figure 1a, HT (total OTUs = 1035), SC (total OTUs = 1410) and CM

Table 1. Individual data of cow's parity, days in milk, somatic cell count and DNA concentration from individual samples in HT (H1, H7, H9, H10), SC (H8, N3, N25, N45, N50) and CM (N21, N23, N32, N36, N40) groups

Clinical status	Samples	Parity	Days in milk	Somatic cell count (cells/mL)	DNA concentration (Mean ng/µL±SD)
Healthy (HT)	H1	1	260	172,000	775.81±10.49*
	H7	2	219	112,000	799.07±6.71*
	H9	1	187	31,000	851.76±5.47*
	H10	1	206	12,000	850.70±3.08*
	N16	1	153	200,000	816.03±9.18*
Sub-clinical mastitis (SC)	H8	2	300	798,000	117.58±2.85
	N3	2	187	406,000	141.28±0.63
	N25	1	621	1,120,000	854.83±8.98
	N45	3	267	903,000	744.27±8.65
	N50	1	191	>2,000,000	100.89±1.49
Clinical mastitis (CM)	N21**	3	162	>2,000,000	1219.43±62.77
	N23**	4	202	>2,000,000	1019.39±2.33
	N32**	2	318	>2,000,000	1334.60±10.22
	N36**	1	385	>2,000,000	1110.46±7.59
	N42**	3	385	>2,000,000	1261.60±19.52

*DNA were amplified by GenomiPhi V2 DNA Amplification Kit;
**Recurrent mastitis in the related quarter

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(total OTUs = 1503), indicated that the number of OTUs assigned in CM is more when compare with the other group. Rare fraction analysis was done and richness of total bacterial communities was predicted (Figure 1b). The number of reads by the number of species in the rare fraction curves diagram indicated that the sequencing depth was completed for microbial richness in the samples. Increasing the number of reads beyond the 100,000 values will have minimal impact on the number of species.

3.3 Taxonomy profile at the phylum level

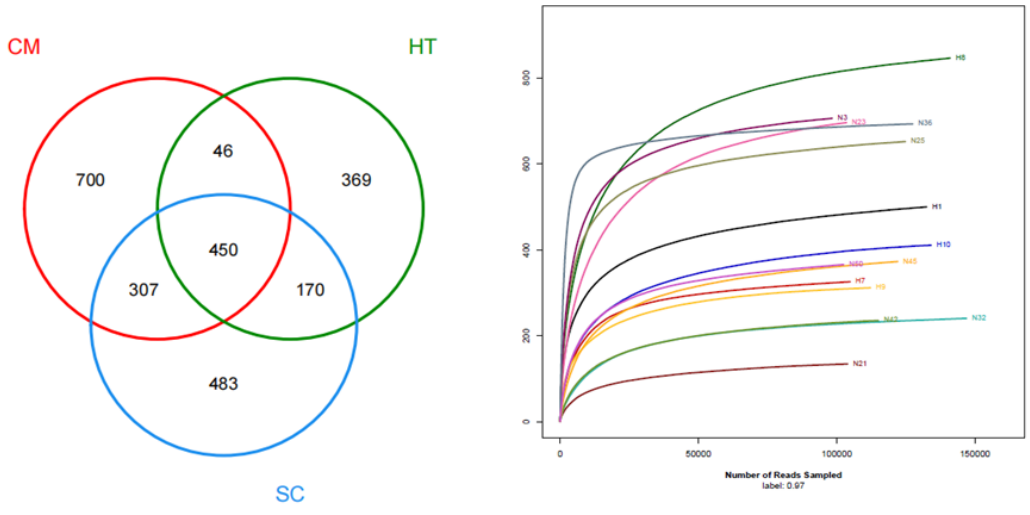
The ten most abundant microbial phyla for each group were identified as shown in Figure 2. Microbial phyla were identified based on 97% 16s rRNA gene sequence identity. *Proteobacteria* are the most abundant in HT and SC (HT 43.8%; SC 63.3%), and the second abundance in CM group (CM 38.6%). On the other hand, *Firmicutes* was the most abundantly present in CM group (CM 45.8%), and the second most abundant in HT and SC groups (HT 39.2%; SC 23%). Subsequently, *Actinobacteriota* (HT 12.3%; SC 4.9%) were the next abundance in both HT and SC group samples. However, CM group only recorded an approximately 1.4% of mean relative abundance of *Actinobacteriota*, which was lesser when compared to *Cyanobacteria* (3.5%), *Campylobacterota* (1.9%), and *Bacteroidota* (1.4%). The rest of the predominant phyla for HT, SC and CM are shown in Figure 2.

3.4 Taxonomy profile at the genus level

At the genus level, distinct bacterial communities were detected in HT, SC and CM, which differed in both composition and abundance. It was found that the prevalent genera were diverse in different groups. An average number of 164.5±31.6 genera was identified in the HT group, 236.0±68.1 in the SC group and 181.2±92.2 in the CM group. A total of 46 pre-dominant genera was observed as shown in Figure 3. The genera percentage showed differences between the microbial communities in quarters of different sample groups. A genus with more than 0.5% of total sequences was defined as predominant and these predominant genera contributed 88.38%, 87.71%, and 92.52% of total sequences in HT, SC and CM groups, respectively (Table 2).

The HT group had more pre-dominant genera than the CM group. There were 24 pre-dominant genera in HT when compared to the CM group which only has 12 genera. The most prevalent microbial genera of HT group included *Ralstonia* (28.7%), *Staphylococcus* (10.6%), *Corynebacterium* (9.4%), *Turicibacter* (5.7%), *Acinetobacter* (4.9%), *Escherichia-Shigella* (3.9%), *Aerococcus* (3.5%), and *Streptococcus* (2.5%). Whereas, *Ralstonia* (40.8%), *Escherichia-Shigella* (13.7%), *Aerococcus* (10.1%), *Corynebacterium* (3.1%), *Streptococcus* (1.9%), *UCG-005* (1.5%), *Sphingomonas* (1.5%), and *Stenotrophomonas* (1.4%) were among the most prevalent microbial genera presents in SC group. On the other hand, the most prevalent microbial genera detected in the CM group included *Streptococcus* (34.0%), *Ralstonia* (21.2%), and *Escherichia-Shigella* (14.1%) (Table 2).

The analysis demonstrated that the CM group had lesser pre-dominant genera, while the HT group showed more balanced microbial profiles. This pattern can be seen in the proportions bar plot (Figure 3) of individual



(a) (b)

Figure 1. Sequencing result analysis. (a) Venn diagram of OTUs and rarefraction graph in healthy (HT), sub-clinical (SC) and clinical (CM) mastitis. OTUs are clustered with a 97% similarity cut-off. The different coloured circles represent different sample groups. The figures in the intersection of two circles represent the number of OTUs in both samples. OTUs are clustered with a 97% similarity cut-off. (b) Rarefaction analysis of the individual samples. Rarefaction curves of OTUs clustered at 95% sequence identity across different samples. Rarefaction curves of OTUs clustered at 95% sequence identity across different samples.

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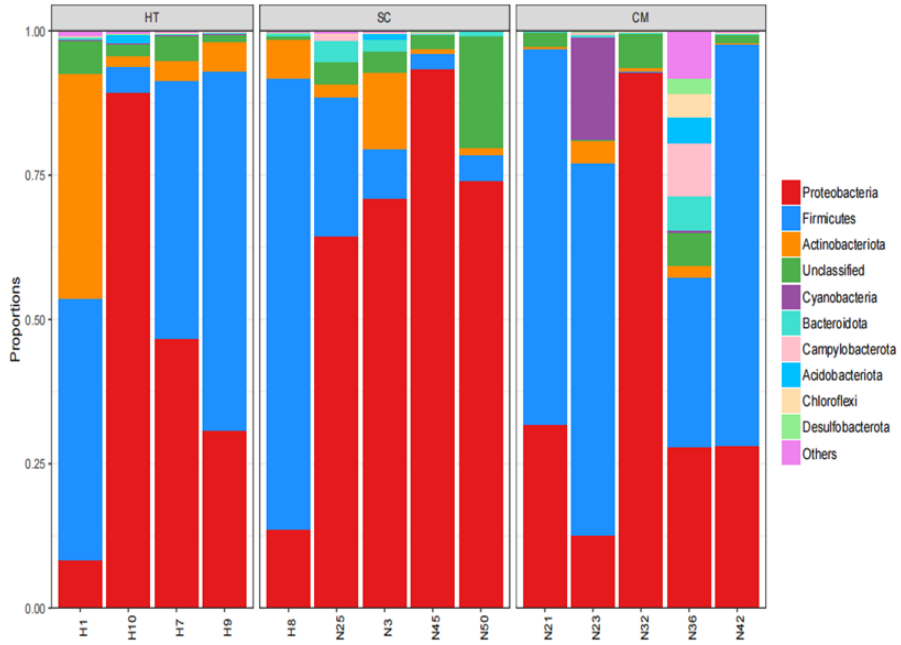

Figure 2. The proportions of the ten most abundance microbial phyla of healthy (HT: H1, H7, H9, H10), sub-clinical (SC: H8, N3, N25, N45, N50) and clinical (CM: N21, N23, N32, N36, N42) mastitis samples

Table 2. The most abundant microbial genera (>0.5%) in healthy (HT), sub-clinical (SC) and clinical (CM) mastitis samples

HT (Mean %)	SC (Mean %)	CM (Mean %)
Ralstonia (28.7%)	*Ralstonia* (40.8%)	*Streptococcus* (34.0%)
Staphylococcus (10.6%)	*Escherichia* (13.7%)	*Ralstonia* (21.2%)
Corynebacterium (9.4%)	*Aerococcus* (10.1%)	*Escherichia* (14.1%)
Turicibacter (5.7%)	*Corynebacterium* (3.1%)	*Jeotgalibaca* (4.4%)
Acinetobacter (4.9%)	*Streptococcus* (1.9%)	*Sulfuricurvum* (4.2%)
Escherichia (3.9%)	*UCG-005* (1.5%)	*Chloroplast_norank* (3.6%)
Aerococcus (3.5%)	*Sphingomonas* (1.5%)	*Globicatella* (1.3%)
Streptococcus (2.5%)	*Stenotrophomonas* (1.4%)	*Helcococcus* (0.7%)
Jeotgalibaca (2.0%)	*Jeotgalibaca* (1.3%)	*Ignavigranum* (0.7%)
Ignavigranum (1.6%)	*Methylobacterium-Methylorubrum* (1.2%)	*Methylobacterium-*
Methylobacterium-Methylorubrum (1.6%)	*Gulbenkiania* (1.0%)	*Methylorubrum* (0.6%)
Helcococcus (1.5%)	*Planococcaceae_Unclassified* (1.0%)	*Corynebacterium* (0.6%)
Weissella (1.4%)	*Staphylococcus* (0.7%)	
UCG-005(1.1%)	*Methyloversatilis* (0.6%)	
Bacillus (0.8%)	*Macrococcus* (0.6%)	
Pseudomonas (0.7%)	*Clostridium sensu stricto 1* (0.6%)	
Micrococcus (0.7%)	*Acinetobacter* (0.5%)	
Guggenheimella (0.7%)		
Erysipelothrix (0.6%)		
Fastidiosipila (0.6%)		
W5053 (0.6%)		
Trueperella (0.6%)		
Macrococcus (0.5%)		
Bifidobacterium (0.5%)		

HM: Health, SM: Subclinical mastitis, CM: Clinical mastitis

samples. CM samples contain a lesser percentage of the relative abundant bacterial community profiles as compared to HT and SC samples.

3.5 Comparison of genera between microbiota populations in HT, SC, and CM group

Principal component analysis (PCoA) was plotted to represent microbial communities that are similar in terms of sequence composition (Figure 4). The ellipse PCoA in the CM group showed more different bacteria sequence compositions when compared to SC and HT groups. Table 3 shows the comparison of mean relative abundance in HT, SC and CM of the selected bacterial genus identified. The prevalence of microbial genera in different samples was diverse. Some genera were more abundant in the specific group when compared with

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Table 3. Comparison of mean relative abundance in healthy, subclinical and mastitis quarters of bacterial genus identified. The bold reading indicated the highest percentage compared to other groups

Genus	Clinical mastitis dominant genus (Mean, %±SE)		
	Healthy	Sub-clinical mastitis	Clinical mastitis
Streptococcus	2.50±2.13	1.94±1.57	**34.03±12.72**
Escherichia	3.94±2.38	13.67±11.88	**14.06±12.56**
Jeotgalibaca	1.98±1.40	1.34±0.87	**4.38±3.49**
Sulfuricurvum	0	0	**4.23±2.39**
Chloroplast_norank	0.01±0.00	0.01±0.00	**3.55±7.84**
Globicatella	0.00 [a]	0.07±0.01 [a]	**1.28±0.00** [b]
Genus	Sub-clinical mastitis dominant genus (Mean, %±SE)		
	Healthy	Sub-clinical mastitis	Clinical mastitis
Ralstonia	28.69±14.31	**40.7±9.24**	21.15±2.98
Escherichia	3.94±2.38	**13.67±11.88**	14.06±12.56
Aerococcus	3.58±2.46	**10.06±7.65**	3.26±2.61
Sphingomonas	0.35±0.23	**1.49±0.54**	0.39±0.03
Stenotrophomonas	0.036±0.023	**1.43±1.02**	0.01±0.00
Methylobacterium	1.55±0.49	**1.25±0.45**	0.62±0.11
Gulbenkiania	0.01±0.00	**1.01±0.01**	0.00±0.00
Methyloversatilis	0.04±0.03	**0.62±0.49**	0.13±0.07
Macrococcus	0.54±0.46	**0.62±0.35**	0.00±0.00
Eubacterium	0.08±0.05	**0.42±0.21**	0.04±0.01
Brevundimonas	0.02±0.01	**0.36±0.30**	0.01±0.00
Lysinibacillus	0.03±0.00	**0.34±0.01**	0.02±0.00
Genus	Healthy mastitis dominant genus (Mean, %±SE)		
	Healthy	Sub-clinical mastitis	Clinical mastitis
Staphylococcus	**10.56±10.48**	0.74±0.31	0.03±0.01
Corynebacterium	**9.38±7.42**	3.08±1.52	0.56±0.49
Turicibacter	**5.74±5.63**	0.14±0.05	0.14±0.09
Acinetobacter	**4.91±2.40** [a]	0.51±0.24 [b]	0.11±0.05 [b]
Ignavigranum	**1.57±1.28**	0.44±0.22	0.65±0.44
Methylobacterium	**1.55±0.49**	1.25±0.45	0.62±0.11
Helcococcus	**1.47±1.25**	0.37±0.21	0.66±0.37
Weissella	**1.36±1.16**	0.11±0.08	0.01±0.00
Bacillus	**0.81±0.79**	0.02±0.00	0.01±0.00
Pseudomonas	**0.68±0.60**	0.24±0.11	0.02±0.01
Micrococcus	**0.67±1.14**	0.23±0.00	0.12±0.14
Guggenheimella	**0.65±1.06**	0.17±0.00	0.02±0.02
Erysipelothrix	**0.63±0.75**	0.17±0.00	0.05±0.08
Fastidiosipila	**0.62±0.84**	0.03±0.00	0.02±0.00
Trueperella	**0.55±0.51**	0.13±0.00	0.06±0.00
Macrococcus	**0.54±0.46**	0.62±0.35	0.00±0.00
Bifidobacterium	**0.52±0.31**	0.32±0.41	0.19±0.25

Values with different superscripts within the same column are significantly different ($p<0.05$).

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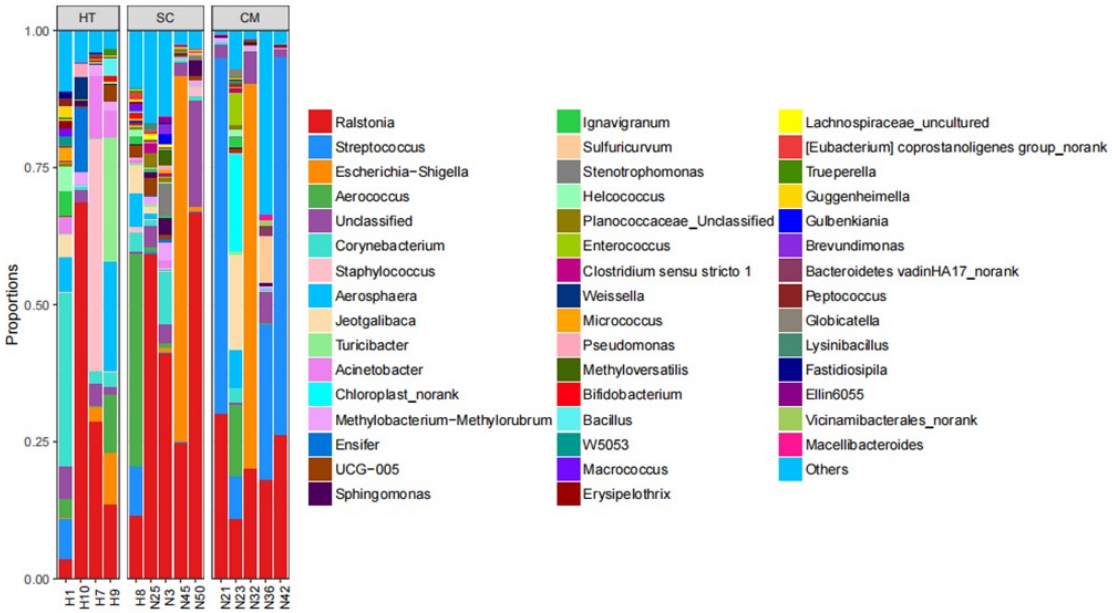

Figure 3. Proportion abundance of microbial genera in healthy (HT: H1, H7, H9, H10), sub-clinical (SC: H8, N3, N25, N45, N50) and clinical (CM: N21, N23, N32, N36, N42) mastitis samples

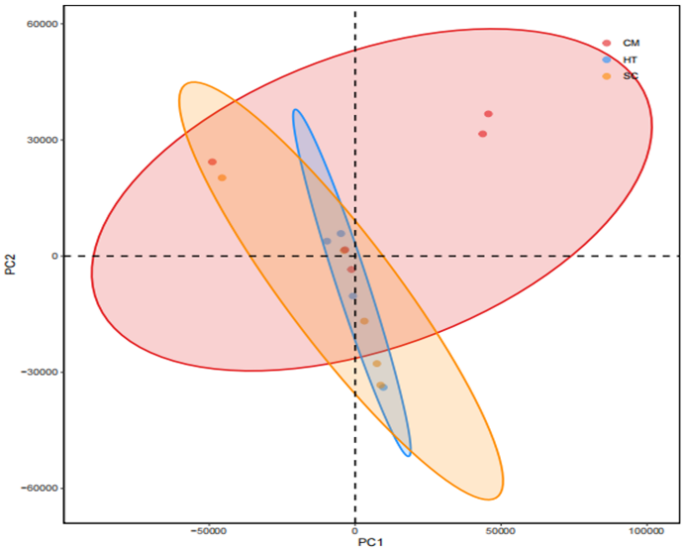

Figure 4. Principle component analysis (PCA) in healthy (HT: H1, H7, H9, H10), sub-clinical (SC: H8, N3, N25, N45, N50) and clinical (CM: N21, N23, N32, N36, N42) mastitis samples. Each dot represents an individual, and colours indicate the populations in three metagenomes.

another group. Even though there was no significant difference and most of the genera were detected in all group, the richness percentage of specific genera were largely distinguishable.

At the genus level, HT group samples possessed relatively more in 17 genera, which were *Staphylococcus* (10.56%), *Corynebacterium* (9.38%), *Turicibacter* (5.74%), *Acinetobacter* (4.91%), *Ignavigranum* (1.57%), *Methylobacterium* (1.55%), *Helcococcus* (1.47%), *Weissella* (1.36%), *Bacillus* (0.81%), *Pseudomonas* (0.68%), *Micrococcus* (0.67%), *Guggenheimella* (0.65%), *Erysipelothrix* (0.63%), *Fastidiosipila* (0.62%), *Trueperella* (0.55%), *Macrococcus* (0.54%), and *Bifidobacterium* (0.52%) in comparison to other groups. Whereas, SC group milk microbiota was enriched in 12 genera, including *Ralstonia* (40.7%), *Escherichia* (13.67%), *Aerococcus* (10.06%), *Sphingomonas* (1.49%), *Stenotrophomonas* (1.43%), *Methylobacterium* (1.25%), *Gulbenkiania* (1.01%), *Methyloversatilis* (0.62%), *Macrococcus* (0.62%), *Eubacterium* (0.42%), *Brevundimonas* (0.36%), and *Lysinibacillus* (0.34%). Moreover, six genera were observed enriched in CM group, which are *Streptococcus* (34.03%), *Escherichia* (14.06%), *Jeotgalibaca* (4.38%), *Sulfuricurvum* (4.23%), *Chloroplast* (3.55%), and *Globicatella* (1.28%), as listed in Table 3. *Escherichia* was found rich in both SC and CM groups, while *Macrococcus* was rich in both HT and SC groups. This result suggested that there is a less prevalent microbial variety in CM, as compared to HT, which showed a more balanced microbial variety profile.

4. Discussion

The understanding of bovine udder microbiota using metagenomic analysis had a growing interest among researchers globally. Various studies were done in Malaysia using culturable methods to unravel the bacteria population and mechanisms in bovine mastitis udder (Othman and Bahaman, 2005; Marimuthu *et al*., 2014; Ariffin *et al*., 2019; Ali *et al.,* 2020). To date, there are no published reports on microbiota using 16S metagenomic analysis to be done locally on mastitis. The study contributes knowledge by exploring the milk microbiota in one of Malaysia's local farms.

The cases that yield no bacteria growth from mastitis milk samples were reported from as low as 10% to as high as 40%, and it was also reported that the incidence might be on the rise (Makoccc and Ruegg, 2003; Taponen *et al.,* 2009; Kuehn *et al*., 2013). Although the real reason behind this is unknown, it was believed to be

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due to the lack of microorganism identification, the inability of the researchers to culture the bacteria responsible for the disease as their presence is below the culturable threshold, or the particular mastitis is caused by non-bacterial microorganisms (Kuehn *et al*., 2013). Metagenomic provides a culture-independent platform to identify all the bacteria using the sequencing method and could be the solution to the above-mentioned setbacks. Furthermore, this approach allows researchers to store the samples at low temperatures for a period of time before bacterial population identification without affecting the DNA quality and results.

4.1 Extraction of DNA

The DNA quality prior to sequencing is very important and might influence the accuracy of data generated. There were various PCR inhibitors present in milk, such as natural proteinases like plasmin and calcium (Lima *et al*. 2018). Previous research comparing different protocols of extraction kits by Quigley *et al.* (2012) determined that among all the methods being used in the research, DNeasy® PowerFood® Microbial Kit was the best approach to extract the total DNA from raw milk and generated highly concentrated and pure DNA. The extraction kits utilized mechanical cell lysis, the shear force which breaks open the gram-positive and negative bacterial cell wall by vibrating bacteria with microbeads at high speed, which had been found to improve detection limits (Odumeru *et al*., 2001). This extraction protocol was optimized for all infected milk samples (HT, SC and CM) to achieve the DNA quality for next-generation sequencing as a downstream process.

Due to the low somatic cells (SCC) count, low DNA yield from HT milk samples was obtained (1.42 – 21.73 ng/μL) with good purity, confirmed by DNA with A260 / 280 readings ranging between 1.72 and 2.0. Healthy milk yielded an insufficient amount of DNA concentration, as reported by Kuehn *et al*. (2013). Therefore, for further processing, Whole genome amplification (GenomiPhi V2 DNA Amplification Kit) was done to all of the samples in the HT group. It was proven that the amplification will have a minimal effect on the samples in terms of taxonomic composition, alpha and beta-diversity (Kuehn *et al*., 2013). Other studies also showed that this method will not contribute to amplification bias that will affect the metagenomic sequencing and analysis, with higher yields of amplified DNA (Pinard *et al*., 2006), and low error rate for genotyping as a downstream process (Han *et al*., 2012).

4.2 16S amplicon metagenomic analysis

The *Streptococcus* (HT:2.50% vs SC:1.94% vs CM:34.03%) was identified in CM group and represents the highest percentage in comparison to HT and SC groups. *Streptococcus i*s gram-positive facultative anaerobe bacteria, known as one of the major pathogens causing bovine mastitis. The relevant species associated with bovine mastitis from this genus included *S. agalactiae*, *S. dysgalactiae*, and *S. uberis* (Kabelitz *et al*., 2021). *Streptococcus agalactiae* is considered to be responsible for 90% of mastitis cases (Ruegg, 2017; Benic *et al*., 2018). *Streptococcus uberis* is occurred in the environment mostly in the milking barn and shed, also known as the most frequent mastitis-causing *streptococci* (Cvetnic *et al.,* 2016; Phuektes *et al*., 2001), with more than one-third in the world (Botrel *et al*., 2009) and approximately 40% in Asia (Kabelitz *et al*., 2021). The current study reported *Streptococcus* as one of the most prevalent and abundant genera detected in CM groups, which has been reported in other studies (Oikonomou *et al*., 2014; Outram *et al*., 2017; Pang *et al*., 2018). It is able to spread within the herd easily, as well as to survive outside the host. Besides, *Streptococcus* is also known as part of the normal microflora of the dairy cow and was detected in small amounts in HT and SC groups as observed in this study.

Escherichia (HT:3.94% vs SC:13.76 vs CM:14.06%) showed the second-highest percentage present in the CM group and the highest percentage of richness in the SC group. *Escherichia* is gram-negative bacteria of the family *Enterobacteriaceae*, and was detected as highly abundant in SC and CM groups. A previous study by Verbeke *et al*. (2014) reported *Escherichia* especially *E. coli* responsible for a high proportion of clinical mastitis, in agreement with the current study. *Escherichia* was found highly in organic material, such as bedding and manure, which indicated *Escherichia* is one of the most common environmental pathogens in mastitis (Rangel, 2009; Liu *et al*. 2018). Milking hygiene and human transmission have been implicated as one of the reasons for *Escherichia* infection (Fahim *et al*., 2019). The clinical sign varies from severe or fatal to mild mastitis (Shpigel *et al*., 2008). The difficulties to treat might be due to the ability of a pathogen to form biofilm (Fernandes *et al*., 2011). The mild infection of clinical coliform is easier to treat and tends to recover naturally without treatment. Both *Streptococcus* and *Escherichia* bacteria might be the main causing pathogens for the incident of clinical mastitis in the group of cows.

Interestingly, the presence of *Jeotgalibaca* (HT:1.98% vs SC:1.34% vs CM:4.38%), *Sulfuricurvum*

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(HT:0.00% vs SC:0.00% vs CM4.23%), *Chloroplast* (HT:0.01 vs SC:0.01 vs CM3.55%), and *Globicatella* (HT:1.28% vs SC:0.00 vs CM:0.07) was detected in clinical mastitis sample and this finding was first reported in Malaysia. *Jeotgalibaca* was found in abundance with the rumen microbial community (Li *et al*., 2020). *Globicatella* bacteria is usually reported as a human pathogen (Miller *et al.,* 2017) instead of in livestock animals. *Sulfuricurvum* and *Chloroplast* bacteria are also not common in bovine mastitis. The above mention genera were not common as mastitis pathogens and were first reported in clinical mastitis samples. The uniqueness of this genera population in the CM group might be used as a biomarker for the detection of clinical mastitis incidents in the particular local farm.

Ralstonia was known to be relatively abundant in non-clinical samples (Kuehn *et al*., 2013). In this study, *Ralstonia* was shown to present in high abundance in all samples, with the highest in the SC group (HT:28.69% SC:40.70% vs CM:21.15%). Bacteria from this genus are unlikely to cause mastitis in dairy cattle (Kuehn *et al*., 2013). However, *Ralstonia* has been 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). *Ralstonia* can withstand extreme environmental conditions with high survival rates (Mergeay *et al*., 2003; Ryan *et al*., 2007; Ryan *et al*., 2011). The local farm is highly reliant on underground water and purified water. Hence, there are possibilities for bacteria from this genus to be found in the milking equipment and environment. Therefore, this could represent a potential source of colonization of mammary tissues in the particular local farm.

Aerococcus, such as *A. viridans*, is an environmental gram-positive opportunistic pathogen and has been reported to be associated with bovine subclinical mastitis (Saishu *et al*., 2015). Coincidently, *Aerococcus* was also found to be enriched in the SC group when compared with HT and CM groups (HT3.58% vs SC:10.06% vs 3.26%). A previous study reported that this genus was highly isolated from cow's mastitis milk related to the contamination from manure, water, and bedding material (Saishu *et al*., 2015; Xi *et al*., 2020). Moreover, Liu *et al.* (2019) showed that *A. viridans* have the ability to adhere with high cytotoxicity. However, the aetiology of *A. viridans* in mastitis infection remains unclear (Xi *et al*., 2020).

Staphylococcus is a well-known gram-positive mastitis pathogen and was found in many mastitis samples (Othman and Bahaman 2005; Oikonomou *et al*., 2012; Outram *et al*., 2013; Ariffin *et al.,* 2019; Ali *et al*., 2020). The genus comprises 45 species and 21 subspecies (Bergeron *et al*., 2011). Among the main species that are associated with mastitis include *S. aureus*, *S. haemolyticus*, *S. chromogenes*, *S. epidermidis*, *S. warneri* and *S. cohnii*, and *S. simulans* (Hosseinzadeh and Saei, 2014). This genus was widely recognized as a cause of subclinical infections; subsequently resulting in an increase of SCC in clinical mastitis cases. In this study, *Staphylococcus* was not the major causing pathogen for mastitis. Surprisingly, *Staphylococcus* was not prevalent in CM group, it plays the role of normal flora and was found in all samples, and enriched in HT samples (HT:0.56% vs SC:0.74% vs CM:0.03%).

Corynebacterium especially *Corynebacterium bovis* is a significant mastitis causing agent (Watts *et al*., 2001). Similar to *Staphylococcus*, infection from *Corynebacterium* will result in an increase in SCC (Joaquim *et al*., 2017). *Corynebacterium bovis* in particular has been associated with subclinical mastitis. Even though *Corynebacterium* was considered a minor pathogen of mastitis (Schukken *et al*., 2009), it can act as a commensal of the bovine mammary gland, and a quarter infected with *Corynebacterium bovis* may be less susceptible compared to another mastitis pathogen (Blagitz *et al*., 2013). However, this study reported the main mastitis infection was not caused by *Corynebacterium.* The genus was detected as prevalent in all samples, and a higher percentage was in the HT group (HT:9.38% vs SC:3.08% vs CM:0.56%).

Turicibacter was found as normal flora, and had high relative abundance in the HT group (HT:5.74% vs SC:0.14% vs CM:0.14%). In this study, *Turicibacter* population was detected higher in the HT group and indicated that the genus is not associated with mastitis in the local farm. This is in agreement with Mein *et al.* (2004) where *Turicibacter* was not associated with mastitis. In contrast, *Turicibacter* was reported present in the clinical mastitis group in several other studies (Khasapane *et al*., 2021; Gryaznova *et al*., 2021). *Turicibacter* was believed to be colonised in the rumen and faeces of cattle (Mao *et al*., 2013), the intestines of pigs, as well as dairy wastewater and whole milk (Gagnon *et al*., 2007). Therefore, it is possible that the *Turicibacter* bacteria can be transferred from an unhygienic environment into the udder.

Acinetobacter is one of the normal flora in milk microbiota and was frequently detected in raw milk (Quigley *et al*., 2013; Kable *et al.,* 2016; Derakhshani *et al*., 2018). Similarly, the presence of *Acinetobacter* was higher in the HT group (HT: 4.91% vs SC:0.51% vs CM:0.11%), *Acinetobacter* was believed to be tolerant to heat (Jain and Danziger, 2004), dry environment (Webster *et al*., 1998), resistant to disinfectant (Gallego and Towner, 2001), as well as resistant to classes of

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antibiotic (Bergogne-Berezin and Towner, 1996). Besides livestock, *Acinetobacter* is a known human pathogen and posed a threat to human health (Gurung *et al*., 2013).

4.3 Bacteria dybiosis associated with bovine mastitis

Predominant genera from CM samples might be the potential causative mastitis pathogens. However, most of the prevalent genera found in CM were present in other groups. From the data, HT group had the most diverse prevalent genera when compared to SC and CM group. In contrast, CM had the least number of prevalent genera than other groups. Prevalence of dysbiosis was detected from samples in CM group, and this represents dynamic changes in microbiota composition and abundance among HT, SC and CM groups through the high throughput next generation sequencing technology.

Previously, milk was believed to be sterile in the mammary gland (Lister, 1978). However, Martín *et al*. (2007) were able to show that there are commensal microbial communities within the human mammary gland. Subsequently, a study by Medrano *et al*. (2018) reported that milk collected from the endogenous environment of the bovine udder, using a cannula, has a large microbial diversity and is not sterile as it was originally believed. Healthy udder quarters showed a higher taxonomical diversity of normal flora compared to those with clinical mastitis, or mastitis history (Falentin *et al*., 2016; Derakhshani *et al.*, 2018). Hogan *et al*. (2003) suggested that the microbiota in the udder that experienced mastitis is altered even after recovery from the infection. Microbiota diversity in udders after the infection might be the reason why cows with mastitis history are more susceptible to getting repetitive mastitis. Even though the theory was not studied thoroughly, during the progression of mastitis, dysbiosis of milk microbiota occurred, with an increase in opportunistic pathogens and a concurrent reduction in healthy milk bacteria (Patel *et al*., 2017; Egilmez *et al*., 2018). The pathogens can easily spread by harbouring bacteria throughout different dairy environments because of wind, water, climate change, human activities, and contact with wild animals (Fitzpatrick and Walsh, 2016).

Overall, the data from this research support the idea that dysbiosis occurred in the mastitis sample. A shared microbiota existed in the three groups when results are aggregated at the genus level. This was confirmed by the overlap of the confidence ellipses PCoA (Figure 4) of the samples from different groups. The prevalence abundance percentage and enriched genera percentage (Table 3) showed that several of the most abundant genera were high in a specific group. The HT group tend to be more diverse and balanced when compared to the CM group. Overall, these data supported the idea that sub-clinical and clinical conditions affect the composition and diversity of udder milk microbiota. The healthy diverse microbiota had been associated to create a protective role against dysbiosis-related diseases (Porcella *et al*., 2020) in the udder. The microbiota balance in the udder is subjected to change due to factors from the host and environment. Once the microbiota is altered, the composition and functionality of the microbiota will be shifted.

5. Conclusion

In conclusion, 16s amplicon metagenomic approach in the present study provided an assessment of descriptive taxonomy bacterial profile in the heathy, sub-clinical and clinical mastitis milk profile of a local dairy farm. The result suggested that clinical mastitis samples have lower microbial diversity, with a higher prevalence of dysbiosis when compared to healthy udder samples. The presence of *Streptococcus*, *Escherichia*, *Jeotgalibaca*, *Sulfuricurvum*, *Chloroplast* and *Globicatella* may indicate the diagnosis of clinical mastitis infection in the respective farm. The diversity of healthy microbiota is believed to provide protection roles against dysbiosis-related issues. The knowledge and information gathered in the study can contribute to the strategies for mitigating the diseases related to dysbiosis, especially in livestock industries in future. Furthermore, the dysbiosis status may be more complex and future research needs to be conducted to clarify how the homeostasis of the milk microbiota can be maintained or recovered as a key regulator to mitigate bovine mastitis.

Conflict of interest

The authors declare no conflict of interests.

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