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Effect of probiotic *Lactobacillus plantarum* CM49 on microbial profile and lactobacilli counts in milk of mastitic cattle

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Abstract

Background Bovine mastitis is a common udder disease in cattle, mainly caused by bacteria and other infectious agents. Traditionally antibiotics are used for their treatment, but the development of antibiotic resistance has increased the importance of using non antibiotic alternative such as probiotic. In current study a previously in vitro characterized isolate *Lactobacillus plantarum* CM49 infused into two groups of cattle suffering from clinical mastitis ($n=5$) and sub-clinical mastitis ($n=5$).

Results The bacterial composition and diversity analysis of milk samples before and after probiotic administration was analyzed using 16S rRNA gene base metagenomic analysis and *lactobacillus* counts were also evaluated using Real time PCR. The results show that there was an increase in abundance of *Proteobacteria* and decrease in *Firmicutes* at phylum level in both groups while major mastitogens genera *Staphylococcus* and *Streptococcus* abundance was reduced after treatment in sub-clinical mastitis group (SCMG) and clinical mastitis group (CMG) respectively. Lactobacilli counts evaluated through Real time PCR showed an increase in number, furthermore diversity indices showed an increase in diversity after treatment with probiotic.

Conclusion It is concluded from the results that *Lactobacillus plantarum* CM49 may serve as promising candidate for improving dysbiosis resulting from mastitis and improving microbial diversity.

Keywords Metagenomics, 16S rRNA, Lactobacilli, Real time PCR

Background

Mastitis is a common disease in cattle that reduces milk production and affects milk quality. It also harms farmers' income and poses risks to human health. Reduced milk output, treatment costs and culling account for 78 percent, 8 percent, and 14 percent of mastitis losses respectively [1, 2]. Mastitis is considered a multifactorial disease. Various etiological agents, including bacteria, viruses, and fungi, have been identified as causes. Among these, bacterial mastitis is the most common [3, 4]. Mastitis is mainly caused by microorganisms like *Staphylococcus* species, *Streptococcus species*, and *Escherichia coli* [2]. Mastitis occurs in two forms: subclinical and clinical. Subclinical mastitis shows no visible symptoms but causes reduced milk production and changes in milk quality. Clinical mastitis causes swollen, painful udders and may result in clots or blood in the milk [5]. Different strategies are used to control bovine mastitis [6]. Among these, antibiotics are the most common method. However, the emergence of antibiotic resistance has raised serious concerns [7] This highlights the need for developing alternative methods [8, 9]. As a result, various

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alternative approaches are being explored to combat mastitogens. These include vaccines [10], nano-based techniques, herbal solutions [11], and bacteriophage-based interventions. Other methods involve bacteriocin therapies and animal-derived proteins [12]. Aiding to these "Probiotics", which are live microorganisms, are emerging as a better option. They offer numerous advantages that help control pathogenic microorganisms [13, 14]. These qualities include adhesion to epithelial cells, colonization, bio-surfactant production, auto-aggregation, and co-aggregation with pathogens. Additionally, they produce antagonistic metabolites such as organic acids, hydrogen peroxide, and bacteriocins [15]. Studies involving probiotic in breastfeeding women and dairy cows have led to assertions of their effectiveness in preventing or treating mastitis [16]. A recent study suggests that *Lb. rhamnosus* GG may help manage sub-clinical mastitis by lowering somatic cell counts and inhibiting mastitis-causing bacteria [17]. Another study highlights probiotics' potential in mammary gland immunobiology, suggesting their use as a preventive strategy against bovine mastitis [18]. Disruption of microbial diversity (dysbiosis) has been observed in mastitis animals as compared to healthy animals [19]. However, it is not confirmed whether this dysbiosis leads to mastitis or is a result of the condition [20]. Using a 16S rDNA metagenomic approach revealed that the milk microbiota of mastitic and healthy cattle and buffalo varies [21], indicating a possible role of udder microbial communities in the prevention of mastitis showing the importance of exploring and enhancing the udder microbiome using probiotic candidates [22]. Therefore, this study aimed to assess the effect of *Lb. plantarum* CM49, (a probiotic strain isolated from healthy cow's milk in 2023), on microbial diversity. The strain, characterized and tested in vitro in our previous study [23], was evaluated using metagenomic analysis of milk samples before and after intramammary infusion in cattle with clinical and subclinical mastitis. Real time PCR was also used to evaluate the lactobacilli count in milk samples before and after probiotic treatment in both groups.

Methods

Sample collection

The study examined the effects of intramammary infusion of the probiotic *Lb. plantarum* CM49 on Sahiwal cattle with clinical mastitis ($n=5$) and subclinical mastitis ($n=5$). All cattle were 3–6 years old, had at least one lactation, and had not received antibiotics in the past 30 days. Similar husbandry and management practices were maintained for all animals. Already characterized probiotic *Lb. plantarum* CM49 strain was obtained from laboratory stock of Probiotic Research Laboratory, Institute of Microbiology (IOM), University of Veterinary and animal Sciences, Lahore. Animal status of clinical mastitis through clinical signs, California mastitis test & Somatic cell count and sub clinical mastitis through California Mastitis test were confirmed prior to treatment (Supplementary Table S3). The probiotic (~ 3 × 10^8 cfu) was infused daily to the diseased animals starting from day zero till day 5 after evening milking using water for injection as a vehicle. The teats and udder surfaces were cleaned with tap water and dried thoroughly. They were then disinfected using a cotton swab dipped in 70% alcohol. After discarding the initial three strips of milk, approximately 15 ml of milk samples were collected before (on zero day) and after use of probiotic (on 7th day) in sterile Falcon tubes following the guidelines of National Mastitis Council [13] in duplicate, correctly labeled and transported to Probiotic Research Laboratory, UVAS Lahore on same day by maintaining cold chain and were stored at a temperature of -80 °C until further processing.

Isolation of DNA from milk samples and its purification

All the milk samples were brought to room temperature before processing for DNA extraction. Samples were gently shaked for uniform distribution and homogenization. Prior to DNA extraction, fat from the samples was removed by the methodology described by Yup et al. [24]. Then metagenomic DNA was extracted using the Genomic DNA purification kit (Thermo Scientific) according to the manufacturer's instructions. Purity and concentration of the DNA extracted from milk samples was evaluated at 260/280 nm wavelength using a Multiscan Sky micro plate spectrophotometer (Thermo scientific, located in Waltham, MA, USA). 16S rRNA gene base metagenomic sequencing was carried out through a commercial company Macrogen in Seoul, South Korea to which properly labelled samples (30ul of DNA having a concentration of 100–200 ng) were sent.

Next-generation sequencing, bioinformatic and statistical analysis of data

NGS is used to identify, quantify microbial communities, their diversity, and their relative abundance. Bioinformatics tools help modify raw sequencing reads into meaningful biological insights. The selected regions (V3-V4) of 16S rRNA gene was targeted using specific primers sets i.e. 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) for generating amplicons [25]. The resulting amplicons were then sequenced with the Illumina DNA Prep Kit (Illumina, San Diego, CA, USA) on the MiSeq platform using 2 × 300 bp paired end reads, following the procedures described in Illumina 16S Metagenomic Sequencing Library Preparation Part #15,044,223 Rev. B document.

For bioinformatic analysis QIIME 2(Version 2.2020.6) was used following the protocols (demultiplexed pair end reads) outlined in online tutorial of QIIME 2 including import of files, filtering of noisy data, trimming (300 bp criteria), cheimra removal and assigned taxonomy using Silva data base (Silva 138) using a 97% threshold level [26]. The bacterial community composition was visualized through heat maps. Alpha and beta diversity metrics were computed to assess the diversity through QIIME q2-diversity plug in" by adopting Core Metrics Phylogenetic method [27].

Frequency tables of OTU (operational taxonomic unit) generated in QIIME 2 for each sample at specific taxonomic level of group were used for abundance (%) calculation and analysis was done using Multiple T-test. Four diversity indices (Shannon, Simpson, Chao 1 and Fisher) were used for calculation of alpha diversity and analyzed using Mann–Whitney U test. Beta diversity was assessed through, Jaccard and Bray–Curti's dissimilarity indices and visualized through PCA plots.

Real time PCR for lactobacilli counting

Real-time PCR was optimized to quantify lactobacilli in cattle milk samples post in vivo trial. *Lactobacillus plantarum* CM49 was cultured on MRS agar, and a microbial suspension (3×10^8 cfu/mL) was prepared in PBS. DNA was extracted using the Thermo Scientific Genomic DNA purification kit and evaluated at tenfold dilutions [28]. PCR amplification employed Maxima SYBR Green qPCR Master Mix (2X) with genus-specific primers of *lactobacillus* [29]. The qPCR conditions were 94 °C for 10 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Using Graph Pad Prism, Ct values were plotted against the logarithm of the initial bacterial concentration (cfu/mL) in standard samples. A calibration curve was generated via linear regression to illustrate the relationship between Ct values and bacterial concentration [30]. After optimizing real-time PCR, DNA from 20 cattle milk samples was amplified, and the Ct values were compared to the standard curve to calculate *lactobacillus* bacterial concentrations (cfu/mL) in each sample.

Results

Effect of probiotic on milk microbial diversity in mastitic cattle

Alpha diversity indices

Alpha diversity measures the microbiome diversity within a sample. It showed a clear increase after probiotic use. Milk samples from clinical mastitis (CMG) and subclinical mastitis (SCMG) animals on day zero, before probiotic use, had lower diversity index values (Chao1, Fisher, Shannon, and Simpson). This suggested potential udder dysbiosis. After probiotic use, the diversity indices were higher, indicating an improvement in udder dysbiosis. In animals with clinical mastitis, diversity indices showed no significant changes before and after probiotic treatment. In animals with subclinical mastitis, three diversity indices (Chao1, Fisher, and Shannon) increased significantly after treatment. However, the Simpson diversity index showed no significant change. This increase in diversity indices after treatment in both groups after treatment was evident both in numerical (Table 1) and graphical representations in Fig. 1.

Beta diversity indices

Diversity indices like Jaccard and Bray Curtis showed that 60% of CMG samples (after probiotic) clustered

Table 1 Alpha diversity indices in Clinical Mastitis and Sub-Clinical Mastitis Groups before and after using probiotic

Clinical Mastitis Group (CMG)			
Alpha Diversity Index	**Before use of probiotic**	**After use of probiotic**	***P*-value**
	Mean ± SEM	**Mean ± SEM**	
Chao 1	147.91 ± 58.72	178 ± 32.44	0.65
Fisher	29.49 ± 12.26	36.25 ± 8.21	0.65
Shannon	5.86 ± 0.67	6.46 ± 0.28	0.67
Simpson	0.96 ± 0.01	0.98 ± 0.004	0.26
Sub-Clinical Mastitis Group (SCMG)			
Alpha Diversity Index	**Before use of probiotic**	**After use of probiotic**	***P*-value**
	Mean ± SEM	**Mean ± SEM**	
Chao 1	56.20 ± 15.17	157 ± 23.77	0.007
Fisher	10.34 ± 2.8	30.25 ± 5.32	0.01
Shannon	4.37 ± 0.64	6.21 ± 0.21	0.02
Simpson	0.86 ± 0.06	0.97 ± 0.02	0.13

P-values < 0.05 were considered significant at $p = 0.05, 0.01, 0.001$

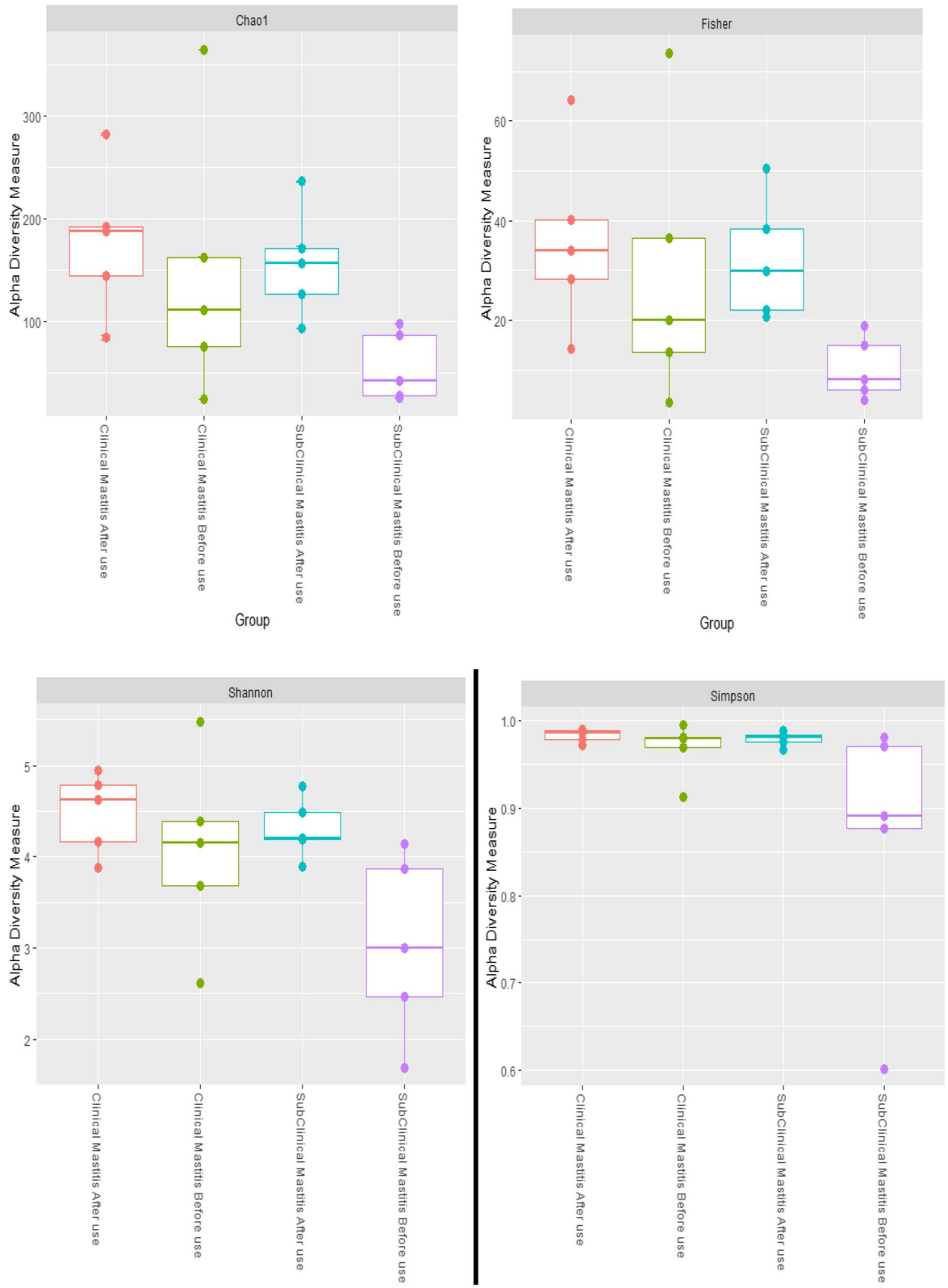

Fig. 1 Alpha diversity analysis of the microbiota in cattle milk before and after use of probiotic: Chao 1 diversity index, Fisher diversity index, Shannon diversity index, Simpson diversity index

separately, along with 20% of CMG samples (before treatment). Similarly, 60% of SCMG samples (after treatment) are clustered with 20% of SCMG (before treatment). Other samples also showed distinct clustering, as shown in Fig. 2.

Effect of probiotic on bacterial taxonomic composition at different levels

16S rRNA gene base metagenomic analysis of samples before and after probiotic administration in both groups of animals i.e. clinical mastitis group (CMG) and subclinical mastitis group (SCMG) yielded different phyla in samples which are represented as heat map in Fig. 3. The group level analysis at phylum level shows that after the use of probiotic in clinical mastitis cases resulted in an increase in abundance of *Acidobacteriota*, *Actinobacteriota*, *Deinococcota*, *Proteobacteria*, while abundance of *Bacteroidota*, *Cyanobacteria*, *Firmicutes*, *Patescibacteria* and *Verrucomicrobiota* decreased as represented in Fig. 4. The distribution of major phyla in CMG before using probiotic was *Proteobacteria* (24.50%), *Firmicutes* (57.37%), *Actinobacteriota* (9.68%) and *Bacteroidota* (6.62) while after using probiotic *Proteobacteria* (44.30%), *Firmicutes* (31.72%), *Actinobacteriota* (16.21%), and *Bacteroidota* (5.38%). The pattern of abundance of phyla across subclinical mastitis group (SCMG) shows that among abundant phyla, *Proteobacteria* increased from 22.88% to 41.91% and *Bacteriodota* increased from 1.15% to 6.102% after treatment with probiotics while the abundance of *Firmicutes* decreased from 62.94% to 39.42% as shown in Table 2.

At class level, *Bacilli* and *Gammaproteobacteria* were predominant in both CMG and SCMG as represented in Supplementary Table S1 and Fig. 5a and b. Class *Bacilli* percentage abundance decreased from 42.01 to 18.19%, while that of *Gammaproteobacteria* class percentage abundance increased from 21.84% to 39.98% after use of probiotic in CMG. In SCMG class *Bacilli* percentage abundance reduced from 60.01% to 27.85% after use of probiotic. Before probiotic treatment in CMG, *Actinobacteria* was detected in high abundance (> 5%) and increased further after treatment. On the other hand, *Bacteroidia* and *Clostridia*, which were present at more than 5% abundance before treatment, showed a decrease after treatment. In SCMG, some classes that were present at less than 5% before treatment, such as *Bacteroidia* and *Clostridia*, increased in abundance to more than 5% after probiotic treatment, as shown in Fig. 5b.

Different orders were observed across both groups samples with varying abundance before and after treatment with probiotic as shown in heat map (Supplementary Fig. 1). *Lactobacillales*, *Staphylococcales* and *Xanthomonadales* were predominant orders in both CMG and SCMG as shown in Supplementary Table S2. *Lactobacillales* percentage abundance increased in SCMG as compared to CM group after use of probiotic while more reduction in mastitogen order *Staphylococcales* was seen in higher abundance in SCMG as compared to CMG after use of probiotic. In addition to these in CMG *Pseudomonadales* and *Oscillospirales* were found in higher abundance (> 5%) and their abundance increased afetr probiotic treatment. Similarly, in SCMG *Corynebacteriales* were higher in abundance (> 5%) while

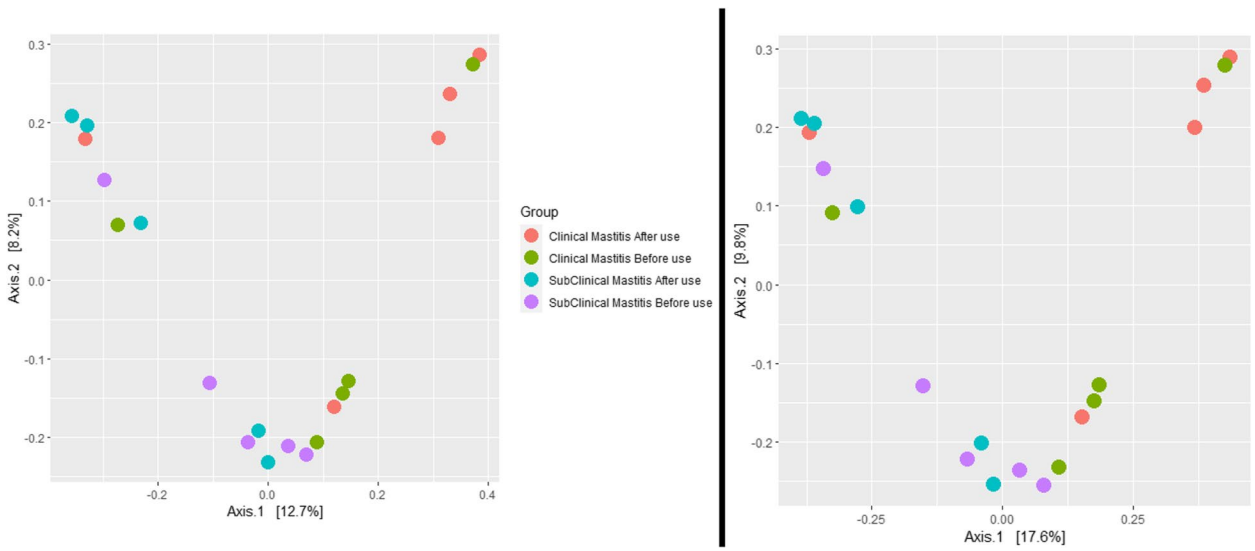

Fig. 2 PCA plots showing beta diversity analysis of the microbiome in cattle milk before and after use of probiotic: Jaccard index (on left side), Bray–Curtis dissimilarity index (on right side)

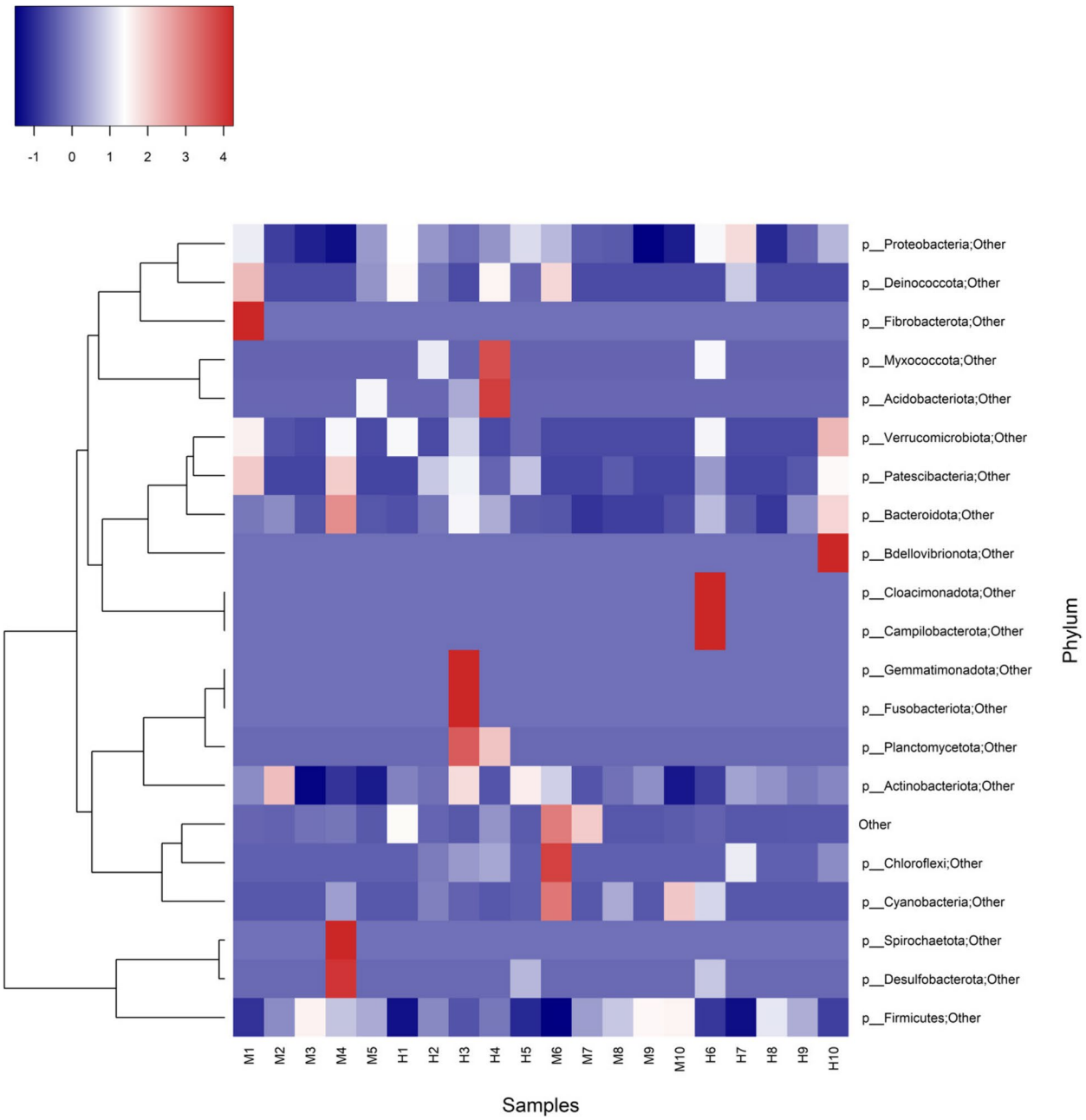

Fig. 3 Distribution of bacterial phyla in samples before and after use of probiotic by Heat map. M1-M5 represents CMG samples (before probiotic use), H1-H5 represents CMG samples (after probiotic use), M6-M10 represents SCMG samples (before probiotic use), H6-H10 represents SCMG samples (after probiotic use)

Micrococcales was low in abundance (< 5%) before treatment the former reduced after treatment while the latter order abundance (> 5%) increased after treatment as shown in Fig. 6.

At the family level, the prominent families identified in all groups included *Corynebacteriaceae*, *Lactobacillaceae*, *Staphylococcaceae*, and *Streptococcaceae*. The abundance of *Corynebacteriaceae* increased in the CMG (2.76% to 4.63%) after use of probiotic as compared to the SCMG where decrease in abundance was seen (from 7.50 to 4.42%) after use of probiotic. A slight decrease in abundance of *Lactobacillaceae* was seen in the CMG (1.98% to 1.03%) compared to the increase in SCM group (0.42 to 3.92%) after use of probiotic.

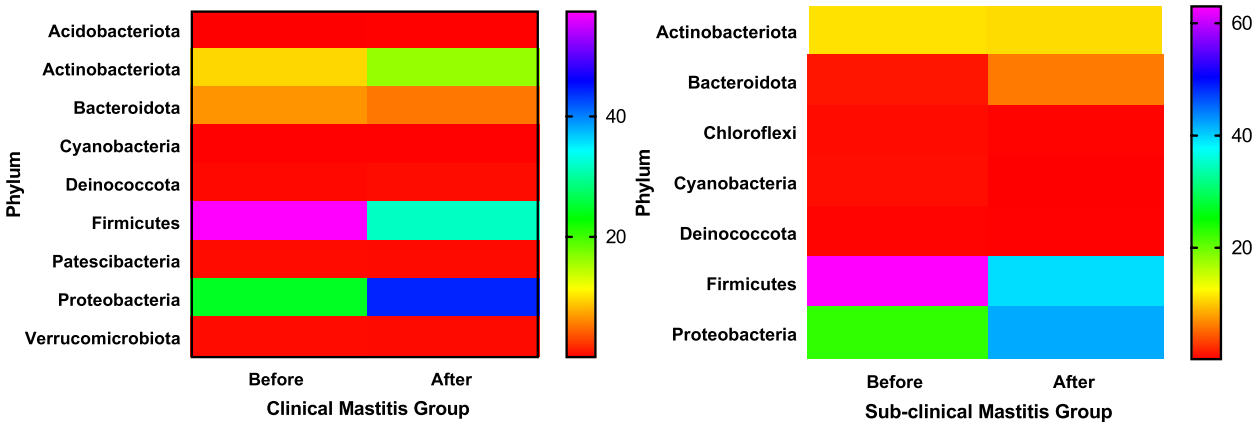

Fig. 4 A heat map representation of phylum-level taxonomic composition of milk microbiome. The color intensity indicates the relative abundance of phyla in the cattle milk microbiome before and after use of probiotic in clinical and sub-clinical mastitis groups

Table 2 Phylum level comparison of percentage abundance (Mean) in clinical mastitis and sub-clinical mastitis groups of cattle before and after use of probiotic

Clinical Mastitis Group					
Before use of Probiotic			**After use of Probiotic**		
Phylum	**Mean**	**SEM**	**Mean**	**SEM**	***P*-Value**
Acidobacteriota	0.03	0.03	0.11	0.09	0.4543
Actinobacteriota	9.68	5.44	16.21	3.86	0.3569
Bacteroidota	6.62	3.38	5.38	1.78	0.7534
Cyanobacteria	0.08	0.08	0.06	0.04	0.8789
Deinococcota	0.38	0.29	0.54	0.26	0.6859
Firmicutes	57.37	10.97	31.72	6.78	0.0818
Patescibacteria	0.50	0.31	0.46	0.16	0.9233
Proteobacteria	24.50	9.90	44.30	7.43	0.1483
Verrucomicrobiota	0.51	0.30	0.48	0.26	0.9458
Sub-Clinical Mastitis Group					
Before use of Probiotic			**After use of Probiotic**		
Phylum	**Mean**	**SEM**	**Mean**	**SEM**	***P*-Value**
Actinobacteriota	11.23	3.56	10.93	1.55	0.9397
Bacteroidota	1.15	0.45	6.10	2.53	0.0905
Chloroflexi	0.67	0.67	0.25	0.19	0.5617
Cyanobacteria	0.77	0.42	0.13	0.13	0.1876
Deinococcota	0.33	0.33	0.14	0.14	0.6216
Firmicutes	62.94	14.15	39.42	12.16	0.2429
Proteobacteria	22.88	9.78	41.91	10.98	0.2318

P-values < 0.05 were considered significant at $p = 0.05, 0.01, 0.001$

Similarly, the abundance of *Streptococcaceae* decreased in both CMG and SCMG after use of probiotic while very less change in *Staphylococcaceae* abundance was observed in CMG and appreciable decrease in abundance of *Staphylococcaceae* (29.60 to 3.64%) in SCMG was seen after use of probiotic as shown in Table 3 and and Fig. 7.

At the genus level, the prominent genera identified in both the groups included *Staphylococcus, Streptococcus, Lactobacillus, Corynebacterium* and *Stenotrophomonas*. The abundance of *Streptococcus* genus decrease in both CMG (27.19% to 1.23%) and SCMG (16.29% to 0.51%) after use of probiotic compared to *Staphylococcus* genus whose abundance was not affected too much in the CMG

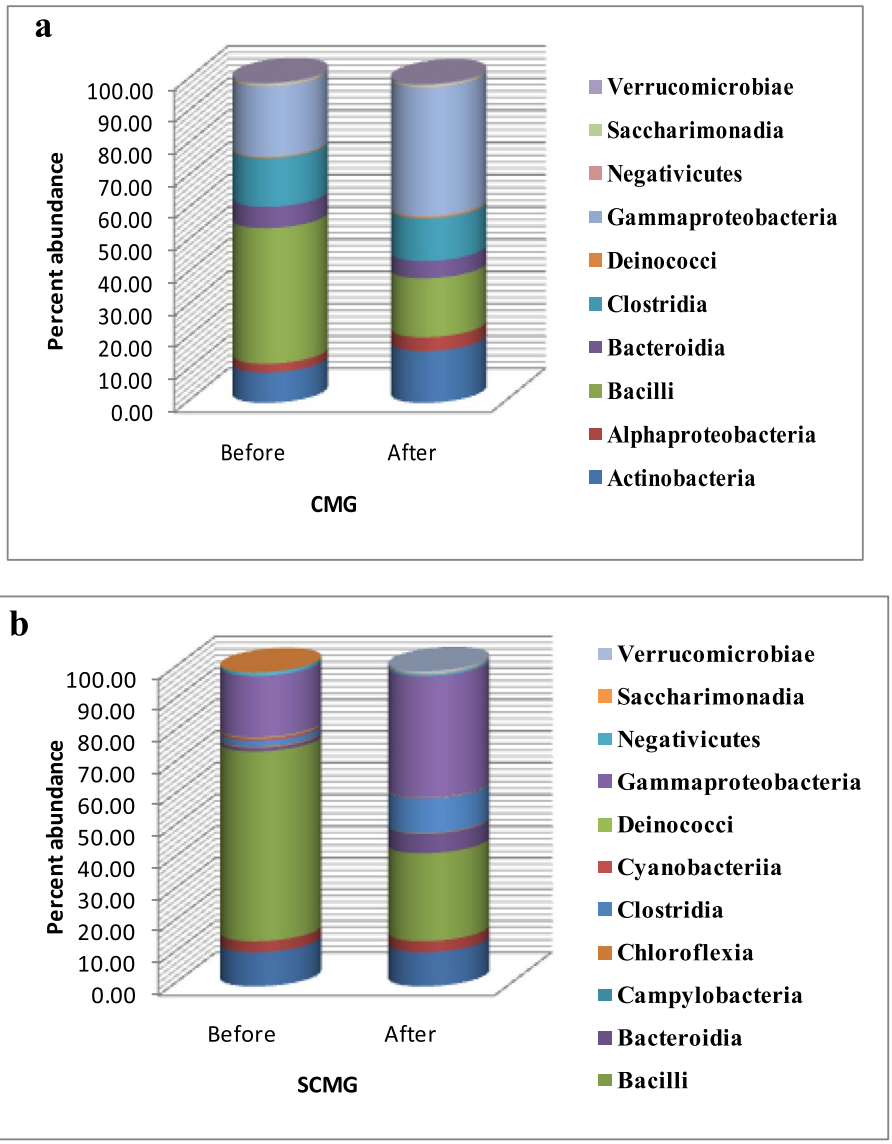

Fig. 5 **a** Representation of different class level taxonomic composition of milk microbiome through taxa bar plot before and after use of probiotic in clinical mastitis group. **b** Representation of different class level taxonomic composition of milk microbiome through taxa bar plot before and after use of probiotic in sub-clinical mastitis group

group (3.16% to 0.92%) while decrease in abundance was more pronounce in SCMG from 29.33% to 0.34% group after use of probiotic Similarly, the abundance of beneficial bacteria genus *Lactobacillus* showed considerable increase in SCMG (0.42% to 4.02%) group after use of probiotic while decreasing a decrease in abundance was s seen in CMG as shown in Table 4 and in Fig. 8.

Our study revealed that a significant portion of the species identified could not be cultured. In certain instances, we were unable to assign a specific taxonomy, categorizing these species as "unknown." In other cases, the assigned taxonomy was more general, such as "uncultured bacterium" or a higher-level bacterium species. It's worth noting that our study's accuracy in classifying species was less precise compared to other taxonomic levels, and many species were not assigned to specific taxonomic ranks as shown in Supplementary Fig. 2.

Development of standard curve and quantification of lactobacilli in milk samples before and after use of probiotic

Real-time PCR was optimized using dilutions of 1 McFarland DNA from *Lb. plantarum* CM49. The Ct

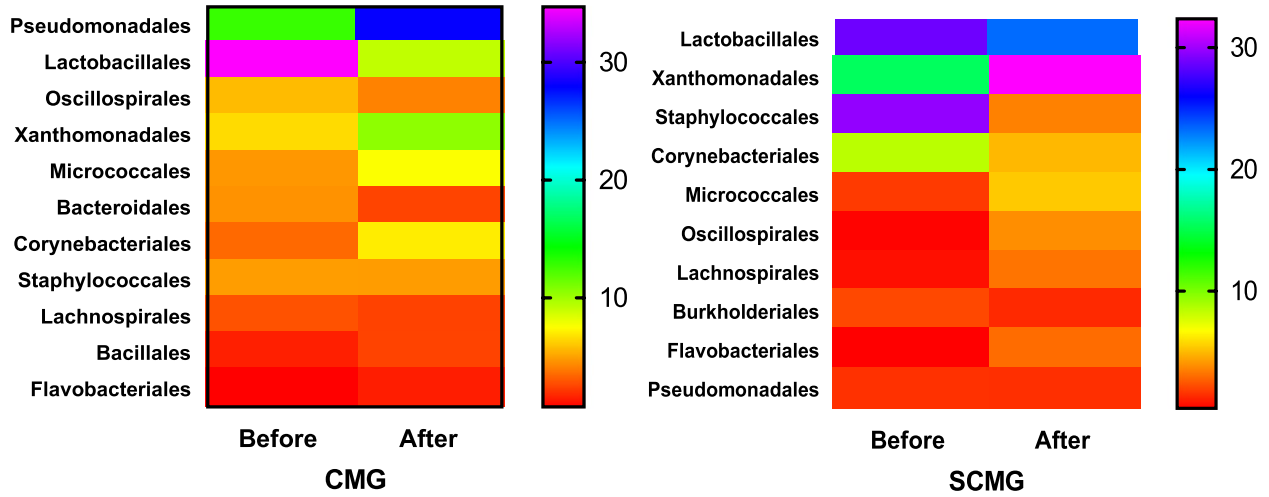

Fig. 6 A heat map representation of order-level taxonomic composition of milk microbiome. The color intensity indicates the relative abundance of different orders in the cattle milk microbiome before and after use of probiotic in clinical and sub-clinical mastitis groups

Table 3 Family level comparison of percentage abundance (Mean) in clinical mastitis and sub-clinical mastitis groups of cattle before and after use of probiotic

Clinical Mastitis Group					
Family	**Before Probiotic**		**After Probiotic**		
	Mean	**SEM**	**Mean**	**SEM**	***P*-Value**
Pseudomonadaceae	11.90	6.82	24.72	10.25	0.3281
Streptococcaceae	27.03	17.52	1.14	0.36	0.1780
Xanthomonadaceae	6.55	6.36	10.49	9.38	0.7369
Staphylococcaceae	4.90	2.42	4.61	1.23	0.9164
Lachnospiraceae	2.92	2.42	2.49	1.36	0.8818
Carnobacteriaceae	4.05	3.28	4.63	0.61	0.8675
Corynebacteriaceae	2.76	2.04	5.86	1.86	0.2942
Micrococcaceae	3.53	2.00	4.53	0.96	0.6645
Bacteroidaceae	1.47	1.16	0.60	0.37	0.4920
Lactobacillaceae	1.98	1.66	1.03	0.34	0.5913
Sub-Clinical Mastitis Group					
Family	**Before Probiotic**		**After Probiotic**		
	Mean	**SEM**	**Mean**	**SEM**	***P*-Value**
Xanthomonadaceae	15.47	6.89	32.43	11.07	0.2295
Staphylococcaceae	29.60	16.70	3.64	0.90	0.1590
Streptococcaceae	27.14	16.75	0.59	0.50	0.1519
Carnobacteriaceae	0.66	0.47	17.30	11.70	0.1929
Corynebacteriaceae	7.50	2.30	4.42	2.04	0.3458
Micrococcaceae	0.99	0.64	3.30	0.95	0.0789
Lactobacillaceae	0.42	0.42	3.92	3.90	0.3977
Pseudomonadaceae	1.57	0.40	1.40	0.43	0.7740
Flavobacteriaceae	0.00	0.00	2.29	1.78	0.2343
Lachnospiraceae	0.73	0.65	3.26	3.06	0.4411

P-values < 0.05 were considered significant at $p = 0.05, 0.01, 0.001$

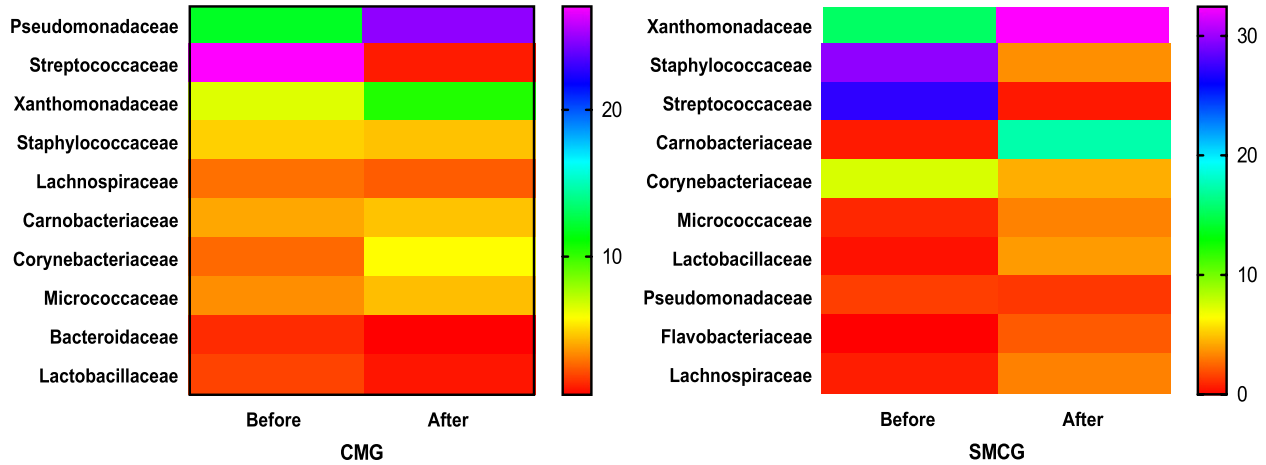

Fig. 7 A heat map representation of Family-level taxonomic composition of milk microbiome. The color intensity indicates the relative abundance of different families in the cattle milk microbiome before and after use of probiotic in clinical and sub-clinical mastitis groups

Table 4 Genus level comparison of percentage abundance (Mean) in clinical mastitis and sub-clinical mastitis groups of cattle before and after use of probiotic

Clinical Mastitis Group					
Genus	**Before Probiotic**		**After Probiotic**		
	Mean	**SEM**	**Mean**	**SEM**	***P*-Value**
Acinetobacter	1.00	0.70	3.25	1.36	0.1818
Bacteroides	1.62	1.27	0.65	0.40	0.4847
Corynebacterium	3.06	2.30	6.08	1.92	0.3445
Escherichia-Shigella	0.69	0.36	0.11	0.10	0.1538
Lactobacillus	2.09	1.81	1.09	0.36	0.6026
Pseudomonas	12.47	7.41	25.75	10.46	0.3306
Staphylococcus	3.16	2.55	0.92	0.29	0.4091
Stenotrophomonas	6.48	6.48	10.56	9.64	0.7348
Streptococcus	27.19	17.53	1.23	0.39	0.1769
Sub-Clinical Mastitis Group					
Genus	**Before Probiotic**		**After Probiotic**		
	Mean	**SEM**	**Mean**	**SEM**	***P*-Value**
Corynebacterium	6.17	1.76	4.54	2.09	0.5694
Dietzia	0.95	0.60	0.64	0.64	0.7348
Lactobacillus	0.42	0.42	4.02	4.00	0.3967
Pseudomonas	1.61	0.42	1.44	0.44	0.7807
Salinicoccus	0.25	0.25	2.28	1.19	0.1331
Staphylococcus	29.33	17.00	0.34	0.21	0.1266
Stenotrophomonas	16.09	7.31	33.27	11.30	0.2375
Streptococcus	16.29	14.98	0.60	0.51	0.3257

P-values < 0.05 were considered significant at $p = 0.05, 0.01, 0.001$

values for each dilution were used to generate a standard curve shown in Fig. 9. This standard curve was used to compare the Ct values of all samples, allowing for the calculation of *lactobacillus* concentration in cfu/mL.

Real-time PCR was performed on milk samples, including CMG (samples 1–5) and SMCG (samples 6–10). The Ct values, shown in Table 5, were compared to the optimized standard curve to calculate the

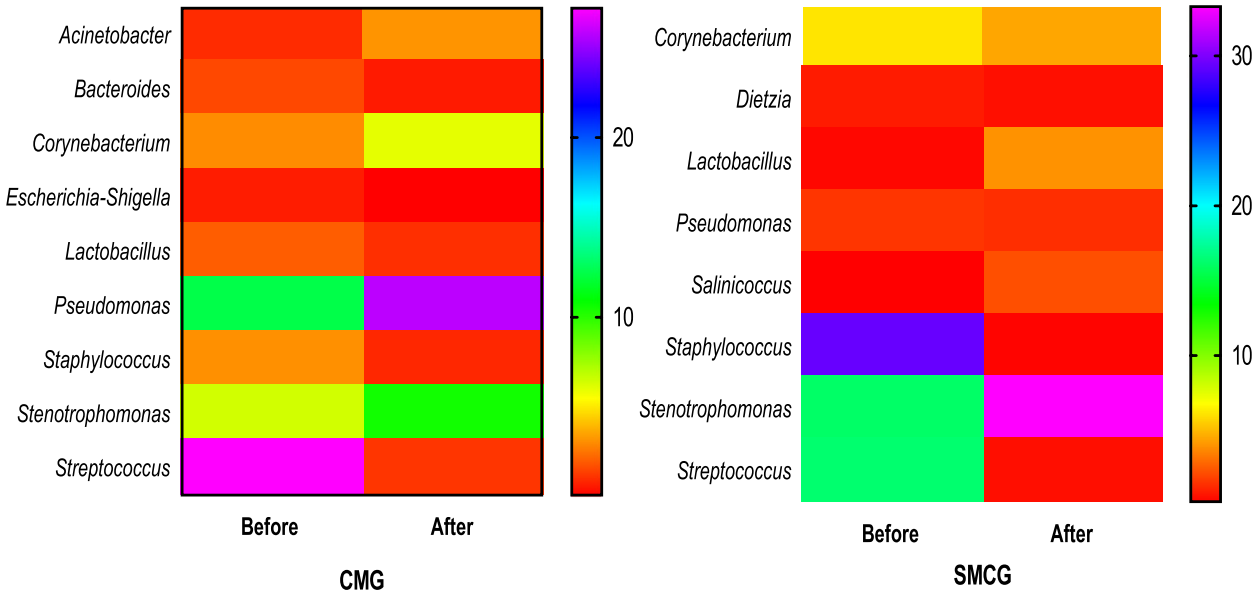

Fig. 8 A heat map representation of Genus-level taxonomic composition of milk microbiome. The color intensity indicates the relative abundance of different genus in the cattle milk microbiome before and after use of probiotic in clinical and sub-clinical mastitis groups

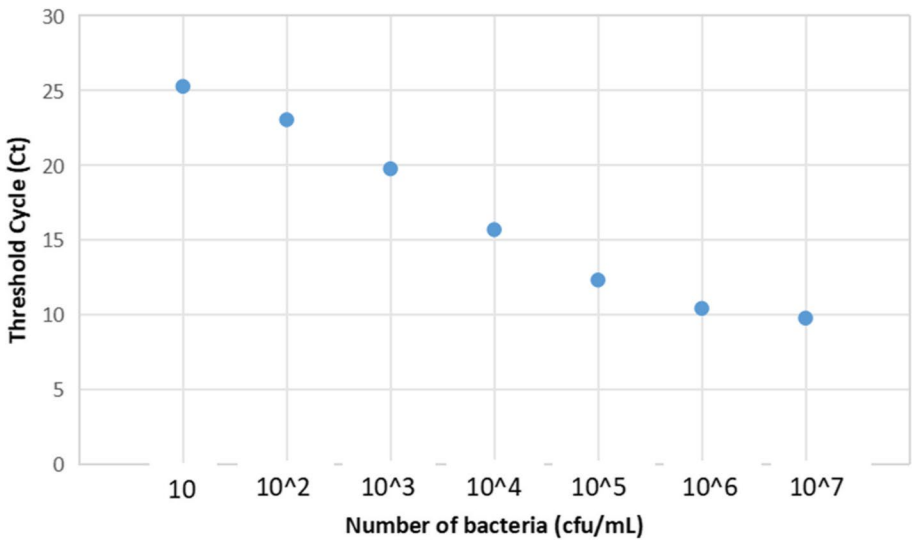

Fig. 9 Standard curve showing the relationship between bacterial count and CT (cycle threshold) values. The curve is generated from quantitative PCR (qPCR) data, where lower CT values indicate higher bacterial abundance

lactobacillus count (cfu/mL). As shown in Table 5, On Day 1, the Ct values ranged from 24.58 to 34.56, with a mean of 26.65 and a standard deviation of 3.05, indicating moderate variability across samples reflecting a broad spread in *lactobacillus* counts before treatment. On Day 7, after administering *Lactobacillus plantarum* CM49 for 5 consecutive days, the Ct values exhibited lower variability, with a range of 18.85 to 20.82 and a mean of 19.81, suggesting a more homogeneous *lactobacillus* population among samples obtained from the animals with clinical mastitis and sub-clinical mastitis after treatment. This indicates an increasing trend in *lactobacillus* counts following probiotic treatment. These results underscore the positive effect of the probiotic intervention on both microbial diversity and *lactobacillus* abundance.

Table 5 Comparison of Ct values and *lactobacillus* counts (cfu/mL) on day one and day 7

Samples no	1st day Ct values	1st day cfu/mL	7th day Ct values	7th day cfu/mL
1	24.58	5.7×10^{1}	20.82	1.4×10^{3}
2	34.56	N/A	20.50	1.9×10^{3}
3	26.07	1.5×10^{1}	18.97	7.1×10^{3}
4	26.19	1.4×10^{1}	N/A	N/A
5	25.5	1.9×10^{1}	20.68	1.6×10^{3}
6	26.40	1.2×10^{1}	19.60	4.1×10^{3}
7	25.12	2.9×10^{1}	19.28	5.4×10^{3}
8	N/A	N/A	18.85	7.9×10^{3}
9	24.8	6×10^{1}	19.4	4.8×10^{3}
10	N/A	N/A	20.16	2.5×10^{3}
Mean ± SD of CT Values (1st day) (26.65 ± 3.05)			**Mean ± SD of CT Values** (7th day) (19.81 ± 0.71)	

Discussion

Probiotics have been used previously for modulating the microbiota and treatment of different bacterial infections of gut and reproductive tract in bovines, poultry and humans [31, 32]. Different mechanisms have been suggested for their effects including modulation of indigenous microbial composition and their activity [16]. Bovine mastitis has been suggested to occur as a result of dysbiosis of microbial communities of mammary gland i.e. change in composition of healthy microbiota and mastitogens [33]. Probiotics have been applied in correcting this dysbiosis in humans and were found to have improved the microbial make up of mammary gland in terms of healthy microbiota [34]. *Lactobacillus* strains are recognized for their ability to counter pathogenic microorganisms through mechanisms such as the production of organic acids, hydrogen peroxide, and bacteriocins [35, 36]. The isolate *Lactobacillus plantarum* CM49 used in this study has been previously characterized for its in vitro characterization our previous study and was found to be effective in reducing *Staphylococcus* and *Streptococcus* by in-vitro settings [23]. A study conducted in buffaloes suffering from mastitis shown improvement in terms of reduction in somatic cell count and mastitogens inhibition by using *Lb. rhamnosus* GG using intra-mammary route [37]. So, in this study we applied probiotic using intra-mammary route and found that after probiotic administration increase in the abundance of, *Proteobacteria* was observed in both groups: CMG and SCMG, suggests that the probiotic might create an environment favoring the proliferation of this phylum while decrease in abundance of *Firmicutes* in CMG and SCMG. This reduction in *Firmicutes* in both groups may reflect a displacement effect caused by the probiotic. These findings correlates with the study conducted on Sahiwal cattle milk microbiota having different udder condition in which high abundance of *Proteobacteria* and low abundance of *Firmicutes* were suggested to be associated with milk of healthy animals [19]. They found a high abundance of *Proteobacteria* in healthy animals (55.99%) and low abundance in clinically infected (2.07%) and sub-clinically infected udder milk (48.06%) while *Firmicutes* was higher in clinical (64.2%) and sub-clinical milk (38.98%) as compared to healthy milk (15.87%). The occurrence of *Proteobacteria*, *Firmicutes*, *Actinobacteriota* and *Bacteroidota* with differences in frequencies of different phyla have been detected [38, 39] with a study having high percent abundance of *Firmicutes* in healthy and mastitic animals but percent abundance of *Proteobacteria* was high in healthy animals as compared to mastitic animals while *Firmicutes* were higher in abundance in mastitic animals as compared to healthy animals milk [40]. The difference may be as a results of use of different breeds or associated factors such as geographical location, weather feeding and managemental practices [39]. The abundance of Genus *Staphylococcus* in SCMG and *Streptococcus* in CMG were found to be higher before probiotic treatment and reduced as a result of probiotic application they have been recognized as major pathogens responsible for causing mastitis [41] and application of probiotic can result in reducing their abundance and controlling them as indicated by different studies [31]. In another study, the probiotic BS C-3102 strain demonstrated a preventive effect against mastitis in dairy cows with a prior history of the mastitis [42] which supports the findings of our study. Abundance of *Lactobacillus* has been found to increase after application with probiotic, this increase was more pronounced in sub-clinical mastitis as compared to clinical mastitis. It has been reported that mastitis result in reducing

the number of lactobacilli in milk [43] and increase in number of *Lactobacillus* which are regarded as beneficial bacteria are found to inhibit mastitogens [44].

Diversity based analysis indicates that diversity indices were found to increase after treatment with probiotic which are consistent with the results of different studies [45, 46] as they also states that healthy milk has microbial diversity as compared to mastitis effected milk. The increase in diversity indicates that udder microbiota is moving towards a balancing state. Beta diversity indices indicates that after treatment with probiotic the clustering pattern of samples in both clinical and sub-clinical mastitis have changed as high clustering between samples of both groups before treatment, this indicates that probiotic have affected the beta diversity which is consistent with the findings of a study wherein they have found that healthy animals milk samples cluster separately from mastitis animals [47]. As in our study with administration of probiotic, there is improvement of dysbiosis in microbiota of udder and shifting to balance as it is normal. Although there was clustering between the groups in the samples before and after treatment which are consistent with the findings of Sokolov et al. [48] in which healthy and mastitic milk samples separation was not clear it might be possible those animals may be in transition phase from mastitis towards healthy condition yet this assumption needs further research.

Quantitative Real Time PCR (qPCR) has been previously used for *lactobacillus* species enumeration in fecal samples of infants receiving probiotic infant formula and found that infants receiving probiotic have resulted an increase in *lactobacillus* count of different species [49] which strengthen findings of our study that upon administering probiotic, lactobacilli counts in a specific niche (udder) increase and lactobacilli have proven to be effective in promoting health of specific niche study [50] also showed the promising outcomes from the introduction of live cultures of specific LAB strains into the bovine udder. These cultures have exhibited the capacity to effectively restrain various mastitis-causing pathogens. According to the results, living culture preparations did not negatively impact mammary tissue and were just as successful at treating intramammary infections as widely used antibiotics [44]. Catozzi also showed that the in vivo intramammary therapy with *Lactobacillus rhamnosus* can alter the microbiota of milk six days after inoculation and has a temporary pro-inflammatory activity as measured by the SCC [51]. Thus, *Lactobacillus plantarum* CM49 may be a promising candidate for improving dysbiosis associated with mastitis by modulating milk microbiota by indirectly inhibiting and reducing mastitogens. However, a key limitation of this study is the relatively small sample size, which may affect the statistical power and generalizability of the findings. Therefore, we recommend conducting further studies to provide a more robust understanding of the probiotic's effects on milk microbiota and mastitis-associated dysbiosis.

Conclusion

The results of our study suggest that *Lactobacillus plantarum* CM49 may serve as promising candidate for improving udder microbiota by inhibiting the growth of mastitogens and promoting the proliferation of beneficial bacteria, contributing to better udder health.

Abbreviations

CMG	Clinical mastitis group
SCMG	Sub-clinical mastitis group
Lb	*Lactobacillus*
cfu	Colony forming units
qPCR	Quantitative Real Time PCR

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

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Clinical trial number

Not applicable.

Authors' contributions

Conceptualization, Muhammad Zeeshan Izhar; Methodology, Muhammad Nawaz; Validation, Muhammad Avais; Formal analysis, Tahir Yaqub; Writing, original draft preparation, Muhammad Zeeshan Izhar; Investigation, Muhammad Zeeshan Izhar; Resources, Muhammad Nawaz and Tahir Yaqub; Writing, review and editing, Muhammad Zeeshan Izhar; Visualization, Muhammad Nawaz; Supervision, Muhammad Nawaz.; project administration, Muhammad Nawaz and Tahir Yaqub.; Funding acquisition, Muhammad Nawaz ; All authors have read and agreed to the published version of the manuscript.

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Data availability

The datasets generated and/or analyzed during the current study are available in the National Center for Biotechnology Information (NCBI) under Bio Project ID PRJNA1044069.

Declarations

Ethics approval and consent to participate

This study was conducted with ethical approval from the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore (Approval No. DR/334). All procedures were carried out in accordance with the institution's guidelines and regulations. Informed consent was obtained from all farm owners for the collection of milk samples and the use of cattle milk in the research. The methods are reported in accordance with the ARRIVE guidelines for the reporting of animal experiments.

Consent for publication
Not Applicable.

Competing interests
The authors declare no competing interests.

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