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Changes in the milk and fecal microbiota in Holstein cows with subclinical intramammary infection

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ABSTRACT

Understanding the relationship between microbial community alterations and disease can provide valuable insights for improving diagnostics, prevention, and treatment strategies. This study used 16S rRNA amplicon sequencing to investigate and compare microbial diversity in the milk and feces of Holstein cows with subclinical mastitis caused by *Streptococcus agalactiae* and *Prototheca* spp. with that of healthy cows. A bacteriological screening identified 50 Holstein cows reared in a commercial dairy farm and classified into 3 experimental groups: (1) animals negative at the bacteriological examination with no history of subclinical mastitis (HLTH; n = 16), (2) animals positive at the bacteriological examination for either *Streptococcus agalactiae* (STRP; n = 22) or *Prototheca* spp. (PRTH; n = 12). The milk microbiota showed significant pathogen-specific alterations, with increased *Firmicutes* in STRP cows (55.6%) and *Cyanobacteria* in PRTH cows (17.3%), compared with healthy cows (39.2% and 0.7%, respectively). Alpha diversity (observed amplicon sequence variants, Shannon, and evenness indices) was significantly lower in infected cows, confirming a microbial imbalance. Beta diversity analysis (PERMANOVA) revealed significant differences in microbial composition between healthy and infected cows, but no significant differences in fecal microbiota composition. Differential abundance analysis identified *Streptococcus* ($\log_2$fold change [FC] = 7.3) as the most enriched taxon in STRP cows and *Cyanobacteria* ($\log_2$FC = 8.9) as the most enriched in PRTH cows in milk matrix, while *Macrococcus caseolyticus* was significantly reduced in both infected groups ($\log_2$FC = −4.5). These findings suggest that subclinical mastitis leads to significant shifts in the milk microbiota but does not alter the fecal microbiome, supporting a localized rather than systemic microbial response. This study provides novel insights into the microbial dynamics of subclinical mastitis and potential biomarkers for disease monitoring.
Key words: milk metabarcoding, feces metabarcoding, mastitis, MiSeq Illumina, *Prototheca* spp

INTRODUCTION

Mastitis is defined as an inflammatory disease mainly resulting from the infection of the udder tissue by a wide range of microorganisms, which causes significant economic losses associated with reduced milk production, a minor probability of conception, premature slaughter, and treatment costs (Ruegg, 2017). Mastitis is still considered the most common and costly health concern in the dairy industry, and for this reason, it remains a research priority to identify tools focused on mammary gland health and mastitis control (Andrews et al., 2019). Mastitis can be categorized as clinical or subclinical, the latter being estimated to be 15 to 40 times more frequent than the former (Martin et al., 2018). This form is characterized by an increase in SCC in milk, above 200,000 cells/mL (IDF, 1997), and represents an important source of infection while going unnoticed. Until recently, previous studies conducted on bovine milk were focused on identifying the main microorganisms involved in mastitis development, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis, Escherichia coli*, *Klebsiella* spp., *Enterobacter aerogenes*, and *Corynebacterium bovis* (Hoque et al., 2019), as well as their effect on the disease. In Italy, where intensive indoor dairy farming is widespread, most IMI are caused by contagious pathogens such as *S. aureus*

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and *Strep. agalactiae*, as well as various environmental bacteria (Bobbo et al., 2017). However, mastitis caused by *Prototheca* spp., a rare and emerging pathogen in dairy herds, has been also detected. This alga represents a significant challenge due to its resistance to conventional antibiotic treatments and limited understanding of its pathogenic mechanisms or detection and control (Pegolo et al., 2022a).

Different studies have already explored the microbiota of milk samples, revealing the presence of a dynamic microbial community that significantly changes with the onset of mastitis from different pathogens (Oultram et al., 2017; Pang et al., 2018; Steinberg et al., 2022). However, information is limited on the role of *Cyanobacteria* in mastitis, and only one study has reported a high prevalence of *Calothrix desertica* in milk from mastitis caused by *Prototheca* spp. (Miura et al., 2019). In addition, sequence-based microbiota analyses were also used to understand the microbial diversity of feces (Oikonomou et al., 2013) and teats (Falentin et al., 2016), to identify possible sources of raw milk contamination (Doyle et al., 2017). Notably, recent evidence has pointed out that the milk microbiota is not the only microbial community altered by the onset of mastitis (Hu et al., 2022; Zhu et al., 2024). Indeed, dysbiosis of the gastrointestinal microbiota might be not only a consequence related to the presence of mastitis, but due to the "entero-mammary pathway hypothesis," could also play a fundamental role in the onset and progression of the disease (Hu et al., 2024; Zhu et al., 2024). In this context, mastitis has been associated with shifts in fecal microbial composition, including decreased abundances of *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia*, and *Firmicutes*, and increased abundances of *Tenericutes*, *Fusobacteria*, and *Bacteroidetes* (Jiang et al., 2023). Notably, the *Firmicutes*:*Bacteroidetes* ratio is considered a key indicator of intestinal dysbiosis and has been linked to impaired gut function and elevated systemic inflammation (Stojanov et al., 2020). However, whether subclinical mastitis specifically alters the fecal microbiota remains poorly understood. In light of these considerations, this study aimed to: (1) characterize the milk and fecal microbiota of cows with subclinical mastitis caused by *Strep. agalactiae* and *Prototheca* spp., using MiSeq Illumina sequencing, and to explore their potential interplay under subclinical infection; (2) compare microbial community alterations between healthy and infected cows, evaluating pathogen-specific differences, in particular for *Prototheca* spp., an emerging mastitis pathogen with limited existing research, and (3) assess whether subclinical mastitis induces shifts in the fecal microbiota, supporting the entero-mammary pathway hypothesis.

MATERIALS AND METHODS

Experimental Design, Farm, and Animals

To compare cows naturally infected by *Strep. agalactiae* or *Prototheca* spp. with healthy cows maintained in the same environment and with the same management, feeding, and sanitary protocols, a commercial herd of 450 lactating cows was selected based on a survey carried out by the national Veterinary Laboratory for northeastern Italy (Istituto Zooprofilattico Sperimentale delle Venezie [**IZSVe**], Legnaro (PD), Italy) aimed at determining the herd prevalence of *Strep. agalactiae*, and *Prototheca* spp. in the Veneto region (northeast Italy).

Cows were housed in freestalls and fed a TMR based mainly on corn silage, sorghum silage, and concentrates. Drinking water was available in automatic water bowls, and milking was carried out twice a day. Animal health was managed by the farmer and local veterinarian, who intervened when needed according to the farm health protocol. All cows were subjected to the same management practices and environment to ensure sample homogeneity; detailed description of the experimental design and the further specifics are reported in Pegolo et al. (2021).

Animals were regularly monitored between January and February 2021 and for inclusion in the study we required the following criteria: (1) absence of clinical signs of infection or other disease, (2) no antibiotic treatment or anti-inflammatory medications in the 6 mo before enrollment, (3) being multiparous and in mid-late lactation (DIM ≥92). Based on these criteria, an initial bacteriological screening (time 0, **T0**) was performed by the IZSVe on 188 lactating cows to identify healthy individuals and cows with subclinical mastitis from either *Strep. agalactiae* or *Prototheca* spp. Details of microbiological analysis are reported by Pegolo et al. (2022b). Then, a second bacteriological examination was conducted 2 wk after T0 (**T1**) to confirm the animals' infection status. Between the T0 and T1 animals underwent daily monitoring by both the farmers and the local veterinarian to ensure they remained free from any visible signs of mastitis, thereby maintaining their subclinical condition. Following the bacteriological results at T0 and T1, we selected 50 multiparous Holstein Friesian cows (ranging from 3 to 7 yr old) and between 92 and 448 DIM and we created 3 experimental groups: (1) healthy (**HLTH**, n = 16) constituted by milk and feces samples collected from healthy individuals with a negative milk bacteriological examination in all mammary quarters both at T0 and T1; (2) *Strep. agalactiae*-infected (**STRP**, n = 22) constituted by milk and feces samples collected from animals naturally infected with *Strep. agalactiae*, and (3) *Prototheca* spp-infected (**PRTH**, n = 12) constituted by milk and

feces samples collected from animals naturally infected with *Prototheca* spp. The distribution of animals according to DIM and parity across the 3 experimental groups is provided in Supplemental Table S1 (see Notes). Animals with coinfection were excluded from the trial. Moreover, we required that animals used as control (HLTH) had no previous history of mastitis. Information was collected from the herd management software (Dairy Comp Sata, Alta Italia Srl, Milan, Italy).

Ethical Statement

This study was part of the LATSAN project that aimed to develop innovative tools for studying and evaluating mammary gland health and improving dairy cows' nutritional milk quality and coagulation properties. The research was approved by the Ethical Animal Care and Use Committee (OPBA—Organismo Preposto al Benessere degli Animali) of the Università Cattolica del Sacro Cuore (Piacenza, Italy) and by the Italian Ministry of Health (Rome, Italy; protocol number 510/2019-PR of 19/07/2019).

Sampling of Milk and Feces for Microbiological Analyses

Milk samples were manually and aseptically collected from each animal according to the National Mastitis Guidelines (Adkins, 2017). Briefly, teat ends were externally cleaned with commercial premilking disinfectants, dried with individual towels, and then washed again with 70% alcohol solution. Pool milk samples of the 4 glands were then collected after discarding the first streams of foremilk from each quarter. Two aliquots of each pool sample (25 mL for quality traits and microbial count analysis and 25 mL for the metabarcoding analyses) were immediately frozen in liquid nitrogen, and then stored at −80°C until analyses in the Research and Innovation Center, Fondazione Edmund Mach (FEM, San Michele all'Adige (TN), Italy) laboratories. Microbial plate counts were carried out within 5 d, and metabarcoding analyses were carried out within 6 mo.

Approximately 30 g of fecal material were collected from the rectum of each cow using individual plastic palpation sleeves and immediately placed in a sterile 50-mL propylene tube, transported in a foam box with ice packs to the laboratories on the same day, and frozen at −80°C until use.

Microbiological Counts of Milk Samples

Microbiological analyses were performed at the Microbiological Laboratory of FEM. Milk samples were decimally diluted in sterile peptone water and placed in the following agar media: plate count agar (**PCA**) with skim milk (10 g/L) for total bacterial count (**TBC**), under aerobic conditions for 24 h at 30°C; Wilkins Chalgren (**WC**) agar, for total anaerobic TBC, incubated under anaerobic conditions (in a jar with an AnaeroGen system) for 48 h at 37°C; violet red bile agar (**VRBA**) for counting coliforms, following the overlay method as suggested by manufacturer's instruction, for 24 h at 37°C; Columbia blood agar (**CBA**) enriched with 5% defibrinated sheep blood for the count of hemolytic streptococci incubated aerobically at 37°C, and only colonies showing greenish discoloration (partial hemolysis) or a clear zone (total hemolysis) around the colonies were counted as hemolytic streptococci after 48 h; and finally, CBA supplemented with 5% defibrinated sheep blood and chloramphenicol 0.7% for the count of *Prototheca* spp., incubated in aerobic conditions at 37°C, and only opaque dark gray colonies were counted after a microscopic inspection for *Prototheca* spp. after 72 h (Marques et al., 2008). All culture media and anaerobic systems were purchased from Oxoid (Thermo Scientific, Waltham, MS). The data regarding microbiological plate counts were expressed as log colony forming unit per milliliter.

Total DNA Extraction from Milk and Fecal Samples

For total genomic DNA extraction, 4 mL of milk were centrifuged at 4,000 × *g* for 10 min at 4°C, and the supernatant was discarded. Genomic DNA was extracted from the pellet using the DNeasyPower Food Microbial Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions and quantified by the Nanodrop8800 fluorospectrometer (Thermo Scientific).

Fecal microbial DNA was extracted starting from 250 mg of thawed fecal sample by using the QIAamp PowerFecal DNA kit (Qiagen, Milan, Italy) according to the kit instructions and quantified by the Nanodrop8800 fluorospectrometer (Thermo Scientific).

Preparation of the MiSeq Library

Amplicon sequencing libraries were prepared and sequenced at the FEM Sequencing Platform (San Michele all'Adige, Trento, Italy) using the Illumina MiSeq system (MiSeq Control Software 2.0.5; Real-Time Analysis 1.16.18, Illumina, San Diego, CA). In brief, the V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified in a 2-stage PCR workflow. In the primary amplification, 2 µL of genomic DNA (10 ng/µL) served as the template in a 25-µL reaction containing 12.5 µL of KAPA HiFi HotStart ReadyMix (2×; Kapa Biosystems Ltd., London, UK), 1 µ*M* each of primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 806R (5′-GACTACNVGGGTWTCTAATCC-3′; Baker

et al., 2003; Claesson et al., 2010), and nuclease-free water to volume. Thermal cycling was performed on a Verity 96-well thermal cycler with an initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 40 s, and a final extension at 72°C for 5 min. Five microliters of each reaction were run in electrophoresis on a 1.5% agarose gel to confirm the expected ≈460 bp amplicon; the remaining 20 µL were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA), following the manufacturer's instructions, and eluted in 25 µL of nuclease-free water.

Purified amplicons were then subjected to indexing PCR to append Illumina Nextera XT dual indices and flow-cell adapters, following manufacturer instructions and according to this protocol: 95°C for 3 min, 7 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. Indexed libraries were purified again with AMPure XP beads (Beckman Coulter) according to the manufacturer's instructions, and eluted in 30 µL of Tris-EDTA buffer. Library concentration and size distribution were assessed before pooling. DNA concentrations were measured with a Quant-iT PicoGreen dsDNA kit (Invitrogen, Thermo Scientific) according to the supplier's protocol, and fragment size profiles were obtained on an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) with the High Sensitivity DNA Kit (Agilent Technologies). Following the quantitation, cleaned amplicons were mixed and combined in equimolar ratios.

Sequencing was carried out on the MiSeq platform in paired-end (**PE**) mode (2 × 300 PE). On-instrument base calling and demultiplexing generated FASTQ files for downstream analysis.

Sequencing Data Analysis

Raw PE FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights into Microbial Ecology (Qiime2, version 2020.11). Sequences were quality-filtered, trimmed, denoised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed using the consensus method in DADA2. Representative sequences were aligned with MAFFT in Qiime2 and used for phylogenetic reconstruction in FastTree using plugin alignment and phylogeny (Price et al., 2009; Katoh and Standley, 2013). Taxonomic and compositional analyses were conducted by using plugin feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pretrained Naive Bayes classifier based on the Greengenes 13_8 99% amplicon sequence variants (**ASV**) database (http://greengenes.secondgenome.com/), which had previously been trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to PE sequence reads to generate taxonomy tables.

Statistical Analysis

The statistical analysis of microbial counts was performed in RStudio (R version 4.0.2, RStudio version 1.4.1106; Posit Team, 2025).

Alpha diversity observed ASV number, evenness and Shannon diversity indexes, and statistical significance ($P < 0.05$) of α diversity metrics were evaluated by the Kruskal–Wallis H test in QIIME2; β diversities were calculated using the unweighted and weighted dissimilarity distance matrix in QIIME2. The β diversity distance matrix indicates differences in taxa composition between samples based on either presence-absence or quantitative species abundance data. The output matrix was ordinated using principal coordinate analysis (**PCoA**) and visualized using EMPeror (Vázquez-Baeza et al., 2013). Statistical significance of β diversity distances between groups was assessed using PERMANOVA with 999 permutations in QIIME2. For the differential abundance test, taxonomy information was provided for each ASV sequence using the ANCOM method (Mandal et al., 2015) implemented in QIIME2.

Pearson correlation analysis was performed to estimate correlations among the microbial communities of milk and feces using the the corr.test() function from the Psych package (Revelle, 2007) applying the Benjamini–Hochberg correction to account for multiple testing (Benjamini and Hochberg, 1995).

In addition, we employed DESeq2 R package (Love et al., 2014) implemented in R Studio to investigate the differentially abundant taxa in different experimental groups through the following pairwise comparisons: (1) HLTH vs. infected cows (regardless of the pathogen), (2) HLTH vs. STRP-infected cows and, (3) HLTH subject vs. PRTH-infected cows. The same analysis was conducted by adding parity and lactation stage (DIM) as batch factors to reduce random noise. The classes of parity and DIM were created as follows: (1) parity = 2, and parity >2; and (2) 92 > DIM ≤ 200, and DIM >200. The level of significance was set using a false discovery rate <0.05. To quantify the changes between the 2 conditions, $\log_2$ fold change (**FC**) was used.

RESULTS AND DISCUSSION

Microbial Counts

Descriptive statistics of the microbial plate count analysis are shown in Table 1. The concentration of coliforms on VRBA was always lower than the limit of detection. These results are in agreement with a pre-

Table 1. Microbial counts (mean ± SE) of raw milk samples[1]

Health status[2]	HLTH	STRP	PRTH
PCA	3.0 ± 0.41^{A}	3.6 ± 0.62^{A}	3.5 ± 0.66^{A}
WC	3.3 ± 0.71^{A}	3.7 ± 0.51^{A}	3.5 ± 0.46^{A}
CBA	1.5 ± 1.2^{A}	3.0 ± 0.49^{B}	2.6 ± 0.86^{B}
CBA-Chl	ND[3]	ND	+[3]
VRBA	ND	ND	ND

[A,B]For each column, bacterial count values with different A and B superscripts are significantly different ($P < 0.05$, one-way ANOVA with post hoc Tukey HSD).

[1]According to the health status, cows were classified in HLTH (healthy cows), STRP (naturally infected with *Streptococcus agalactiae*), and PRTH (naturally infected with *Prototheca* spp.

[2]PCA = total mesophilic aerobic bacteria grown on PCA; WC = total mesophilic anaerobic bacteria grown on WC agar; CBA = hemolytic streptococci grown on CBA; CBA-Chl = *Prototheca* spp. grown on CBA supplemented with 5% defibrinated sheep blood and 0.7% chloramphenicol; VRBA = total coliforms counted on VRBA.

[3]ND = not detected; no colony was grown on the plate. A plus sign (+) indicates presence in the plate but uncountable.

vious study by Martin et al. (2016) where the authors found coliforms representing a minor population if compared with other bacterial groups. Considering the mesophilic microbial population, the health status of the dairy cows did not show any significant effect on PCA (aerobic mesophilic bacteria) and WC (anaerobic mesophilic bacteria) plate counts (Table 1).

Columbia blood agar is used for the isolation of streptococci from bulk tank milk and mastitis milk (Sawant et al., 2002). Only plate counts on CBA were significantly affected when the milk was sampled from STRP dairy cows: hemolytic streptococci were significantly higher in STRP (3 log cfu/mL, $P < 0.05$) than in HLTH milk (1.5 log cfu/mL); no significant differences were observed when comparing PRTH and HLTH samples. *Prototheca* spp. can be aerobic or microaerobic and grow aerobically in various standard laboratory culture media, such as Columbia agar supplemented with 5% sheep blood (blood agar), Sabouraud dextrose agar, and brain heart infusion broth, among others (Marques et al., 2008, 2010; Jagielski et al., 2019). Because contaminating bacteria can overgrow these algae, chloramphenicol must be added to the culture media to suppress bacterial growth. On CBA, these algae usually show white to grayish, opaque, pasty, yeast-like colonies. In our case, grayish colonies were enhanced by microscopic observations and we considered the presence or absence in milk samples (symbol "+" in Table 1; Marques et al., 2008, 2010).

Taxonomic Analysis of Milk and Fecal Microbiota

MiSeq sequencing of 16S rRNA gene amplicons from raw milk and fecal samples yielded a total of 795,173 and 1,480,228 quality-filtered reads, respectively. The taxonomic profile of the raw milk bacterial microbiome revealed that at the phylum level (Table 2), the samples were dominated by *Firmicutes* (range: 36%–56%), *Actinobacteria* (range: 11%–39%), and *Proteobacteria* (range: 4%–7%). In samples from HLTH animals, the most prevalent phyla were *Firmicutes* and *Actinobacteria* (39.67% and 38.55%, respectively). In STRP animals, the predominance of *Firmicutes* was higher than in HLTH animals (55.66%) followed by *Actinobacteria*, which, conversely, were lower than in HLTH animals (16.79%). In PRTH animals, *Firmicutes* relative abundance was similar to the one recorded in HLTH animals (35.90%) and *Actinobacteria* were much lower than in the HLTH group (11.03%). Gram-positive *Firmicutes*, belonging to *Streptococcus* spp. and *Staphylococcus* spp., have already been related to contagious mastitis (Bhatt et al., 2012; Oikonomou et al., 2014; Rodrigues et al., 2017). In contrast, some genera belonging to *Firmicutes*, such as *Lactobacillus*, are cited as important dairy-related microbes, especially in fermented dairy products (Widyastuti et al., 2021) and are considered beneficial probiotics. The higher relative abundance of *Firmicutes* in STRP milk samples is probably due to the streptococci presence in the subclinical mastitis milk samples and this result was in agreement with a recent study by Steinberg et al. (2022) that reported the effects of subclinical mastitis on the milk microbiota, revealing that the bacterial communities were primarily composed of *Firmicutes*, *Proteobacteria*, and *Actinobacteria*. The study found an increased abundance of *Firmicutes* in cows with subclinical mastitis, and *Proteobacteria* were more prevalent in healthy animals. Many studies have already reported that the microbiota of raw bovine milk is dominated by *Firmicutes*, *Actinobacteria*, *Bacteroides*, and *Proteobacteria* (Falentin et al., 2016; Bonsaglia et al., 2017; Taponen et al., 2019). Members of the *Firmicutes* phylum represent the most abundant phylum in the dairy cow raw milk microbiota, although its specific role in the milk remains to be determined (Bonsaglia et al., 2017). Although the presence of *Firmicutes* in milk seems to be associated with a forage-based diet, the presence of *Bacteroidetes* in milk is usually more abundant in diets consisting primarily of concentrate (Secchi et al., 2023). The reduction of the relative abundance of *Bacteroidetes* could indicate a reduced nutritional supply and decreased immunity of the organism (Kim et al., 2011; Liu et al., 2023). *Proteobacteria* consists of a wide variety of gram-negative species including pathogenic and nonpathogenic bacteria (Parente et al., 2020). *Actinobacteria* includes gram-positive bacteria that are regularly found in the milk of healthy cows (Parente et al., 2020), although a deeper investigation is needed on the ecology of this phylum in milk. Interestingly, a well-known pro-biotic genus, *Bifidobacterium* is a member of *Actinobacteria* phylum. However, we found a different

Table 2. Phyla composition (expressed as percent)[1] by health status (HLTH, STRP, and PRTH) in individual cows' samples (milk and feces) as revealed by high-throughput sequencing analysis

	Milk			Feces		
Sample	HLTH	STRP	PRTH	HLTH	STRP	PRTH
Archaea	0.062	0.012	0.016	0.047	0.072	0.025
Acidobacteria	0.087	ND[2]	ND	ND	ND	ND
Actinobacteria	38.55	16.79	11.03	1.144	0.807	0.694
Bacteroidetes	5.299	4.256	2.824	42.07	41.67	42.55
Chlamydiae	0.007	ND	ND	ND	ND	ND
Chloroflexi	0.662	0.544	0.121	ND	ND	ND
Cyanobacteria	0.027	0.177	17.19	0.146	0.176	0.045
Elusimicrobia	ND	ND	ND	0.006	0.023	0.007
Fibrobacteres	0.006	0.007	0.015	0.087	0.135	0.049
Firmicutes	39.67	55.66	35.90	43.18	44.08	42.81
Fusobacteria	ND	0.002	ND	ND	ND	ND
Gemmatimonadetes	0.002	ND	ND	ND	ND	ND
Lentisphaerae	0.005	0.001	ND	0.005	0.003	0.004
Planctomycetes	0.062	0.110	0.059	0.014	0.065	0.027
Proteobacteria	6.154	6.591	4.399	0.801	0.700	1.070
Spirochaetes	0.017	0.056	0.018	2.603	3.078	3.691
TM7	0.538	1.189	0.510	0.057	0.052	0.040
Tenericutes	0.017	0.136	0.013	0.539	0.521	0.588
Verrucomicrobia	0.079	0.150	0.104	0.274	0.465	0.309
[*Thermi*]	0.041	0.087	0.015	ND	ND	ND

[1]Only detectable phyla are reported. Brackets indicate taxonomic status is provisional.

[2]ND = not detected.

pattern of relative abundances among different groups of subclinical mastitis animals. The PRTH group showed a *Cyanobacteria* relative abundance of about 3 orders of magnitude higher (17.3%) than the HLTH (0.68%) and STRP (0.72%) groups, in which their presence was negligible. To our knowledge, this is one of the few studies reporting this high presence of *Cyanobacteria* in raw milk samples, probably because of the *Prototheca* spp. presence in the PRTH raw milk samples. *Cyanobacteria* have been identified in cow milk samples, but at low levels (Ganda et al., 2016; Taponen et al., 2019). Microalgae, such as *Prototheca* spp., are conventionally thought to exhibit characteristics of both eukaryotic and prokaryotic cells (Mur et al., 1999), and the DNA sequence data suggest that algal phylogeny encompasses both eukaryotic and prokaryotic major phyla including in prokaryotic *Cyanobacteria* and *Prochlorobacteria*. In a previous study, Miura et al. (2019) reported a higher prevalence of *Calothrix desertica* (a cyanobacterium) in milk from cows with protothecal mastitis than in milk from healthy cows. *Chloroflexi* was also detected in the milk samples, which was consistent with previous findings (Verdier-Metz et al., 2012). This phylum includes bacteria with diverse metabolic traits, such as aerobic thermophiles that utilize oxygen for growth and anoxygenic phototrophs that perform photosynthesis using light (Ward et al., 2018). However, their specific role in the milk microbiota remains unclear. Regarding the fecal microbiota, the samples shared most of the phyla that were already identified in the milk samples, except for the *Elusimicrobiota* phylum that was present only in fecal samples. The presence of phyla in milk samples that are also found in the fecal microbial population suggest that the fecal microbiota may inform the milk population, providing support for the hypothesis of the existence of an entero-mammary pathway. It was indeed demonstrated that gut bacteria could reach the mammary gland by endogenous entero-mammary pathway (Addis et al., 2016) or through contamination by herd feces (Doyle et al., 2017). The entero-mammary pathway may have implications for understanding how factors like diet, health status, and antibiotic use can influence both the gut and milk microbiotas, potentially affecting milk quality and safety. as well as the development of diseases such as mastitis. *Bacteroidetes* showed the highest abundance in the fecal samples, and it was found to be one order of magnitude higher (in the range of 42%–43%) than in milk (in the range of 2.8%–5.3%) samples. By contrast, *Actinobacteria* showed a lower abundance in fecal (in the range of 0.69%–1.1%) than in milk samples (in the range of 11%–39%). The *Bacteroidetes* presence in the gastrointestinal tract of dairy cows has been correlated with many functions, including degradation of carbohydrates, such as complex plant cell walls, and production of butyrate (Thomas et al., 2011; Miguel et al., 2019). Phylum *Chloroflexi* was only present in milk microbiota, but *Fibrobacteres* were present in both milk and fecal microbiota. *Fibrobacteres* has been previously identified as a core rumen bacterial taxon in dairy cows and linked to a higher forage-to-concentrate ratio (Xue et al., 2018).

The composition of the most abundant taxa in the milk and fecal microbiome are shown in Figures 1A and 1B, respectively. In HLTH milk samples, the most abundant taxa were *Clostridia* (23.5%), *Corynebacterium* (15.1%), *Intrasporangiceae* (7.0%), and *Arthrobacter* (8.4%). In contrast, in samples of animals with subclinical mastitis, the most abundant genera were *Corynebacterium* (10.1% and 6.3% in STRP and PRTH groups, respectively), and *Clostridia* (19.4% and 29.8% in STRP and PRTH groups, respectively). In addition, *Intrasporangiceae* and *Arthrobacter* taxa present in the HLTH group were replaced by *Streptococcus* (28%), in the STRP group and by *Cyanobacteria* in the PRTH group (17.3%). *Corynebacterium* and *Clostridia* taxa have recently been frequently associated with bovine milk core microbiota (Rodrigues et al., 2017; Oikonomou et al., 2020) as found in the current study. In other studies, the milk bacterial microbiome of cows with increased SCC was dominated by *Streptococcus* (Oikonomou et al., 2014; Angelopoulou et al., 2019).

Milk and fecal populations shared many families belonging to the *Clostridia* class (i.e., *Clostridiaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae*; Figure 1B), which were present in higher levels in fecal microbiota (ranging from 35.59% to 37.56%) than in the milk microbiota (ranging from 19.35% to 29.75%; Figures 1A and 1B). All these families of obligate anaerobes have been previously isolated in beef cattle with increased residual-feed intake (Welch et al., 2021). The common presence of bacterial genera in milk and fecal samples is, again, in agreement with the entero-mammary pathway, as explained above. The dominant genera identified in milk samples collected in the present study included *Corynebacterium*, *Staphylococcus*, and *Streptococcus* (Oikonomou et al., 2014; Rodrigues et al., 2017; Derakhshani et al., 2018). *Corynebacterium* was identified as the second most abundant genus in milk samples and is frequently detected within cow's milk and can assist in the development of cheese flavor and aroma (Duthoit et al., 2003; Quigley et al., 2013; Oikonomou et al., 2014). *Streptococcus* has been identified mainly in STRP milk samples, and although many of these species are considered pathogenic, some are frequently isolated in milk and used as starter cultures in the manufacture of dairy products (Randazzo et al., 2002; Quigley et al., 2013; Oikonomou et al., 2014). In particular, in dairy fermentations, *Streptococcus thermophilus* is widely used mixed with lactobacilli as a starter culture because of its rapid acidification capacity in milk, a technological feature of considerable importance in ensuring successful milk processing, fermented food, and improving food safety by preventing the development of pathogenic bacteria (Grizon et al., 2023; Zhao et al., 2023).

Correlations between the milk and fecal bacterial communities are presented in Supplemental Table S2 (see Notes). The only moderate correlation ($r = 0.51$, $P = 0.1088$) was between *Macrococcus caseolyticus* in milk and *Bifidobacteriaceae* in feces, suggesting no evident relationships between gut and mammary microbial communities in our population.

Bacterial Community Diversity in Milk and Feces Samples

Alpha diversity indices (observed ASV number, Shannon diversity, and evenness diversity) were calculated for each of the experimental groups using health status (HLTH, STRP, and PRTH) as criterion. Significant differences ($P < 0.05$) in observed ASV number and Shannon and evenness indices were detected among milk bacterial microbiomes from animals with different health statuses (Table 3) when tested by Kruskal–Wallis tests. Indeed, HLTH animals exhibited higher microbial richness compared with those with subclinical mastitis (STRP and PRTH groups). Several publications described the milk samples from animals with mastitis characterized by lower richness (observed ASV number) and diversity (Shannon and evenness) indices than healthy animals' samples (Oikonomou et al., 2014; Falentin et al., 2016; Derakhshani et al., 2018). The decrease in α diversity has been reported as a clear indication of dysbiosis of the mammary gland bacterial microbiome (Parente et al., 2020): our data corroborate these findings because the averaged values of the observed ASV and the Shannon and evenness diversity indices showed significantly higher richness values in HLTH animals.

Beta diversity analysis was employed in the construction of 2 dendrograms using both weighted and unweighted pair group methods based on unique fraction (**UniFrac**) distance metrics, which quantified overall taxonomic composition similarities between the different groups of samples. Two distance matrixes were created based on weighted and unweighted UniFrac indexes, which were used to calculate distances between pairs of samples, representing how closely related samples are. The PCoA based on weighted (Figure 2A) and unweighted (Figure 2B) UniFrac distance matrixes showed similar results. The combination of the first 3 axes explained 75.84% and 22.66% of the variance based on the weighted and unweighted distance matrix, respectively. Samples were colored according to health status. It is worth noting that with the use of the unweighted UniFrac distance matrix PCoA it was possible to appreciate the samples clustering according to cow health status. Looking at the visualization of the unweighted UniFrac distance matrix (Figure 2B), the STRP and PRTH samples were more closely grouped, while most of HLTH samples were spread more widely across the PCoA second component. In the weighted distance matrix PCoA

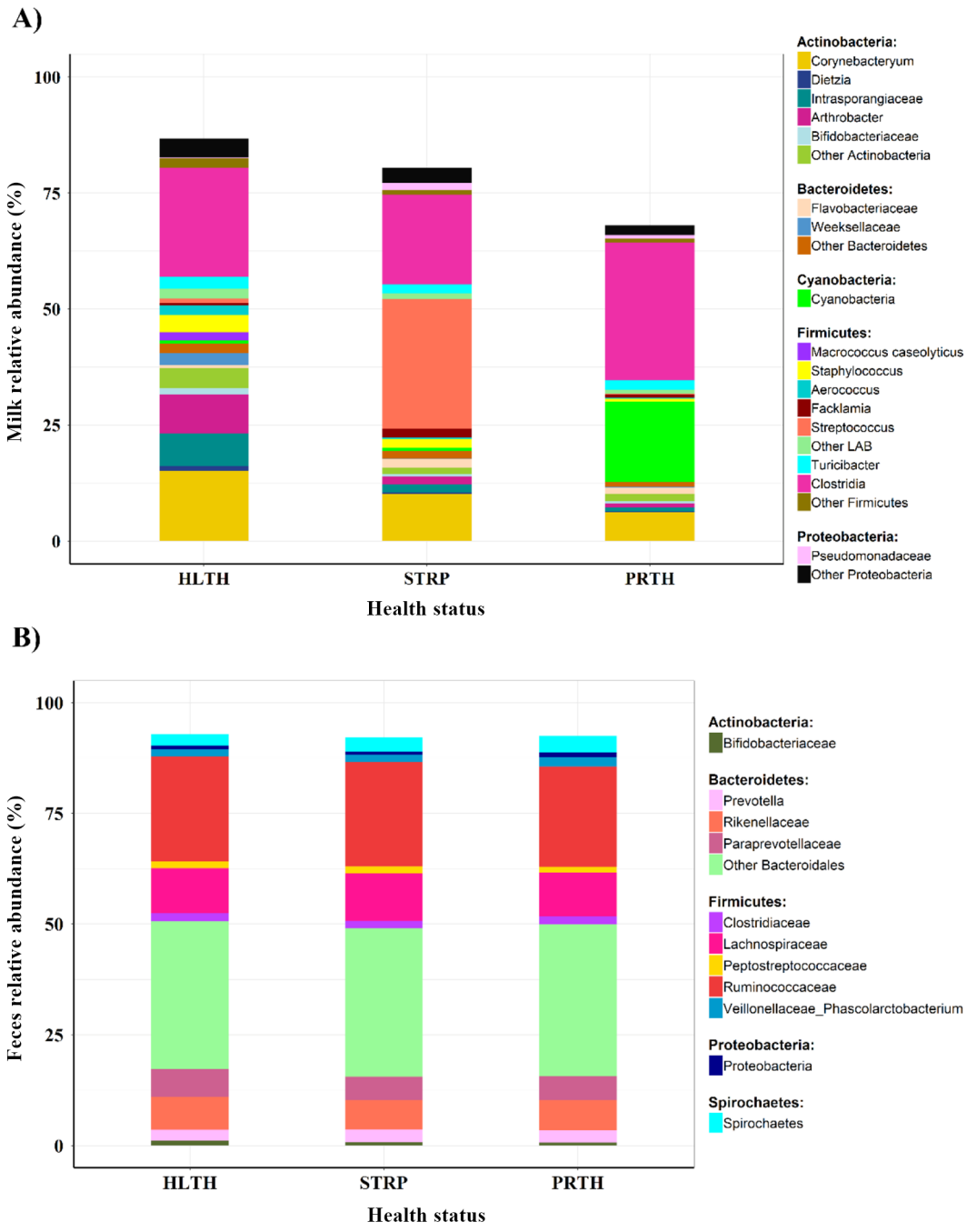

Figure 1. Relative abundances (%) of bacterial taxa of the major phyla identified by MiSeq Illumina in raw milk (A) and feces (B) samples collected from individual cows. Health status: HLTH = healthy dairy cows; STRP = cows naturally infected with *Streptococcus agalactiae*; PRTH = cows naturally infected with *Prototheca* spp. Only taxa with relative abundances >0.5% were reported. The bacterial taxa were grouped by phyla (bold in legend). LAB = lactic acid bacteria.

there was no clear grouping based on health status, and the samples were indistinctly spread in the 3 components apart from 3 PRTH samples. Because the unweighted UniFrac distance accounts for the presence or absence of ASV, whereas the weighted UniFrac accounts for abundance too, the results of these analyses suggest that the microbial presence rather than the individual microbial abundance drives the distance between healthy (HLTH) and not healthy (STRP and PRTH) samples. The PERMANOVA analysis revealed significant (P-value) microbial composition differences between HLTH and STRP milk samples (unweighted UniFrac pseudo-F = 2.14, P

Table 3. Comparison of α diversity indices (observed ASV, Shannon index, and evenness; ±SE) among milk bacterial communities from healthy (HLTH) and subclinical mastitis–affected (STRP and PRTH) cows

	Health status		
Item	HLTH	STRP	PRTH
Observed ASV	255 ± 74^{A}	195 ± 84^{B}	128 ± 77^{C}
Shannon	7.092 ± 0.776^{A}	5.931 ± 1.42^{B}	4.727 ± 1.78^{C}
Evenness	0.895 ± 0.058^{A}	0.771 ± 0.133^{B}	0.683 ± 0.196^{C}

$^{A–C}$Within a row different uppercase letters indicate significant differences ($P < 0.05$) in mean values (Kruskal–Wallis test).

= 0.001; weighted UniFrac pseudo-F = 5.40, P = 0.002), and between HLTH and PRTH milk samples (unweighted UniFrac pseudo-F = 1.91, P = 0.003; weighted UniFrac pseudo-F = 4.17, P = 0.017), confirming again that the health status had a high and significant effect on the milk microbial community. Regarding microbial composition differences between PRTH and STRP milk samples, the PERMANOVA analysis revealed no significance according to the unweighted UniFrac (P = 0.1875); and a significant difference according to the weighted UniFrac (pseudo-F = 4.767, P = 0.036). As we have reported here, because the weighted UniFrac distance accounts for both presence or absence of ASV and abundance (the unweighted UniFrac accounts only for presence or absence of ASV), the results of these analyses suggest that the abundance rather than microbial presence drives the distance between STRP and PRTH samples. In contrast to milk, fecal samples revealed no differences ($P > 0.05$) for any calculated diversity metrics (data not shown). Thus, although there were differences in milk microbiota based on the infection status of the dairy cow, this was not reflected in the fecal samples of the dairy cattle. Past research found a significant shift in fecal microbiota associated with inflammation during mastitis, and a decrease in diversity of fecal microbiota in cows with subclinical mastitis when compared with healthy animals (Wang et al., 2022). Although an association of health status with fecal microbiota is not present in the current study, further investigation on the fecal microbial diversity on a larger population will be fundamental to assessing differences in the health status of animals.

Differentially Abundant Taxa in the Investigated Matrixes

The milk differentially abundant taxa obtained with DESeq2 are displayed in Figure 3 (HLTH vs. infected animals), Figure 4 (HLTH vs. STRP) and Figure 5 (HLTH vs. PRTH).

When comparing microbiota composition in mid- and late-lactating cows the only notable result emerged for *Cyanobacteria*, which exhibited a reduced relative abundance in late-lactation cows (DIM > 200 d). To our knowledge, no previous studies have specifically investigated the dynamics of *Cyanobacteria* in milk across lactation stages. However, Marcos et al. (2024) observed a comparable trend in the ruminal microbiota, suggesting a potential systemic modulation of this phylum during lactation. However, it is worth noting that DIM and parity adjustment did not substantially overturn the differentially abundant taxa observed in the first analysis with no adjustment factors (Supplemental Table S3, see Notes); therefore, we decided to focus only on the differential analysis without the inclusion of these factors in the analyses. This finding might be the result of the exclusion from the current study of periparturient and primiparous cows to minimize any possible source of variation that could have influenced the outcome of our findings.

When comparing HLTH versus infected animals (both STRP and PRTH in Figure 3) we observed a total of 6 underrepresented and 5 overrepresented taxa. Among these, *Macrococcus caseolyticus* (Log_2FC = −4.5491), *Cyanobacteria* (Log_2FC = 8.090), and *Streptococcus* (Log_2FC = 5.213) were the most significant taxa.

In the HLTH versus STRP comparison (Figure 4), *Streptococcus* was the most overrepresented (Log_2FC = 7.332) taxa observed, and *Macrococcus caseolyticus* was the most underrepresented one (Log_2FC = −5.085) in the STRP-infected samples. Intramammary infections can result in a significant alterations of the udder microbiota, characterized by a marked reduction in bacterial diversity in infected animals compared with healthy ones (Parente et al., 2020). In particular, it has been observed that bacteria such as *S. aureus* establish negative interactions with other bacteria of the phylum *Bacteroidetes* and the genus *Aereococcus* (Park et al., 2022), in agreement with our data. Regarding *Macrococcus caseolyticus*, a commensal bacterium that can become an opportunistic pathogen (Carroll et al., 2023), our hypothesis is that, like other bacteria, it is outcompeted by *S. aureus.*

Finally, when we investigated the differentially abundant taxa in the HLTH versus PRTH-infected animals (Figure 5), we observed 4 significant underrepresented

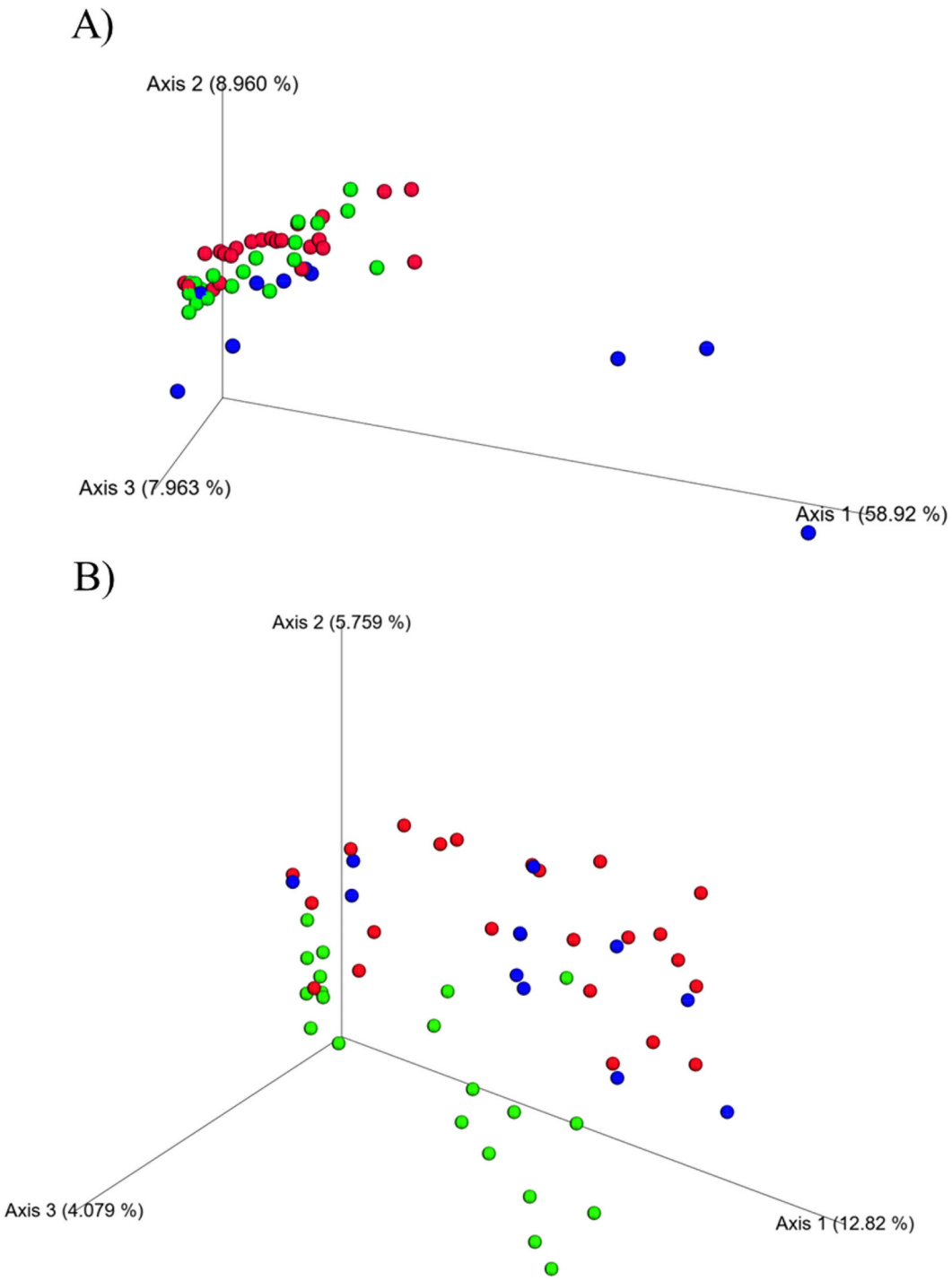

Figure 2. Beta diversity microbiota changes based on health status of sampled cow. Two principal coordinate analysis (PCoA) ordination using (A) weighted UniFrac and (B) unweighted UniFrac distances were performed to visualize microbial community ASV differences across the 3 health statuses (green = healthy dairy cows; red = cows naturally infected with *Streptococcus agalactiae*; blue = cows naturally infected with *Prototheca* spp.).

taxa and 5 overrepresented taxa, with *Macrococcous* and *Cyanobacteria* being the most significant ones ($Log_2FC = -3.798$ and $Log_2FC = 8.878$, respectively). *Cyanobacteria* and *Streptococcus*, which were the most represented microorganisms in the infected group (regardless of the pathogen) were found also to be the main etiological

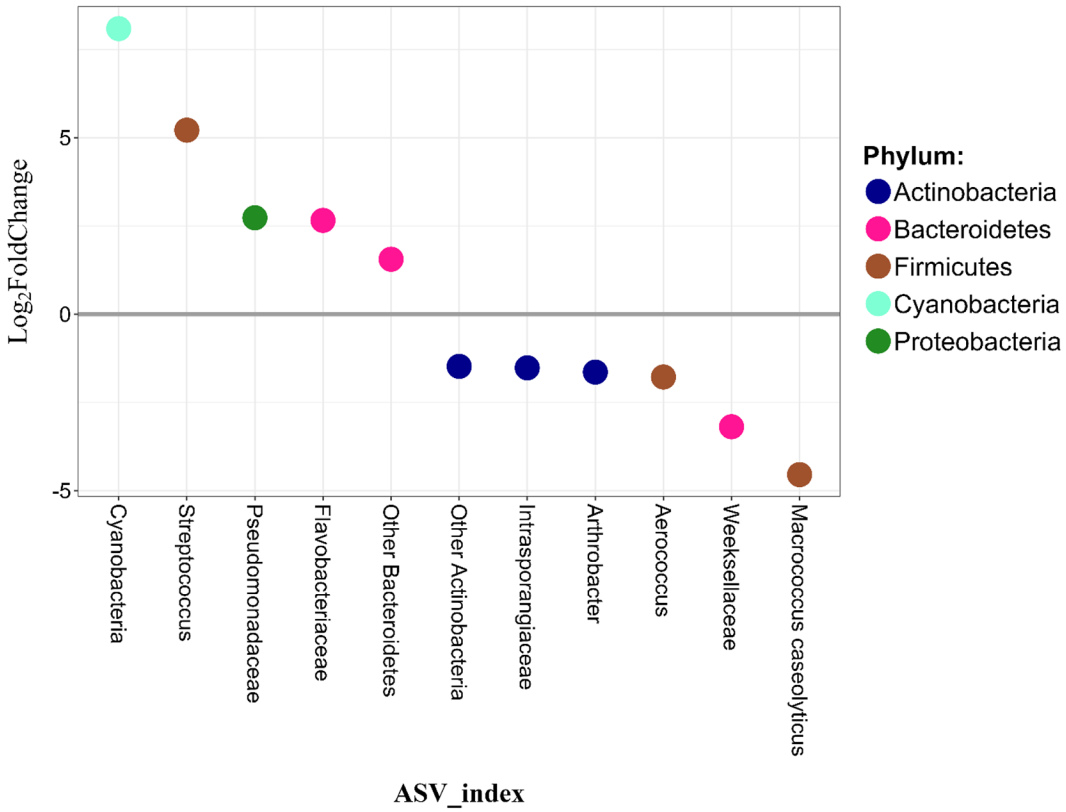

Figure 3. Log_2FoldChanges (DESeq2) in milk samples for unhealthy (STRP + PRTH) versus healthy cows. STRP = cows naturally infected with *Streptococcus agalactiae*; PRTH = cows naturally infected with *Prototheca* spp.

agents for the PRTH and STRP groups. Among Cyanobacteria, *Prototheca* is recognized as an emerging threat for animals, especially considering the lack of effective treatments, which often results in the premature culling of affected animals (Bisutti et al., 2023). Concerning *Strep. agalactiae*, its contribution as one of the main agents of mastitis has been widely recognized in the literature (Kabelitz et al., 2021; Tamba et al., 2022; Bonsaglia et al., 2023). The *Pseudomonadaceae* family was always overrepresented in the infected groups, even if the relative abundance of *Proteobacteria* was not the highest. The *Pseudomonadaceae* family was found to be slightly positively correlated with the increase of SCC (Cremonesi et al., 2018). Although our taxonomic classification does not reach the species level, it is not surprising to find the family *Pseudomonadaceae* among the overrepresented in the infected group because species such as *Pseudomonas aeruginosa* are known opportunistic pathogens (Schauer et al., 2021). Like *Pseudomonadaceae*, the *Flavobacteriaceae* family was also found to be overrepresented in all infected groups, and recently a bacterial strain of *Elizabethkingia* spp, identified as a member of *Flavobacteriaceae*, was isolated from an bovine mastitis outbreak (Pan et al., 2020). *Bacteroidetes*, which was overrepresented in the STRP and PRTH pairwise comparisons, is a phylum generally found in abundance in the gastrointestinal tract. However, some genera like *Prevotella* or *Bacteroides* were also found in milk by other authors (Pang et al., 2018) and our study confirmed their presence in this matrix. Nevertheless, changes in these genera were never associated with the onset of mastitis before, so further studies are needed to confirm these findings. The *Clostridiaceae* family was found to be overrepresented only in PRTH group. This family is considered to be part of the milk core microbiota (Oikonomou et al., 2014), but it includes some genera, such as *Clostridium perfringens,* that have been associated with mastitis (Abo Elyazeed et al., 2024).

The *Weeksellaceae* family and *Aerococcus* spp. were both among the most underrepresented taxa. For the former, despite not being a pathogen associated with mastitis, a positive association with increased SCC was found, although this was not associated with the presence of infection (Nguyen et al., 2020). Instead, for the latter, although there is evidence linking the presence of *Aerococcus* spp. in milk with a SCC content <200,000

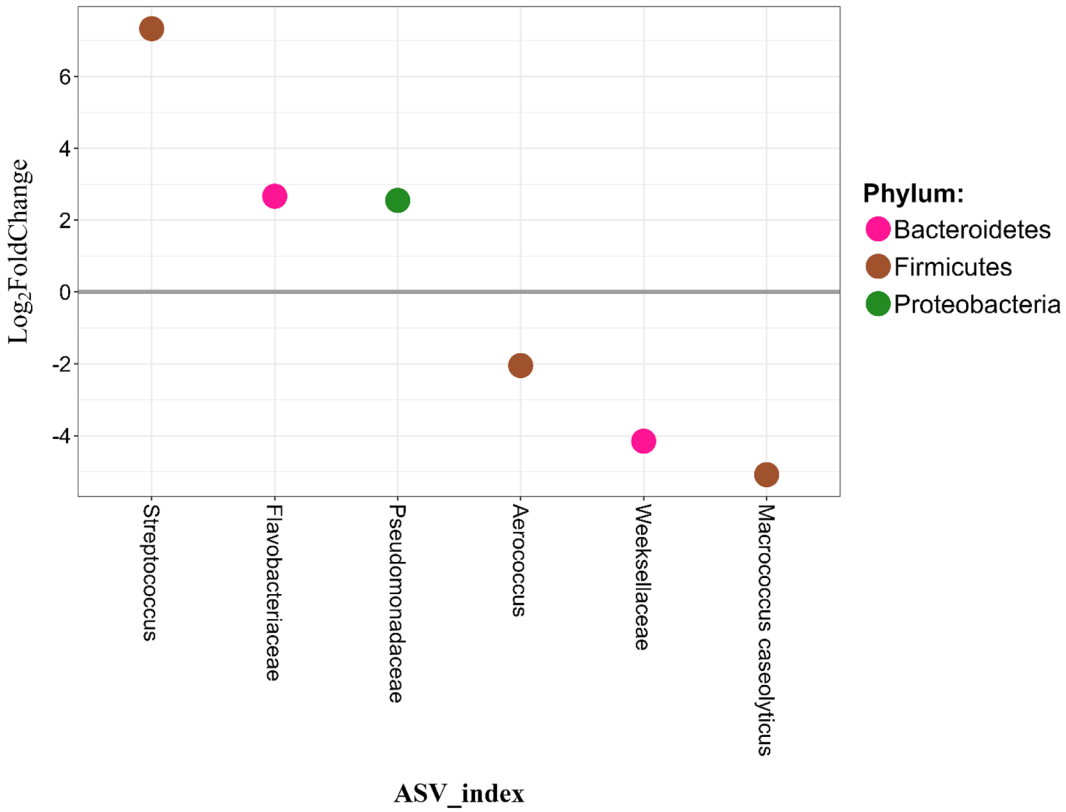

Figure 4. Log_2FoldChanges (DESeq2) in milk samples for STRP-infected cows versus healthy cows. STRP = cows naturally infected with *Streptococcus agalactiae*.

cells/mL (Park et al., 2022), there are also species (e.g., *Aerococcus viridians*) that are considered pathogens causing mastitis (Sun et al., 2017).

Minor underrepresented taxa like *Arthrobacter* spp. are normally part of the microbial communities of raw milk and are rarely found predominant in this matrix. The competition with other microorganisms, or pH changes, limit the growth of this bacteria taxa in milk (Sutthiwong et al., 2023), explaining its underrepresentation in the present study in samples from animals having IMI. Another underrepresented taxa found in our research was the I*ntrasporangiaceae* family, which Du et al. (2020) found in teat skin and bedding samples of different Chinese herds (Du et al., 2020). Considering the high relative abundance observed by these authors in bedding samples (20.6%), we can speculate that these bacteria might originate from the environment, but their role in milk remains unclear. Further studies are needed to assess whether this family plays a specific role in udder health.

Interestingly, no differentially abundant taxa were observed when comparing STRP with PRTH groups, which is in agreement with the results found by PERMANOVA that revealed significant differences according only to abundance (weighted UniFrac) and not the ASV presence or absence (unweighted UniFrac). This finding was in line with previous results by Bisutti et al. (2023) that evaluated changes in gene expression in the same cohort of animals and reported no differentially enriched pathways when comparing the 2 types of infection.

Although this study evidenced changes in milk microbiota associated with subclinical mastitis, we did not observe significant differences in the abundance of taxa in the fecal microbiome of infected animals with respect to the healthy ones, as also confirmed by the correlation analysis. Similarly, Zhu et al. (2024) reported significantly lower microbial community richness and diversity in the feces of cows with clinical mastitis than in the fecal samples of healthy cows. Zhu et al. (2024) reported Shannon index values significantly lower only in animals with clinical infection but not in those with subclinical mastitis, suggesting a stronger systemic effect when the mastitis is in its clinical form. Given the subclinical form of infection, we hypothesized that the immune response was primarily localized within the udder, which may explain why the gut microbiota remained unaffected. Furthermore, the absence of systemic signs in cows with subclinical mastitis reinforces the idea that physiological

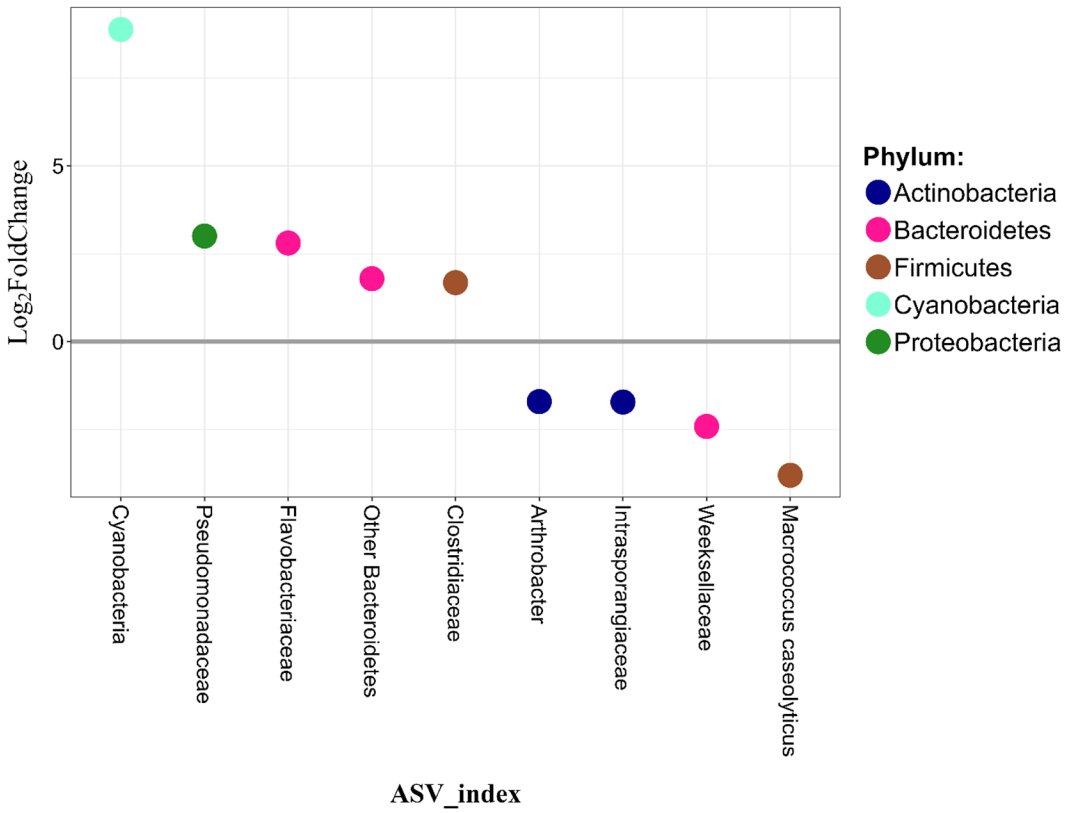

Figure 5. Log_2FoldChanges (DESeq2) in milk samples for PRTH-infected cows versus healthy cows. PRTH = cows naturally infected with *Prototheca* spp.

host homeostasis, including gut microbial composition, remains largely undisturbed. These findings highlight the complexity of subclinical infections and underscore the need for further investigation of the interaction between localized immune response and systemic microbial homeostasis in dairy cows.

Our study has some limitations that should be considered. One potential limitation is the use of composite milk samples from the 4 quarters, which may result in a dilution effect if only one quarter was affected and the others remained healthy. However, the mixed sample was chosen to provide a sampling more similar to the one done routinely by veterinarians. Future research should consider also individual quarter milk sampling to better capture local microbial changes associated with the mastitis. In addition, the observational and cross-sectional design of the study provides only a snapshot of microbial diversity at a single time point, meaning it does not account for changes in the microbiota over time, such as during disease progression. Moreover, despite efforts to control for potential sources of variation associated with parity and lactation stage, samples testing positive for *Prototheca* spp. exhibited the highest degree of variability. Nevertheless, considering the emerging status of *Prototheca* spp. as a pathogen and the commercial nature of the farm setting, all positive samples were retained for analysis to maximize the opportunity for investigation. Finally, the study focused on taxonomic profiling using 16S rRNA metabarcoding, which provides valuable insights into microbial diversity but lacks the comprehensive resolution offered by metagenomic sequencing. Although metabarcoding is faster, more cost-effective, and capable of detecting shifts in microbial composition that may be associated with infection even in the absence of visible clinical symptoms, metagenomic sequencing, although more expensive, can offer deeper insights into the functional roles of specific microorganisms in disease progression.

CONCLUSIONS

The data of the present study contribute to a better understanding of the mechanism of mastitis in lactating dairy cows. Despite the absence of clinical signs of disease, we highlighted that milk microbiota profiles diverged among healthy and subclinical mastitis–affected cows. Potential proinflammatory bacteria (*Streptococcus* spp. and *Cyanobacteria*) were enriched in the milk of

cows with subclinical infection from *Strep. agalactiae* or *Prototheca* spp. respectively, whereas *Actinobacteria* accounted for a decrease in the community of milk microbiota. Moreover, milk samples from cows with subclinical mastitis showed a decreased α diversity of the microbial population. In addition, our results showed that the fecal and milk microbiotas of dairy cows behaved differently upon infection, likely due to the subclinical characteristic of the infection. Further research is required to better investigate the biological pathways connecting the fecal microbiome to the resident milk microbiota. Moreover, it will be pivotal to integrate these results with host molecular and phenotypic information (i.e., gene expression, metabolome) to better comprehend the host-microbiome relationships involved in the pathogenesis of mastitis.

NOTES

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Nonstandard abbreviations used: ASV = amplicon sequence variant; CBA = Columbia blood agar; CBA-Chl = CBA supplemented with 5% defibrinated sheep blood and 0.7% chloramphenicol; FC = fold change; HLTH = healthy cows (negative milk bacteriological examination in all mammary quarters); IZSVe = Istituto Zooprofilattico Sperimentale delle Venezie; LAB = lactic acid bacteria; PCA = plate count agar; PCoA = principal coordinate analysis; PE = paired end; PRTH = cows naturally infected with *Prototheca* spp.; STRP = cows naturally infected with *Strep. agalactiae*; T0 = initial bacterial screening of cows; T1 = second bacteriological screening 2 wk after T0; TBC = total bacteria count; UniFrac = unique fraction; VRBA = violet red bile agar; WC= Wilkins Chalgren.

REFERENCES

Abo Elyazeed, H., M. Elhariri, N. E. Eldeen, D. A. Aziz, and R. Elhelw. 2024. nGenetic diversity and phylogenetic relationships of *Clostridium perfringens* strains isolated from mastitis and enteritis in Egyptian dairy farms. BMC Microbiol. 24:157. https://doi.org/10.1186/s12866-024-03260-1.

Addis, M. F., A. Tanca, S. Uzzau, G. Oikonomou, R. C. Bicalho, and P. Moroni. 2016. The bovine milk microbiota: Insights and perspectives from -omics studies. Mol. Biosyst. 12:2359–2372. https://doi.org/10.1039/C6MB00217J.

Adkins, P. R. F. 2017. Laboratory Handbook on Bovine Mastitis. 3rd ed. J. R. Middleton, L. K. Fox, G. Pighetti, and C. Petersson-Wolfe, ed. National Mastitis Council, New Prague, MN.

Andrews, T., D. A. Neher, T. R. Weicht, and J. W. Barlow. 2019. Mammary microbiome of lactating organic dairy cows varies by time, tissue site, and infection status. PLoS One 14:e0225001. https://doi.org/10.1371/journal.pone.0225001.

Angelopoulou, A., R. Holohan, M. C. Rea, A. K. Warda, C. Hill, and R. P. Ross. 2019. Bovine mastitis is a polymicrobial disease requiring a polydiagnostic approach. Int. Dairy J. 99:104539. https://doi.org/10.1016/j.idairyj.2019.104539.

Baker, G. C., J. J. Smith, and D. A. Cowan. 2003. Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods 55:541–555. https://doi.org/10.1016/j.mimet.2003.08.009.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. R. Stat. Soc. Series B Stat. Methodol. 57:289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

Bhatt, V. D., V. B. Ahir, P. G. Koringa, S. J. Jakhesara, D. N. Rank, D. S. Nauriyal, A. P. Kunjadia, and C. G. Joshi. 2012. Milk microbiome signatures of subclinical mastitis-affected cattle analysed by shotgun sequencing. J. Appl. Microbiol. 112:639–650. https://doi.org/10.1111/j.1365-2672.2012.05244.x.

Bisutti, V., N. Mach, D. Giannuzzi, A. Vanzin, E. Capra, R. Negrini, M. E. Gelain, A. Cecchinato, P. Ajmone-Marsan, and S. Pegolo. 2023. Transcriptome-wide mapping of milk somatic cells upon subclinical mastitis infection in dairy cattle. J. Anim. Sci. Biotechnol. 14:93. https://doi.org/10.1186/s40104-023-00890-9.

Bobbo, T., P. L. Ruegg, G. Stocco, E. Fiore, M. Gianesella, M. Morgante, D. Pasotto, G. Bittante, and A. Cecchinato. 2017. Associations between pathogen-specific cases of subclinical mastitis and milk yield, quality, protein composition, and cheese-making traits in dairy cows. J. Dairy Sci. 100:4868–4883. https://doi.org/10.3168/jds.2016-12353.

Bonsaglia, E. C. R., M. S. Gomes, I. F. Canisso, Z. Zhou, S. F. Lima, V. L. M. Rall, G. Oikonomou, R. C. Bicalho, and F. S. Lima. 2017. Milk microbiome and bacterial load following dry cow therapy without antibiotics in dairy cows with healthy mammary gland. Sci. Rep. 7:8067. https://doi.org/10.1038/s41598-017-08790-5.

Bonsaglia, E. C. R., R. S. Rossi, G. Latosinski, B. F. Rossi, F. C. Campos, A. F. Junior, J. C. F. Pantoja, and V. L. M. Rall. 2023. Relationship between biofilm production and high somatic cell count in *Streptococcus agalactiae* isolated from milk of cows with subclinical mastitis. Pathogens 12:311. https://doi.org/10.3390/pathogens12020311.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13:581–583. https://doi.org/10.1038/nmeth.3869.

Carroll, L. M., R. Pierneef, T. Mafuna, K. Magwedere, and I. Matle. 2023. Genus-wide genomic characterization of *Macrococcus*: Insights into evolution, population structure, and functional potential. Front. Microbiol. 14:1181376. https://doi.org/10.3389/fmicb.2023.1181376.

Claesson, M. J., Q. Wang, O. O'Sullivan, R. Greene-Diniz, J. R. Cole, R. P. Ross, and P. W. O'Toole. 2010. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res. 38:e200. https://doi.org/10.1093/nar/gkq873.

Cremonesi, P., C. Ceccarani, G. Curone, M. Severgnini, C. Pollera, V. Bronzo, F. Riva, M. F. Addis, J. Filipe, M. Amadori, E. Trevisi, D.

Vigo, P. Moroni, and B. Castiglioni. 2018. Milk microbiome diversity and bacterial group prevalence in a comparison between healthy Holstein Friesian and Rendena cows. PLoS One 13:e0205054. https://doi.org/10.1371/journal.pone.0205054.

Derakhshani, H., K. B. Fehr, S. Sepehri, D. Francoz, J. De Buck, H. W. Barkema, J. C. Plaizier, and E. Khafipour. 2018. *Invited review:* Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility. J. Dairy Sci. 101:10605–10625. https://doi.org/10.3168/jds.2018-14860.

Doyle, C. J., D. Gleeson, P. W. O'Toole, and P. D. Cotter. 2017. Impacts of seasonal housing and teat preparation on raw milk microbiota: A high-throughput sequencing study. Appl. Environ. Microbiol. 83:e02694-16. https://doi.org/10.1128/AEM.02694-16.

Du, B., L. Meng, H. Liu, N. Zheng, Y. Zhang, X. Guo, S. Zhao, F. Li, and J. Wang. 2020. Impacts of milking and housing environment on milk microbiota. Animals (Basel) 10:2339. https://doi.org/10.3390/ani10122339.

Duthoit, F., J. J. Godon, and M. C. Montel. 2003. Bacterial community dynamics during production of registered designation of origin Salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. Appl. Environ. Microbiol. 69:3840–3848. https://doi.org/10.1128/AEM.69.7.3840-3848.2003.

Falentin, H., L. Rault, A. Nicolas, D. S. Bouchard, J. Lassalas, P. Lamberton, J. M. Aubry, P. G. Marnet, Y. Le Loir, and S. Even. 2016. Bovine teat microbiome analysis revealed reduced alpha diversity and significant changes in taxonomic profiles in quarters with a history of mastitis. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.00480.

Ganda, E. K., R. S. Bisinotto, S. F. Lima, K. Kronauer, D. H. Decter, G. Oikonomou, Y. H. Schukken, and R. C. Bicalho. 2016. Longitudinal metagenomic profiling of bovine milk to assess the impact of intramammary treatment using a third-generation cephalosporin. Sci. Rep. 6:37565. https://doi.org/10.1038/srep37565.

Grizon, A., S. Theil, C. Callon, P. Gerber, S. Helinck, E. Dugat-Bony, P. Bonnarme, and C. Chassard. 2023. Genetic and technological diversity of *Streptococcus thermophilus* isolated from the Saint-Nectaire PDO cheese-producing area. Front. Microbiol. 14:1245510. https://doi.org/10.3389/fmicb.2023.1245510.

Hoque, M. N., A. Istiaq, R. A. Clement, M. Sultana, K. A. Crandall, A. Z. Siddiki, and M. A. Hossain. 2019. Metagenomic deep sequencing reveals association of microbiome signature with functional biases in bovine mastitis. Sci. Rep. 9:13536. https://doi.org/10.1038/s41598-019-49468-4.

Hu, X., Z. He, C. Zhao, Y. He, M. Qiu, K. Xiang, N. Zhang, and Y. Fu. 2024. Gut/rumen-mammary gland axis in mastitis: Gut/rumen microbiota–mediated "gastroenterogenic mastitis". J. Adv. Res. 55:159–171. https://doi.org/10.1016/j.jare.2023.02.009.

Hu, X., S. Li, R. Mu, J. Guo, C. Zhao, Y. Cao, N. Zhang, and Y. Fu. 2022. The rumen microbiota contributes to the development of mastitis in dairy cows. Microbiol. Spectr. 10:e02512-21. https://doi.org/10.1128/spectrum.02512-21.

International Dairy Federation (IDF). 1997. Recommendations for presentation of mastitis-related data. Int. Dairy J. 7:II. https://doi.org/10.1016/S0958-6946(97)87641-8.

Jagielski, T., H. Krukowski, M. Bochniarz, T. Piech, K. Roeske, Z. Bakuła, Ł. Wlazło, and P. Woch. 2019. Prevalence of *Prototheca* spp. on dairy farms in Poland—A cross-country study. Microb. Biotechnol. 12:556–566. https://doi.org/10.1111/1751-7915.13394.

Jiang, C., X. Hou, X. Gao, P. Liu, X. Guo, G. Hu, Q. Li, C. Huang, G. Li, W. Fang, W. Mai, C. Wu, Z. Xu, and P. Liu. 2023. The 16S rDNA high-throughput sequencing correlation analysis of milk and gut microbial communities in mastitis Holstein cows. BMC Microbiol. 23:180. https://doi.org/10.1186/s12866-023-02925-7.

Kabelitz, T., E. Aubry, K. van Vorst, T. Amon, and M. Fulde. 2021. The role of *Streptococcus* spp. in bovine mastitis. Microorganisms 9:1497. https://doi.org/10.3390/microorganisms9071497.

Katoh, K., and D. M. Standley. 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol. Biol. Evol. 30:772–780. https://doi.org/10.1093/molbev/mst010.

Kim, M., M. Morrison, and Z. Yu. 2011. Status of the phylogenetic diversity census of ruminal microbiomes. FEMS Microbiol. Ecol. 76:49–63. https://doi.org/10.1111/j.1574-6941.2010.01029.x.

Liu, L., P. Wu, A. Guo, Y. Yang, F. Chen, and Q. Zhang. 2023. Research progress on the regulation of production traits by gastrointestinal microbiota in dairy cows. Front. Vet. Sci. 10:1206346. https://doi.org/10.3389/fvets.2023.1206346.

Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15:550. https://doi.org/10.1186/s13059-014-0550-8.

Mandal, S., W. Van Treuren, R. A. White, M. Eggesbø, R. Knight, and S. D. Peddada. 2015. Analysis of composition of microbiomes: A novel method for studying microbial composition. Microb. Ecol. Health Dis. 26:27663. https://doi.org/10.3402/mehd.v26.27663.

Marcos, C. N., M. D. Carro, M. Gutiérrez-Rivas, R. Atxaerandio, I. Goiri, A. García-Rodríguez, and O. González-Recio. 2024. Ruminal microbiome changes across lactation in primiparous Holstein cows with varying methane intensity: Heritability assessment. J. Dairy Sci. 107:7064–7078. https://doi.org/10.3168/jds.2023-24552.

Marques, S., E. Silva, J. Carvalheira, and G. Thompson. 2010. Phenotypic characterization of mastitic *Prototheca* spp. isolates. Res. Vet. Sci. 89:5–9. https://doi.org/10.1016/j.rvsc.2009.12.017.

Marques, S., E. Silva, C. Kraft, J. Carvalheira, A. Videira, V. A. R. Huss, and G. Thompson. 2008. Bovine mastitis associated with *Prototheca blaschkeae*. J. Clin. Microbiol. 46:1941–1945. https://doi.org/10.1128/JCM.00323-08.

Martin, P., H. W. Barkema, L. F. Brito, S. G. Narayana, and F. Miglior. 2018. *Symposium review:* Novel strategies to genetically improve mastitis resistance in dairy cattle. J. Dairy Sci. 101:2724–2736. https://doi.org/10.3168/jds.2017-13554.

Martin, N. H., A. Trmcic, T. H. Hsieh, K. J. Boor, and M. Wiedmann. 2016. The evolving role of coliforms as indicators of unhygienic processing conditions in dairy foods. Front. Microbiol. 7:1549. https://doi.org/10.3389/fmicb.2016.01549.

Miguel, M. A., S. S. Lee, L. L. Mamuad, Y. J. Choi, C. D. Jeong, A. Son, K. K. Cho, E. T. Kim, S. B. Kim, and S. S. Lee. 2019. Enhancing butyrate production, ruminal fermentation and microbial population through supplementation with *Clostridium saccharobutylicum*. J. Microbiol. Biotechnol. 29:1083–1095. https://doi.org/10.4014/jmb.1905.05016.

Miura, A., T. Kurumisawa, R. Kano, T. Ito, K. Suzuki, and H. Kamata. 2019. Next-generation sequencing analysis of bacterial flora in bovine protothecal mastitic milk and feces. J. Vet. Med. Sci. 81:1547–1551. https://doi.org/10.1292/jvms.18-0649.

Mur, L. R., O. M. Skulberg, and H. Utkilen. 1999. Cyanobacteria in the environment. Pages 15–40 in Toxic Cyanobacteria Water: A Guide to Their Public Health Consequences, Monitoring, and Management. 1st ed. I. Chorus and J. Bartram, ed. CRC Press, London.

Nguyen, Q. D., T. Tsuruta, and N. Nishino. 2020. Examination of milk microbiota, fecal microbiota, and blood metabolites of Jersey cows in cool and hot seasons. Anim. Sci. J. 91:e13441–11. https://doi.org/10.1111/asj.13441.

Oikonomou, G., M. F. Addis, C. Chassard, M. E. F. Nader-Macias, I. Grant, C. Delbès, C. I. Bogni, Y. Le Loir, and S. Even. 2020. Milk microbiota: What are we exactly talking about? Front. Microbiol. 11:60. https://doi.org/10.3389/fmicb.2020.00060.

Oikonomou, G., M. L. Bicalho, E. Meira, R. E. Rossi, C. Foditsch, V. S. Machado, A. G. V. Teixeira, C. Santisteban, Y. H. Schukken, and R. C. Bicalho. 2014. Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. PLoS One 9:e85904. https://doi.org/10.1371/journal.pone.0085904.

Oikonomou, G., A. G. V. Teixeira, C. Foditsch, M. L. Bicalho, V. S. Machado, and R. C. Bicalho.; 2013. Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium* species with health and growth. PLoS One 8:e63157. https://doi.org/10.1371/journal.pone.0063157.

Oultram, J. W. H., E. K. Ganda, S. C. Boulding, R. C. Bicalho, and G. Oikonomou. 2017. A metataxonomic approach could be considered

for cattle clinical mastitis diagnostics. Front. Vet. Sci. 4:36. https://doi.org/10.3389/fvets.2017.00036.

Pan, Z., Q. Zhou, H. Ma, Q. Gong, S. Wang, H. Yao, J. Ma, and K. Wang. 2020. Identification of a novel bacterial taxon associated with bovine mastitis showing a close evolutionary relationship with *Elizabethkingia* sp. Microbiol. Res. 236:126443. https://doi.org/10.1016/j.micres.2020.126443.

Pang, M., X. Xie, H. Bao, L. Sun, T. He, H. Zhao, Y. Zhou, L. Zhang, H. Zhang, R. Wei, K. Xie, and R. Wang. 2018. Insights into the bovine milk microbiota in dairy farms with different incidence rates of subclinical mastitis. Front. Microbiol. 9:2379. https://doi.org/10.3389/fmicb.2018.02379.

Parente, E., A. Ricciardi, and T. Zotta. 2020. The microbiota of dairy milk: A review. Int. Dairy J. 107:104714. https://doi.org/10.1016/j.idairyj.2020.104714.

Park, S., D. Jung, I. Altshuler, D. Kurban, S. Dufour, and J. Ronholm. 2022. A longitudinal census of the bacterial community in raw milk correlated with *Staphylococcus aureus* clinical mastitis infections in dairy cattle. Anim. Microbiome 4:59. https://doi.org/10.1186/s42523-022-00211-x.

Pegolo, S., D. Giannuzzi, V. Bisutti, R. Tessari, M. E. Gelain, L. Gallo, S. Schiavon, F. Tagliapietra, E. Trevisi, P. Ajmone Marsan, G. Bittante, and A. Cecchinato. 2021. Associations between differential somatic cell count and milk yield, quality, and technological characteristics in Holstein cows. J. Dairy Sci. 104:4822–4836. https://doi.org/10.3168/jds.2020-19084.

Pegolo, S., R. Tessari, V. Bisutti, A. Vanzin, D. Giannuzzi, M. Gianesella, A. Lisuzzo, E. Fiore, A. Barberio, E. Schiavon, E. Trevisi, F. Piccioli Cappelli, L. Gallo, P. Ruegg, R. Negrini, and A. Cecchinato. 2022a. Quarter-level analyses of the associations among subclinical intramammary infection and milk quality, udder health, and cheese-making traits in Holstein cows. J. Dairy Sci. 105:3490–3507. https://doi.org/10.3168/jds.2021-21267.

Pegolo, S., A. Toscano, V. Bisutti, D. Giannuzzi, A. Vanzin, A. Lisuzzo, F. Bonsembiante, M. E. Gelain, and A. Cecchinato. 2022b. *Streptococcus agalactiae* and *Prototheca* spp. induce different mammary gland leukocyte responses in Holstein cows. JDS Commun. 3:270–274. https://doi.org/10.3168/jdsc.2022-0216.

Posit Team. 2025. RStudio: Integrated development environment for R. Posit Software, PBC, Boston, MA. Accessed Dec. 13, 2024. http://www.posit.co/.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2009. Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. Mol. Biol. Evol. 26:1641–1650. https://doi.org/10.1093/molbev/msp077.

Quigley, L., O. O'Sullivan, C. Stanton, T. P. Beresford, R. P. Ross, G. F. Fitzgerald, and P. D. Cotter. 2013. The complex microbiota of raw milk. FEMS Microbiol. Rev. 37:664–698. https://doi.org/10.1111/1574-6976.12030.

Randazzo, C. L., S. Torriani, A. D. L. Akkermans, W. M. De Vos, and E. E. Vaughan. 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Appl. Environ. Microbiol. 68:1882–1892. https://doi.org/10.1128/AEM.68.4.1882-1892.2002.

Revelle, W. 2007. psych: Procedures for psychological, psychometric, and personality research. Accessed Dec. 13, 2024. https://CRAN.R-project.org/package=psych.

Rodrigues, M. X., S. F. Lima, S. G. Canniatti-Brazaca, and R. C. Bicalho. 2017. The microbiome of bulk tank milk: Characterization and associations with somatic cell count and bacterial count. J. Dairy Sci. 100:2536–2552. https://doi.org/10.3168/jds.2016-11540.

Ruegg, P. L. 2017. *A 100-Year Review:* Mastitis detection, management, and prevention. J. Dairy Sci. 100:10381–10397. https://doi.org/10.3168/jds.2017-13023.

Sawant, A. A., S. R. Pillai, and B. M. Jayarao. 2002. Evaluation of five selective media for isolation of catalase-negative gram-positive cocci from bulk tank milk. J. Dairy Sci. 85:1127–1132. https://doi.org/10.3168/jds.S0022-0302(02)74174-X.

Schauer, B., R. Wald, V. Urbantke, I. Loncaric, and M. Baumgartner. 2021. Tracing mastitis pathogens—Epidemiological investigations of a *Pseudomonas aeruginosa* mastitis outbreak in an Austrian dairy herd. Animals (Basel) 11:279. https://doi.org/10.3390/ani11020279.

Secchi, G., N. Amalfitano, I. Carafa, E. Franciosi, L. Gallo, S. Schiavon, E. Sturaro, F. Tagliapietra, and G. Bittante. 2023. Milk metagenomics and cheese-making properties as affected by indoor farming and summer highland grazing. J. Dairy Sci. 106:96–116. https://doi.org/10.3168/jds.2022-22449.

Steinberg, R. S., L. C. Silva e Silva, M. R. de Souza, R. B. Reis, P. C. L. da Silva, G. A. Lacorte, J. R. Nicoli, E. Neumann, and Á. C. Nunes. 2022. Changes in bovine milk bacterial microbiome from healthy and subclinical mastitis affected animals of the Girolando, Gyr, Guzera, and Holstein breeds. Int. Microbiol. 25:803–815. https://doi.org/10.1007/s10123-022-00267-4.

Stojanov, S., A. Berlec, and B. Štrukelj. 2020. The influence of probiotics on the *Firmicutes*/*Bacteroidetes* ratio in the treatment of obesity and inflammatory bowel disease. Microorganisms 8:1715. https://doi.org/10.3390/microorganisms8111715.

Sun, M., J. Gao, T. Ali, D. Yu, S. Zhang, S. U. Khan, S. Fanning, and B. Han. 2017. Characteristics of *Aerococcus viridans* isolated from bovine subclinical mastitis and its effect on milk SCC, yield, and composition. Trop. Anim. Health Prod. 49:843–849. https://doi.org/10.1007/s11250-017-1271-2.

Sutthiwong, N., S. Lekavat, and L. Dufossé. 2023. Involvement of versatile bacteria belonging to the genus *Arthrobacter* in milk and dairy products. Foods 12:1270. https://doi.org/10.3390/foods12061270.

Tamba, M., R. Rocca, A. Prosperi, G. Pupillo, P. Bassi, G. Galletti, E. Martini, A. Santi, G. Casadei, and N. Arrigoni. 2022. Evaluation of control program against *Streptococcus agalactiae* infection in dairy herds during 2019–2021 in Emilia-Romagna region, northern Italy. Front. Vet. Sci. 9:904527. https://doi.org/10.3389/fvets.2022.904527.

Taponen, S., D. McGuinness, H. Hiitiö, H. Simojoki, R. Zadoks, and S. Pyörälä. 2019. Bovine milk microbiome: A more complex issue than expected. Vet. Res. 50:44. https://doi.org/10.1186/s13567-019-0662-y.

Thomas, F., J. H. Hehemann, E. Rebuffet, M. Czjzek, and G. Michel. 2011. Environmental and gut *Bacteroidetes*: The food connection. Front. Microbiol. 2:93. https://doi.org/10.3389/fmicb.2011.00093.

Vázquez-Baeza, Y., M. Pirrung, A. Gonzalez, and R. Knight. 2013. EMPeror: A tool for visualizing high-throughput microbial community data. Gigascience 2:2047-217X-2-16. https://doi.org/10.1186/2047-217X-2-16.

Verdier-Metz, I., G. Gagne, S. Bornes, F. Monsallier, P. Veisseire, C. Delbès-Paus, and M. C. Montel. 2012. Cow teat skin, a potential source of diverse microbial populations for cheese production. Appl. Environ. Microbiol. 78:326–333. https://doi.org/10.1128/AEM.06229-11.

Wang, Y., X. Nan, Y. Zhao, L. Jiang, H. Wang, F. Zhang, D. Hua, J. Liu, L. Yang, J. Yao, and B. Xiong. 2022. Discrepancies among healthy, subclinical mastitic, and clinical mastitic cows in fecal microbiome and metabolome and serum metabolome. J. Dairy Sci. 105:7668–7688. https://doi.org/10.3168/jds.2021-21654.

Ward, L. M., J. Hemp, P. M. Shih, S. E. McGlynn, and W. W. Fischer. 2018. Evolution of phototrophy in the Chloroflexi phylum driven by horizontal gene transfer. Front. Microbiol. 9:260. https://doi.org/10.3389/fmicb.2018.00260.

Welch, C. B., J. M. Lourenco, T. R. Krause, D. S. Seidel, F. L. Fluharty, T. D. Pringle, and T. R. Callaway. 2021. Evaluation of the fecal bacterial communities of Angus steers with divergent feed efficiencies across the lifespan from weaning to slaughter. Front. Vet. Sci. 8:597405. https://doi.org/10.3389/fvets.2021.597405.

Widyastuti, Y., A. Febrisiantosa, and F. Tidona. 2021. Health-promoting properties of lactobacilli in fermented dairy products. Front. Microbiol. 12:673890. https://doi.org/10.3389/fmicb.2021.673890.

Xue, M., H. Sun, X. Wu, L. L. Guan, and J. Liu. 2018. Assessment of rumen microbiota from a large dairy cattle cohort reveals the pan and

core bacteriomes contributing to varied phenotypes. Appl. Environ. Microbiol. 84:e00970-18. https://doi.org/10.1128/AEM.00970-18.
Zhao, R., Z. Chen, J. Liang, J. Dou, F. Guo, Z. Xu, and T. Wang. 2023. Advances in genetic tools and their application in *Streptococcus thermophilus*. Foods 12:3119. https://doi.org/10.3390/foods12163119.
Zhu, C., Y. Zhao, F. Yang, Q. Zhang, X. Zhao, Z. Yang, X. Dao, and L. Laghi. 2024. Microbiome and metabolome analyses of milk and feces from dairy cows with healthy, subclinical, and clinical mastitis. Front. Microbiol. 15:1374911. https://doi.org/10.3389/fmicb.2024.1374911.

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