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Original article

Milk microbiota of Holstein Friesian cattle reared in Lahore: Association with mastitis

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ABSTRACT

The dairy industry is reshaping itself and becoming commercialized in Pakistan due to the increased demand for milk to overcome the shortage. Exotic breeds such as Holstein Friesian, a high milk producing breed have started being reared more on farms in Pakistan. Along with other issues, mastitis does affects the milk production of this breed. The objective of this study was to evaluate the milk composition in terms of bacterial communities in Holstein Friesian reared in Punjab, Pakistan and alteration in microbial composition with healthy and mastitic udder. Milk samples (n = 36) from farms rearing Holstein Friesian were collected. Among these samples, 05 samples from each three groups, HHF(healthy), CHF (clinical mastitis) and SHF (subclinical mastitis), based on their udder health condition, were processed using the 16 S r=RNA gene based technique. Diversity assessment as carried out by alpha diversity indices showed that milk samples from the udder infected with clinical mastitis were the least diverse and those from the healthy udder were more diverse. Beta diversity across samples showed a scattered pattern suggesting overlap amongst bacterial communities across different groups samples as depicted by PCA plots of beta diversity indices. The taxonomic profile revealed that Proteobacteria Firmicutes, Bacteroidota and Actinobacteriota were the major phyla detected across all groups. Proteobacteria dominated the HHF and SHF group while abundance of Firmicutes was higher in CHF group. Differences at other levels including order, genus and species were also recorded. The overall picture concludes that diverse microbiota is associated with different udder health conditions.

1. Introduction

Punjab is known as the most densely populated province with a population of about 90 million and Lahore which is its capital is ranked as the 2nd largest city in Pakistan (Maheshwari et al., 2016). The majority of households in Punjab are dependent on livestock and crop production for their livelihood (Hussain et al., 2011). The dairy industry is mostly dominated by commercial farmers and their interest relies on high milk production and to meet the local requirement farmers also keep exotic or non-native cattle breeds (Ashfaq et al., 2015), especially Holstein Friesian and Jersey which were initially imported by the government of Pakistan (Suhail et al., 2010), and now private companies also import these Holstein Friesian is a well known cattle breed and found across the globe, highlighting its significance at international level rather than confined to a region (Windsor and Agerholm, 2009). Holstein Frisian have been reported to perform well in terms of milk production performance in Pakistan (Bilal et al., 2008). The production potential of lactating animals may be affected by many factors and one of the most important is an inflammatory condition of the udder known as mastitis which not only affects animal production but also implicates economic impact on farmers (Abebe et al., 2016). It has been reported that under tropical conditions the economic impact inflicted by mastitis on Holstein cattle herds is a result of production losses (54.9 %), milk disposal (22 %), culling (13.2 %) and medication (9.8 %) (Guimarães et al., 2017).

Mastitis occurs as a result of any injury or infection resulting from pathogens such as *Staphylococcus aureus, Streptococcus* spp. *and E.coli* (Schukken et al., 2011), which are mainly isolated by routine culture methods (Abdeen et al., 2021; Bashir et al., 2020; Ruk et al., 2021; Surya et al., 2021). In addition to the common pathogens, an imbalance in

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udder microbiota may also predispose the udder to infection by the common pathogens (Park et al., 2022). Recent studies have suggested that the teat canal and apex are important contributors of the microbiota to the sterile healthy milk produced from alveoli (Derakhshani et al., 2018b), thus milk obtained from healthy animals comprised of a range of bacterial commensals (Oikonomou et al., 2020). In addition, various environmental and physiological factors affect the microbial communities present in the milk (Cremonesi et al., 2018; Metzger et al., 2018; Taponen et al., 2019). The occurrence of mastitis is also known to increase with the increase in temperature and humidity index (Bokharaeian et al., 2023). It has been reported that in hot weather, the somatic cell count and microbial load increases in milk while immunity is reduced making dairy animals more susceptible to infections (Ataallahi et al., 2022; Igono et al., 1988; Ranjan et al., 2011). The bedding material and skin microbial load have also been reported to rise with the increase in temperature specifically in summer (Hogan et al., 1989) and pathogens from these extra mammary sources can adopt the udder and result in intra mammary infections (Cheng et al., 2021).

Traditionally in laboratories, the pathogens are isolated using routine culture methods but many bacteria cannot be grown and isolated by these methods due to their requirements for more strict growth conditions (Derakhshani et al., 2018a), thus their role in the development of any infection remains unexplored. The advance molecular techniques have opened a gateway for the exploration of the fastidious microbial communities across different habitats.

Different studies on Holstein Friesian microbial composition have been carried out in different countries but to our knowledge, it is the first study that focuses on udder microbial diversity of Holstein Friesian from Pakistan. This study may help in exploring the core microbiota of milk of this breed and the variations observed in its microbial composition with the health status of the udder. It may untimely help in devising strategies to control mastitis, especially caused by pathogens that cannot be identified by routine culture methods and indirectly help the farmer communities in minimizing the economic losses resulting from mastitis.

2. Materials and methods

2.1. Milk sample collection from animals

Initially, a total of 36 milk samples were collected from four dairy farms in Lahore, Punjab. Similar management and husbandry practices were focused for the inclusion of farms in the study. Criteria for the selection of Holstein Friesian cattle's in these farms is outlined in supplementary Table S1. The animals included in this study were divided into three different groups (healthy, clinical mastitis and subclinical mastitis) based on the condition of udder/teat and milk characteristics. Field test i.e California mastitis test was used for detection of mastitis and somatic cell count was used in laboratory for its confirmation. Animals with inflamed udder/teat or abnormal milk were added to clinical mastitis group (MHF). Animals having normal udder and teats showing no inflammatory condition, normal milk with no abnormality noticed and tested negative on California mastitis test along with somatic cell counts (SSC) below the set criteria (200,000 cells/ml) were enlisted in healthy group (HHF) (Poudel et al., 2021). Subclinical mastitis group (SHF) carried animals having normal udder, teats and milk whereas their milk tested positive on the California mastitis test and the somatic cell counts were above the set criteria. To avoid contamination, the dirt and loose hairs were removed from the udder with a brush, followed by cleaning the udder and teats with fresh water and allowing it to dry. Teats were dipped in a teat dip for a contact time of 30 seconds. Using a separate towel, each teat was dried. Initially, streaks (two) of milk were discarded and the milk sample (15 ml) was taken in a sterile tube in duplicate and properly labeled (NMC, 2017). Samples were transported by maintaining a cold chain (4 °C) to the Probiotics Research Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences (UVAS, Lahore). Somatic cell counts were carried out using the direct microscopic count method upon arrival from one tube amongst the duplicate while the other tube containing milk sample was stored at −80 °C for metagenomic studies. A total of 15 samples (05 from each group) were selected randomly for carrying out metagenomic analysis (Supplementary Table.S2).

2.2. DNA extraction

The biosafety cabinet (BSC Class II type A2) present in the Probiotics Research laboratory, UVAS, Lahore specified for DNA extraction for metagenomic studies was used. Extraction of DNA was performed with DNeasy PowerSoil Kit (Qiagen) adopting the instructions enlisted in the user manual with slight modifications. Milk pellets extracted from whole milk samples were used for DNA extraction while phosphate buffer saline was used as negative control. For extracting the pellet, milk samples were subjected to centrifugation (4500 × g for 20 min at 4 °C), washing (with phosphate buffer saline), centrifugation (13,000 × g for 1 min at 4 °C), washing (PBS), centrifugation (13,000 × g for 1 min at 4 °C) and washing steps (Yap et al., 2021). Quality checks (concentration and purity) of extracted DNA were carried out with Multiskan Sky microplate spectrophotometer (Thermoscientific). Samples passing quality check (Optical density values falling between 1.6 and 1.9 at 260/280 nm) were utilized for 16S rRNA (V3 and V4 region) gene-based metagenomic analysis and was shipped to Macrogen (Seoul, South Korea) for carrying out sequencing.

2.3. Bioinformatics analysis of sequences

QIIME2 version 2.2020.6 (Quantitative Insight into Microbial Ecology), which is an open-source online Linux-based software used for analyzing and visualizing the sequence data (Bolyen et al., 2019), was used for carrying out bioinformatics analysis following the user online available manual of QIIME2. In the first step the sequence data, which was provided in demultiplexed form was used. The files were imported into QIIME2 by the command outlined as manifest file import method adopting PairedEndFastqManifestPhred33 input format. The q2-dada2 plugin offered by QIIME 2 for removing noisy data was employed that apply the DADA2 algorithm for estimating and correcting errors in sequences using statistical models and also removing low quality reads (Callahan et al., 2016). A 300 bp (base pair) criteria was chosen for trimming of reads. Chimera's and reads not fulfilling this criterion were removed. Taxonomic labels for the operational taxonomic units (OTUs) were assigned using the classify-sklearn method, a machine learning approach that incorporates a naive Bayes classifier against the reference database specifically Silva database (Silva 138) with an identity threshold of 99 % to obtain frequency base taxonomic tables at different levels (Bokulich et al., 2018). The bacterial communities' profiles at different taxonomic levels for the samples were graphically represented using heat maps. The taxonomic profiles of the processed samples were illustrated at various taxonomic levels through heat maps and bar plots. The data of raw sequences of all the samples associated with this study has been deposited in the National Center for Biotechnology Information (NCBI) and is available for retrieval under the unique accession number (PRJNA998186).

2.4. Statistical analysis

Rarefactions were performed for all samples, with the threshold set to the minimum value. The diversities were calculated utilizing alpha and beta diversity indices using R software. Shannon diversity index (H), Simpson's index (D), Chao 1 and Observed features were used to calculate alpha diversity and analyzed using Kruskal–Wallis test. Amongst these, the first two are based on species richness and evenness while the latter two takes into account species richness. The beta diversity was measured with two non-phylogentic base indices i.e. Jaccard index and Bray Curtis dissimilarity index, and two phylogenetic base

indices i.e. weighted and unweighted UniFrac metrics. These indices were evaluated by employing permutational multivariate analysis of variance (PERMANOVA) and visualized by generating Principal Component Analysis (PCA) graphs.

The output received in the form of the number of OTUs (frequencies) was processed for calculating percentage abundance. The percentage abundance at each taxonomic level was analyzed to check for any variation among groups using Analysis of Variance (ANOVA). Microbial diversities were assessed using "QIIME q2-diversity plug in" by adopting Core Metrics Phylogenetic method. For graphical presentation of the abundance data GraphPad Prism version 8.0.1. (GraphPad Software, San Diego, CA, USA)and Microsoft Excel (Microsoft Corp., Redmond, WA) softwares were utilized. Venn diagrams were prepared using online tool (Heberle et al., 2015).

3. Results

3.1. Diversity: Comparison of alpha and beta diversity in healthy and mastitic groups in HF milk

The diversity analysis was carried out to assess diversity within samples using four diversity indices i.e. Richness and evenness base indices such as Shannon and Simpson, and abundance base indices including observed species and Chao 1. The HHF group samples appeared to have a higher diversity values based on all four used indices. The diversity pattern observed in the CHF group appeared to be lowest amongst all the group samples with the used indices except Simpson where its value was found to be a little higher than SHF group samples. The microbial diversity depicted in the SHF group samples using the aforementioned indices shows that it was lower than the HHF group and higher than the CHF group samples (except Simpson). Although variation was observed in alpha diversity but no significant difference was observed as shown in Table.1 and Fig. 1.

The beta diversity indices including non-phylogenetic abundance base indices such as Jaccard index, Bray Curtis dissimilarity index and phylogenetic base indices such as weighted and unweighted UniFrac distance metrics were used to evaluate diversity across samples. The PCA plots used for visualizing Jaccard index and Bray Curtis dissimilarity index exhibited that a clear separation was not observed between the groups but a distribution pattern in which 40 % of samples of both HHF and SHF clustered distantly from the rest. Similarly, the other 60 % of HHF samples clustered with 40 % SHF and CHF samples and 40 % of CHF were also distantly placed from each other and the rest of the CHF samples. Weighted UniFrac Principal Component Analysis (PCA) plot resulted in clustering of all the HHF samples with 4(80 %) samples of CHF and SHF groups. The unweighted UniFrac Principal Component Analysis (PCA) plot exhibited a dispersed or non-clustered arrangement of all samples (Fig. 2).

Table 1
Alpha diversity indices (mean ± SEM) observed in Holstein Friesian cattle milk microbiota.

Group	Alpha diversity indices			
	Shannon	Simpson	Chao1	Observed features
HHF (mean ± SEM)	7.25 ± 1.08	0.97 ± 0.02	396.28 ± 147.39	396.20 ± 147.37
CHF (mean ± SEM)	6.48 ± 1.01	0.96 ± 0.02	219.90 ± 73.57	219.60 ± 73.76
SHF (mean ± SEM)	6.59 ± 1.21	0.95 ± 0.04	288.00 ± 121.84	288.00 ± 121.84
P –values	0.4317	0.357	0.5945	0.5945

†p-values were considered significant at p = 0.05.

3.2. Comparison of microbial profile through venn diagram

The distribution of taxa at different levels exhibited a variable pictures in terms of udder health status. Various taxa were shared amongst the groups and in addition every group also exhibited a different number of taxa at each level (Fig. 3). The taxonomic assignment of OTUs at the phylum level resulted in a total of 39 phyla of which 29 phyla were detected in each HHF and SHF group whereas the number of phyla was 22 in CHF. The highest number of distinct phyla were presented in the SHF group (n = 6), followed by CHF (n = 4) and HHF (n = 3) groups. Upon analysis; at the order level, a total of 204 orders exhibiting varying abundances across all the groups were identified. The total number of orders allocated based on OTUs to the HHF group were 151, CHF group 107 and 146 orders to the SHF group. Interestingly, a total of 74 orders were detected in all three groups, indicating their presence in the milk microbiota across all udder's health conditions. Intriguingly, the HHF group exhibited 32 orders that were not detected in the other groups, suggesting the presence of unique microbial taxa specific to the udder's healthy state. Similarly, the CHF and SHF groups harbored 19 and 27 orders, respectively, that were not observed in the respective contrasting groups. The shared order picture revealed a high number (38) of order sharing between HHF and SHF groups, whereas order sharing among CHF and HHF, and CHF and SHF group only confined to 7 orders. Genus analysis revealed the presence of 660 different genera across various groups. Among these groups, 106 genera were commonly found in all groups. The HHF group exhibited the highest number of unique genera (194), followed by the SHF and CHF groups with 113 and 54 distinct genera, respectively. Additionally, 42 genera were shared between the HHF and CHF groups, while 132 genera were shared between the HHF and SHF groups. Lastly, there were 21 genera common to both the CHF and SHF groups. A total of 422 species were identified across the different groups. Among these, 49 species were found to be common across all groups. Notably, the HHF group exhibited the highest number of distinct species (149), while the SHF and CHF groups displayed 80 and 57 distinct species, respectively. There were 26 species shared between the the HHF and CHF groups, and 13 species shared between HHF and SHF. Additionally, 26 species were common between the SHF and CHF groups.

3.3. Differences in milk microbial profile at various levels for each udder health status

Assessment of the milk microbial profile at different levels showed variance across the three groups. Phylum level variations are depicted in Fig. 4. The phylum level abundance based picture of the HHF group revealed different phyla, in which Proteobacteria (40.14 %) accounted for the major proportion of bacterial communities. The other phyla having high abundance (>5%) included Firmicutes (25.06 %), Bacteroidota (17.92 %) and Actinobacteriota (5.76 %). These four phyla accounted for 88.88 % of the assigned OTUs. On the other hand, the milk microbiota of the CHF group was majorly occupied by Firmicutes (29.20 %), Bacteroidota (26.33 %), Proteobacteria (21.97 %) and Actinobacteriota (5.58 %) accounting for 83.08 % for the assigned OTUs. In the SHF group, the major portions of OTUs were from Proteobacteria (46.51 %) as shown in Fig. 5. whereas Firmicutes (23.29 %), Bacteroidota (13.56 %) and Actinobacteriota (5.77 %) were also found in high proportions as presented in Table.2. As a whole 89.13 % of the OTUs belong to these four phyla in this group. The distinct phyla detected in HHF included Abditibacteriota, Thermotogota and RCP2-54, in CHF group Sva0485, Deferribacterota, Nitrospinota and Halanaerobiaeota whereas in SHF group Armatimonadota, SAR324_clade, Cloacimonadota, Calditrichota, Halobacterota and Marinimicrobia were the distinct phyla.

Variations in abundance were also depicted at order level with HHF group mainly comprised of Burkholderiales (14.28 %), Flavobacteriales (8.83 %), Bacteroidales (6.67 %) and Lactobacillales (5.91 %), and a low abundance (<5%) of Pseudomonadales, Rhizobiales, Corynebacteriales,

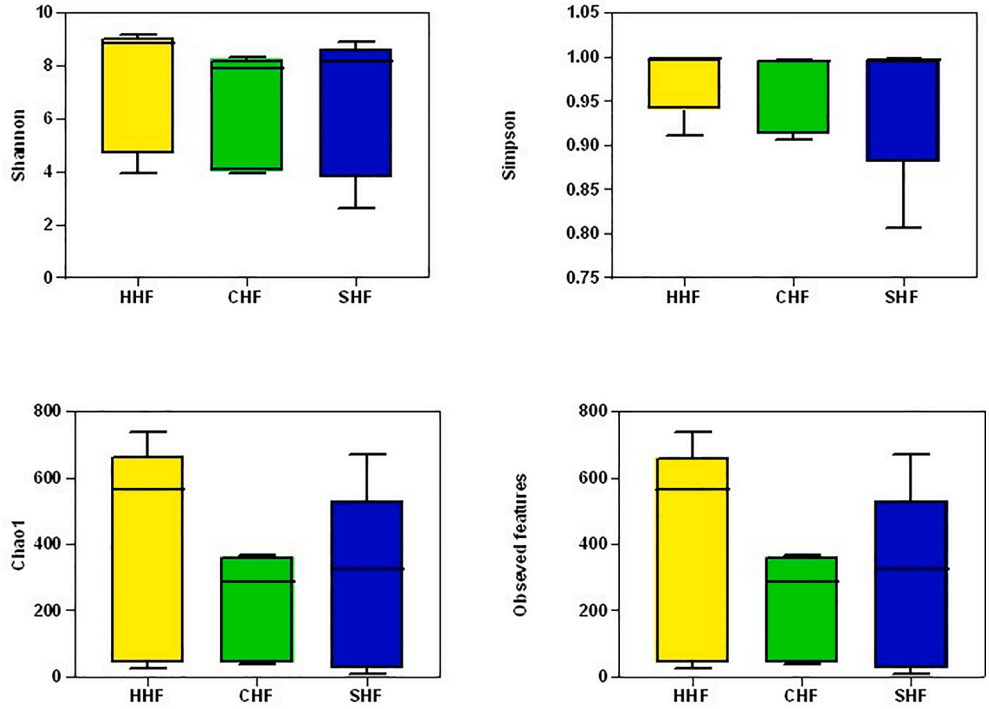

Fig. 1. Alpha diversity indices observed in milk of HHF (Healthy, Yellow), MHF (Clinical mastitis, green), SHF (Subclinical mastitis, blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Bacillales, Staphylococcales and Micrococcales. The major orders detected in the CHF group were Bacillales (18.50 %), Bacteroidales (16.88 %), Flavobacteriales (8.12 %) and Pseudomonadales (5.62 %) while orders with less than 5% abundance included Burkholderiales, Lactobacillales, Rhizobiales, Corynebacteriales, Micrococcales and Staphylococcales. The abundance of these major orders varied in the SHF group compared to the other two groups, with Burkholderiales (31.30 %), Bacteroidales (6.03 %) and Pseudomonadales (5.14 %) while Flavobacteriales, Bacillales, Lactobacillales, Rhizobiales, Micrococcales, Corynebacteriales and Staphylococcales were detected with low abundance (<5%) (Supplementary Table.S3 and Supplementary Fig. S1).

Different genera were detected across the study groups which shows that the percentage abundance of *Corynebacterium, Sphingomonas and Staphylococcus* were high in the HHF group compared to other groups. *Bacteroides, Flavobacterium, Lactobacillus, Pseudomonas* and uncultured bacterial genera dominated the CHF group in terms of abundance as compared to the other two groups while *Acinetobacter* and *Streptococcus* were the genera whose abundance was high in SHF group. (Supplementary Table S4 and Supplementary Fig. S2).

Based on assigned OTUs, species-level picture showed that most of species remained unassigned or were placed into un culturable group possibly due to the low resolution. The species that were detected in the HHF group included *Acinetobacter gerneri, Acinetobacter guillouiae, Acinetobacter harbinensis, Acinetobacter indicus, Acinetobacter kyonggiensis, Acinetobacter wuhouensis, Corynebacterium bovis, Corynebacterium efficiens, Corynebacterium humireducens, Corynebacterium marinum, Corynebacterium mari, Corynebacterium pollutisol, Lachnospiraceae bacterium, Lactobacillus intestinalis, Streptococcus dysgalactiae, Flavobacterium cloacae, Flavobacterium frigidarium, Flavobacterium arsenatis, Flavobacterium buctense, Flavobacterium fryxellicola, Flavobacterium gelidilacus, Flavobacterium granuli, Flavobacterium jumunjinense, Flavobacterium limicola, Flavobacterium luticoct, Flavobacterium ponti, Flavobacterium psychrolimnae and Akkermansia muciniphila.* In CHF group, the species that were detected included *Acinetobacter celticus, Acinetobacter harbinensis, Corynebacterium bovis, Lactobacillus intestinalis, Streptococcus vestibularis, Flavobacterium frigidarium, Flavobacterium gelidilacus, Flavobacterium buctens, Flavobacterium granuli, Flavobacterium aquatile, Flavobacterium cloacae, Flavobacterium jejuense, Flavobacterium limicola, Flavobacterium orientale, Flavobacterium psychrolimnae and Akkermansia muciniphila.* In SHF group, *Acinetobacter bohemicus, Acinetobacter harbinensis, Corynebacterium marinum, Corynebacterium maris, Corynebacterium pollutisoli, Corynebacterium simulans, Corynebacterium variabile, Lactobacillus fermentum, Lactobacillus intestinalis, Streptococcus dysgalactiae, Flavobacterium cloacae, Flavobacterium frigidarium, Flavobacterium gelidilacus, Flavobacterium gilvu, Flavobacterium hydatis, Flavobacterium lacus, Flavobacterium limicola, Flavobacterium luticocti, Flavobacterium ponti* and *Akkermansia muciniphila* were detected (Supplementary Fig. S3).

4. Discussion

The milk microbiota of Holstein Friesian has been previously characterized using 16S rRNA gene base metagenomics (Cremonesi et al., 2018; Curone et al., 2018), but to the best of our knowledge, this is the first study of such kind in which milk composition of Holstein Friesian reared in Pakistan is explored. The focus of our study was to evaluate the composition of bacterial communities prevalent in milk microbiota of HF with different udder health statuses. As mentioned earlier many studies have reported HF milk microbiota and specifically keeping emphasis on udder health status yet it seems important to have a look into this outstanding milk producing breed as many studies conducted on it are culture or PCR-based (Ali et al., 2021; Lubna et al., 2023).

The microbial composition of the milk is affected by many factors. It has been reported that variations in milk microbial profile occur due to factors such as breed, geographical location, weather and herd (Espeche et al., 2012; Tarrah et al., 2022). The production capacity of dairy animals is known to be affected by mastitis which may be manifested in clinical or subclinical form (Bansod et al., 2021). The major pathogens including *Staphylococcus aureus, E.coli and Streptococcus species* can be identified using classical routine culture-based methods (Ahmed et al., 2022; Alkhouly et al., 2023; Sarwar et al., 2021) but it has been reported that these method are less effective when it comes to identifying

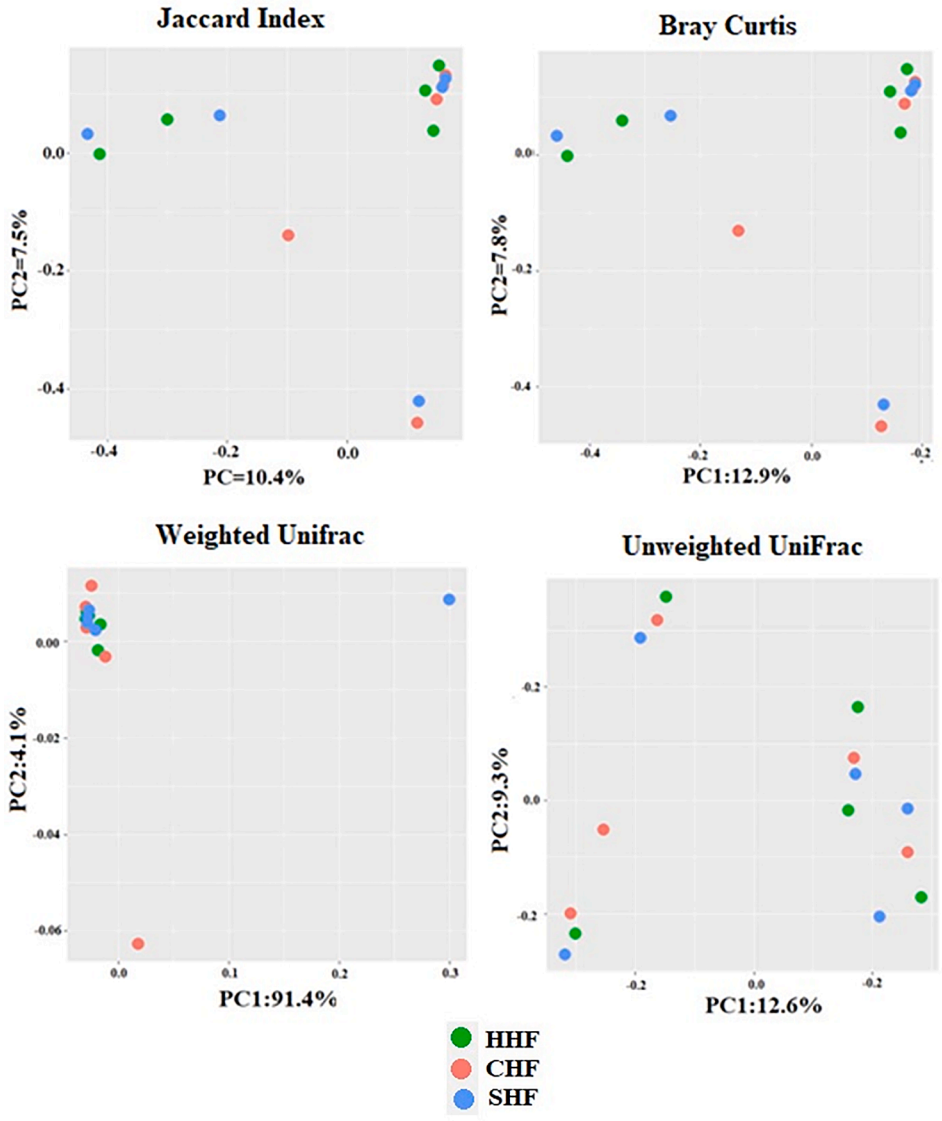

Fig. 2. Distribution of samples of different groups based on beta diversity indices.

pathogens with fastidious growth requirements (Gelgie et al., 2022). The advancement in molecular biology, specifically the use of next generation sequencing has helped in exploring the microbial communities associated with different niches (Gilbert et al., 2014; Huttenhower et al., 2012).

Many studies have been conducted to assess the diversity in different bovine species targeting different hyper variable regions and it has been concluded that during mastitis diversity is known to be less in the milk of animals infected with intra-mammary infection (Catozzi et al., 2017; Falentin et al., 2016; Kaczorowski et al., 2022) which is also observed in this study, in which the V3 and V4 hyper variable regions of the 16S rRNA gene is used as a target. A similar finding to our study regarding non significant differences is also reported about alpha diversity indices (Oikonomou et al., 2014). Mastitis majorly occurs when an imbalance occurs in the intra-mammary bacterial communities and pathogenic bacteria dominate the niche which results in alteration of the commensal bacteria and results in reducing diversity (Khalil et al., 2022a) which is also depicted by the diversity indices in this study.

The dispersion and clustering of the samples of the three groups show a mixed pattern as depicted by the PCA plots of beta diversity indices as demonstrated in various studies conducted on different bovine species (Sokolov et al., 2021). The clustering pattern shows a clearer picture especially in the case of non phylogenetic base indices after removing clinical mastitis group samples and it shows that the microbiota between healthy and subclinical mastitis group samples are close enough and may be the animals are in the transition phase from infection to recovery state. The overlapping of samples of different groups also shows that various bacterial taxa are shared across groups.

Different numbers of taxa have been reported in milk across different udder health status in different studies and it has been found that at the phylum level Proteobacteria, Firmicutes, Actinobacteriota, Bacteroidota and Acidobacteriae constitute the core taxa with varying levels of abundance (Addis et al., 2016; Derakhshani et al., 2018a; Kaczorowski et al., 2022; Ruegg, 2022). Similarly, differences across different taxonomic levels have been reported in different udder health conditions with differences in sharing and distinction of taxa that may be due to breed, species, geography, age, lactation stage, milk portion used, sequencing method used and weather (Bhatt et al., 2012; Lima et al., 2018; Patel et al., 2017a; Polveiro et al., 2022; Toquet et al., 2021).

The observed phyla associated with udder health statuses showed that Proteobacteria dominated the HHF and SHF groups while Firmicutes representation was recorded in higher proportion in the CHF group. Phylum Proteobacteria has been reported to vary with udder health status and breed, it has been described as a major contributing

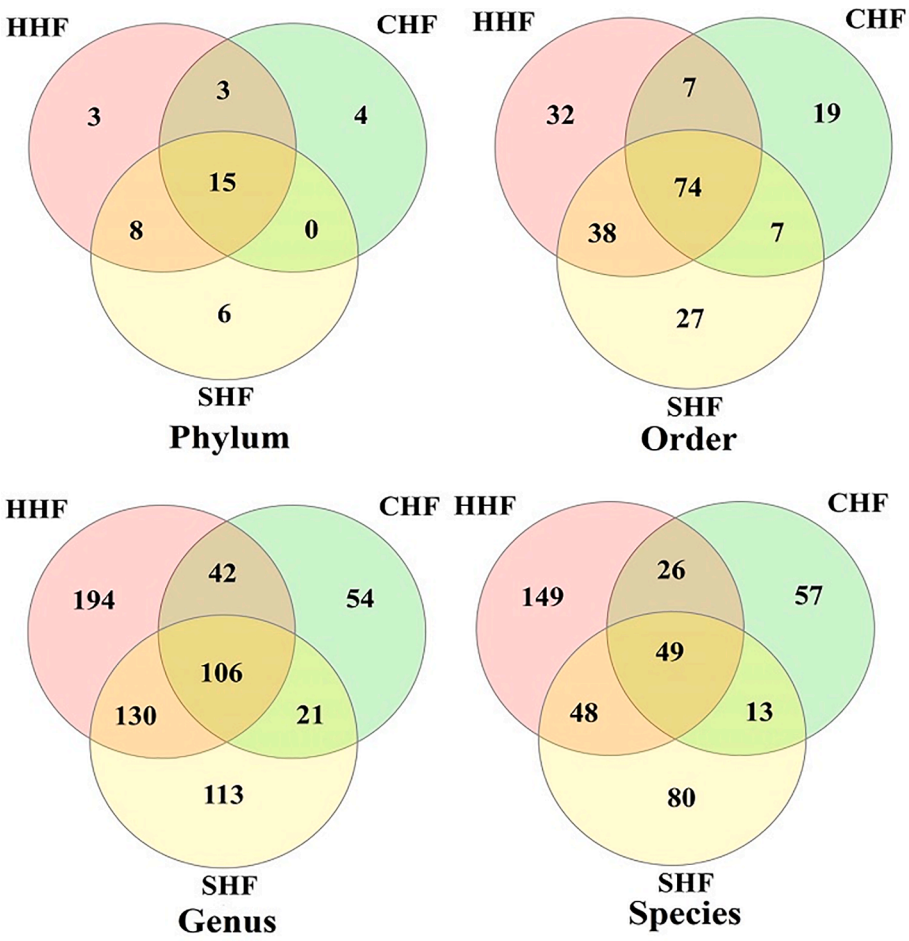

Fig. 3. Distribution of different taxonomical levels across groups represented by venn diagram.

phyla to the milk microbiota of healthy animals (Cremonesi et al., 2018; Steinberg et al., 2022) which has also been observed in our study, although a different pattern of occurrence of Proteobacteria is also reported (Lima et al., 2018). Firmicutes were identified as abundant phyla in the CHF group in our study but in different studies Firmicutes abundance is mostly less than Proteobacteria in mastitic milk (Khalil et al., 2022b; Patel et al., 2017b). In another study, it was also found that milk microbiota of mastitis resulting from *E.coli* and *Klebsiella* was majorly comprised of Proteobacteria whereas *Streptococcus* caused mastitis samples were dominated by Firmicutes (Lima et al., 2018). In subclinical mastitis animals' milk microbiota similar findings have been reported in a study (Kaczorowski et al., 2022) which states that either of the major phyla Proteobacteria or Firmicutes may dominate the samples and it specifically depends upon the causative agent of mastitis such as in case of *E.coli* Proteobacteria dominates whereas in case of *S.agalactiae* infection Firmicutes dominate. In our study, the findings are similar to the latter although we have not identified the causative agent specifically. Thus, it can be interpreted that in both types of mastitis etiological agent plays an important role in shaping the udder microbiota.

Genus *Sphingomonas* has been detected in all groups in low abundance but the pattern of occurrence is different with respect to udder health status as already reported where it's abundance was high in mastitic milk (Jiang et al., 2023) but in our study its abundance was high in healthy milk. They are opportunistic pathogens and contain glycosphingolipids in their outer membrane instead of lipopolysaccharide (Hashimoto et al., 2004). They are also known for their ability to survive and grow in harsh environments (Baraniecki et al., 2002). Their presence in milk is suggested to be a transfer from milking parlor and stable (Vacheyrou et al., 2011). In Pakistan, among other transportation methods used for delivery of milk, horse-drawn carriage is also used and the presence of *Sphingomonas* may be attributed to the regular visit of horse drawn carriage yet it needs further investigation. While fodder is also transported via donkey cart and mostly they are kept on farms, *Sphingomonas* has been reported in endometrial and milk microbiome of donkeys (Li et al., 2022; Papademas et al., 2020; Papademas et al., 2021).

Flavobacterium contains psychotropic opportunistic bacteria and have been detected in raw milk previously (Delbès et al., 2007). In a recent study a novel bacterial taxon that exhibits evolutionary relationship with member of *Flavobacteriaceae* was isolated from milk of mastitis affected Holstein Friesian (Pan et al., 2020). Although *Flavobacterium* has been detected as a major genera in clinical mastitis group in current study yet its role in causing mastitis needs further exploration.

Interestingly at the genus level, *Staphylococcus* was not detected in any sample of the subclinical mastitis HF group while they were detected in the other two groups but with low abundance, being higher in HHF group followed by CHF group. This is in contrast with the findings of the previous study (Steinberg et al., 2022), although family Staphylococcaceae was identified in all the samples with decreasing abundance across HHF, SHF and CHF groups. In a study conducted in different breeds of cattle (Bhanderi et al., 2014), the metagenomic approach was not able to identify the genus *Staphylococcus* in two breeds. The detection of *Staphylococcus* in a very low percentage in the clinical mastitis group and its absence in the subclinical mastitis group may be explained by the antagonistic property of *Pseudomonas* against *Staphylococcus* species specifically *S.aureus*. due to the production of pseudomonic acid (Machan et al., 1991).

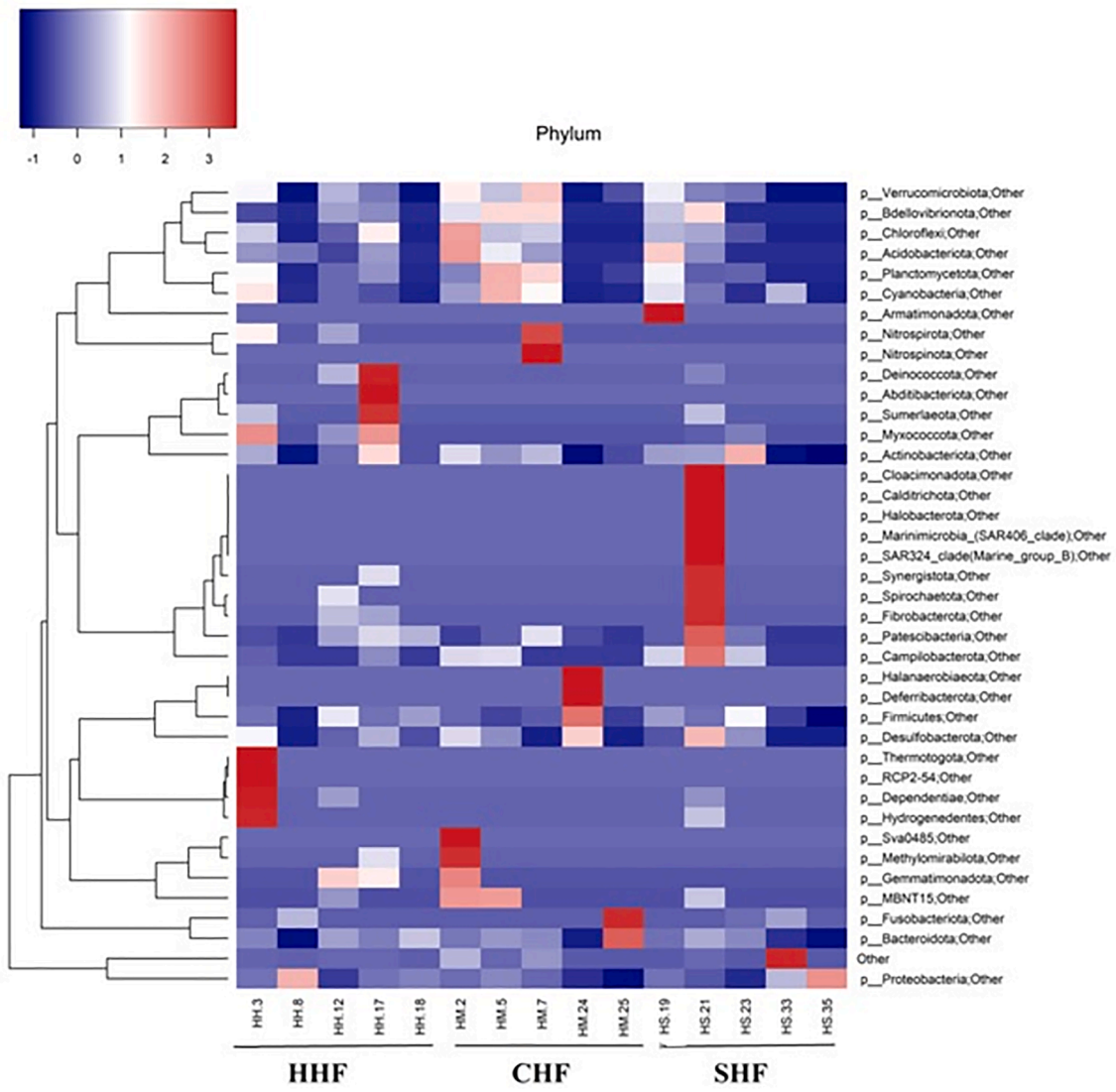

Fig. 4. Heat map representing phyla distribution across milk samples of study groups.

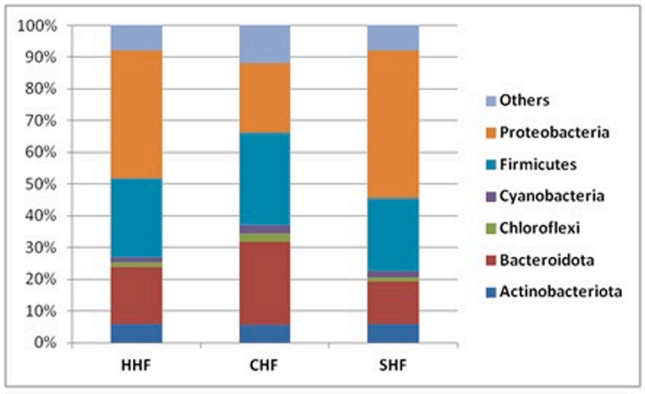

Fig. 5. Group wise phyla distribution represented by bar plot.

Table 2
Variation in percentage abundance (mean) in different abundant phyla across the study groups.

Taxa	HHF		CHF		SHF	
Phylum	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Acidobacteriota	0.25[a]	0.09	0.54[a]	0.27	0.30 [a]	0.21
Bacteroidota	17.92[a]	4.93	26.33[a]	11.22	13.56 [a]	4.44
Chloroflexi	1.74[a]	0.88	2.57[a]	1.23	1.22 [a]	0.58
Cyanobacteria	1.39[a]	0.81	2.48[a]	1.12	1.82 [a]	0.79
Firmicutes	25.06[a]	6.33	29.20[a]	13.17	23.29 [a]	7.79
Proteobacteria	40.14[a]	12.88	21.97 [a]	5.98	46.51 [a]	16.37

Different letters (a,b) in the same rows denotes differences between means for p-value < 0.05.

5. Conclusion

In the current study evaluation of milk microbiota of Holstein Friesian, the taxonomic profile reveals alterations in microbial composition with udder health. The microbial composition of HF reared in Pakistan varied considerably from other reported studies and it might be due to many factors that may vary based on host or environment, which may be responsible for shaping the microbiota under specific managmental, and geographic conditions that need further exploration. Specific microbiota that are identified in healthy and mastitic milk must be taken into consideration for devising strategies in improving milk production, for overcoming mastitis and improving milk production of exotic breeds such as HF reared under different conditions not similar to their native

ones.

Ethics approval

This study was conducted after obtaining ethical approval from the "Ethical review committee, University of Veterinary and Animal Sciences Lahore" (No.DR/81 dated 14-01-2020) and all the methods were performed in accordance with the institutional guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

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CRediT authorship contribution statement

Mian Muhammad Salman: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Muhammad Nawaz:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Tahir Yaqub:** Data curation, Software, Visualization, Validation. **Muhammad Hassan Mushtaq:** Software, Visualization, Writing – review & editing, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2024.103984.

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