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Comparison of milk microbiota between healthy and mastitic cows

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Abstract: Mammary gland infections occur due to bacterial changes in the mammary tissue. Studies conducted in recent years have reported variations in the most common bacteria differ according to geographical locations. California mastitis test (CMT), somatic cell count (SCC), and aerobic colony count (ACC) analyses were performed on approximately 50 mL of hygienically collected raw milk samples. Raw milk was also subjected to conventional bacteriological isolation and identification. Bacterial diversity and rates in raw milk were compared through metagenome analysis. Two samples, one from healthy milk and another from subclinical milk with mastitis, were independently tested to determine whether there were differences in the percentages (%) of bacterial phylum and genera detected as a result of metagenome analysis. As a result of the conventional isolation and identification of raw milk, *Escherichia-Shigella*, *Acinetobacter*, *Vibrio*, *Streptococcus*, *Pseudomonas*, *Lactococcus*, *Glutamicibacter* and *Bacillus* genera, and *Enterobacteriaceae* family were frequently detected, respectively. As a result of metagenome analysis, the following phyla were detected in healthy raw milk: *Firmicutes* and *Proteobacteria* (7/7), *Bacteroidota* (6/7), and *Actinobacteriota* (4/7). In raw milk with subclinical mastitis, the detected phyla were *Firmicutes* and *Proteobacteria* (27/29), *Actinobacteriota* (11/29), and *Bacteroidota* (10/29). As a result of the statistical analysis, the frequency of *Bacteriodata* in healthy milk samples, as well as *Enhydrobacter*, *Enterobacteriaceae*, *Paenibacillus*, *Macrococcus*, *Spingobacterium,* and "Others", were significantly higher than the incidence in milk samples with subclinical mastitis. The only exception was observed in *Escherichia-Shigella* genera, where the opposite situation was evident. As a result of metagenome studies conducted on the raw milk of animals with both healthy and subclinical mastitis, significant differences were detected in some phyla and genera. The findings of our study will shed light on mastitis treatment studies by improving the microbiota.

Key words: Cow, milk, mastitis, microbiota

1. Introduction

Mastitis is one of the most common infectious diseases in dairy cows [1]. It is also a significant animal welfare issue, as it is associated with pain, reduced welfare, and behavioral changes in animals [2]. Moreover, mastitis poses problems in terms of both food safety and the economy [3].

Somatic cell count (SCC) is an important determinant of intramammary infection. The level of subclinical mastitis can be monitored with SCC [3]. SCC is $< 1 \times 10^5$ cells/mL in milk from a healthy cow; however, if there is a bacterial infection, this number rises above 1×10^6 cells/mL [4].

The most common organisms that cause mastitis are infectious host pathogens and environmentally transmitted pathogens. The infectious host pathogens *Staphylococcus aureus* (*S. aureus*) and *Streptococcus agalactiae* (*S. agalactiae*) are generally associated with the most common elevated scores of SCC. Although the environmentally pathogens *Streptococcus dysgalactiae* (*S. dysgalactiae*), *Streptococcus uberis* (*S. uberis*), *Corynebacterium bovis* (*C. bovis*), and coagulase-negative *Staphylococcus* cause some increase in SCC, their levels of SCC are lower than those caused by infectious pathogens [3]. The California mastitis test (CMT) is another method that detects intramammary infection caused by important mastitis pathogens in the early stages of lactation. The CMT is more effective in detecting subclinical mastitis [5]. CMT is a qualitative

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measurement of somatic cell count in milk and serves as a screening test to detect subclinical mastitis [6]. The gold standard method for this purpose is the bacteriological culture test [7].

By improving the mammary microbiota, inflammation in the mammary gland can be resolved without the use of antibiotics. This sheds light on the fact that economic costs can be reduced, and animal welfare can be ensured. Mammary microbiota, currently a significant issue, continues to gain popularity as research reveals that milk is not sterile, and new generation sequencing methods replace conventional ones. It was believed that the contents of the mammary gland and milk were sterile [8], and the belief was that microorganisms in the milk contaminated it from the outside [9]. This understanding has changed due to the development of sensitive molecular methods [10]. The theory that milk in a healthy mammary gland is germ-free dates back to the 1870s [11]. It has been suggested that the udder is associated with the normal flora, consisting of bacteria found around it [12]. With culture-independent microbial identification methods, the concept of a sterile intramammary environment has been reintroduced, and studies have shown that the healthy mammary gland contains many diverse bacterial populations [11].

There is increasing evidence that clinical mastitis is associated with reduced microbial diversity and altered composition of the intramammary microbiota (i.e. dysbiosis). However, whether microbiota dysbiosis is the cause or consequence of infectious mastitis is a matter of debate [13]. Young et al. [14] reported that intestinal bacteria are transferred to the mammary gland during the lactation period in cows, thus supporting the existence of an endogenous entero-mammary pathway. As milking hygiene improves and etiologies shift towards environmental pathogens, there has been an observed increase in the proportion of milk samples that do not grow bacteria in culture [15, 16].

Researchers have now begun to question the concept of sterile milk because early studies, using culture-independent sequencing technologies, have shown that there is a wide variety of bacterial DNA in both healthy and mastitis milk samples [17]. They also stated that although bacterial DNA was found in culture-negative samples, its origin is not yet known [18]. The first microbiota study conducted with cow's milk, using pyrosequencing, was published in 2012. Researchers examined the microbiota of cattle subclinically infected with culture-positive milk. *Pseudomonas, Shigella*, *S. aureus* and *Escherichia coli* (*E. coli*) were found among the operational taxonomic units (OTUs) through sequencing and culture methods [19].

In our study, we aimed to identify healthy and subclinical mastitic animals using conventional bacteriological isolation and identification methods along with CMT, SCC, and ACC. Additionally, we aimed to determine the mammary microbiota of healthy and subclinical mastitic animals through metagenome analysis, to determine the diversity and proportions of bacteria, and to investigate differences in the microbiota between healthy and subclinical mastitic animals.

2. Materials and methods

2.1. Sample collection

A total of 36 raw milk samples from dairy farms in the İzmir Region were analyzed. The raw milk samples were obtained from Holstein cows on two different farms. No clinical signs of mastitis were detected in any of the animals, and they were approximately 1.5 years old. After being disinfected with 70% alcohol, the teats were dried. To identify healthy or subclinical mastitis in animals, middle milking streams of raw milk samples taken from all udders of the same animal were considered one sample. Raw milk samples, taken hygienically, were placed in approximately 50 mL sterile tubes with screw caps and delivered to the laboratory through a cold chain.

2.2. CMT

Two milliliters of raw milk samples were placed in plastic petri dishes, and an equal amount of CMT reagent was added. The mixture was then stirred in circular movements for 15–20 s, followed by an evaluation based on the precipitation situation [7]. According to Kandeel, in the negative reaction (0), the mixture remains liquid with no precipitate. In the trace reaction, there is light precipitation that tends to disappear with the constant movement. One positive reaction is characterized by an obvious precipitate with no gel tendency. For two positives, the mixture thickens immediately upon movement, with some gel formation and a tendency to move towards the center. In three positives, there is pronounced gelation, with a tendency to stick to the bottom of the palette and the formation of a distinct central peak when rotated [20].

2.3. SCC

Analysis of raw milk arriving at the laboratory was performed under a microscope using the Standard (Breed) Method [21]. According to the "Regulation on special hygiene rules for foods of animal origin," the valid limit value was determined as 400,000 cells/mL for cow's milk. Raw milk with an SCC of ≤400,000 cells/mL was considered healthy, while a count >400,000 cells/mL was indicative of mastitis [22].

2.4. ACC

After performing serial dilutions of raw milk for the total bacterial count, plantings were carried out on Plate Count Skim Milk Agar. The mixture was then incubated at 30 ^{0}C for 48 h. At the end of the incubation period, the colonies formed on the medium were counted, and the results were evaluated. According to the "Regulation on special hygiene

rules for foods of animal origin," the maximum legal limit for the number of bacteria colony-forming unite (cfu) is ≤100,000/mL [22].

2.5.Conventional bacteriological isolation and identification

The raw milk that came to the laboratory was plated on Blood Agar, MacConkey Agar and Nutrient Agar. It was then incubated at 37 ^{0}C in both aerobic and anaerobic environments. Additionally, PPLO Agar, Brucella Agar and Trypticase Soy Agar (TSA) were used for *Brucella* spp. and *Mycoplasma* spp. identification. These were aerobically and microaerophilically incubated at 37 ^{0}C. The identification of isolates was carried out using conventional methods [23, 24].

2.6. Metagenome analysis

The milk samples from the groups determined for metagenome analysis were compared in terms of bacterial diversity and ratios.

2.7. DNA isolation

For the extraction protocol, the Quick Gene (Tissue DNA isolation kit, Kurabo) extraction device was used. As a result of the extraction process, an average of 30–40 ng of genomic DNA was obtained and diluted with 50 µL of elution buffer. The V3-V4 region of 16S rDNA in the extracted bacterial DNA was amplified by PCR, and sequencing was performed on the HiSeq platform (Illimuna) by following the 2 × 250 bp pair-end protocol. Read pairs with unique molecular barcodes were separated, and pair-end reads assembled using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/).

According to the QIIME (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html) quality control process, it was carried out under specific filtering conditions to obtain clean tags with a high-quality filter on raw tags. These tags are part of the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) used for detecting chimera sequences with the UCHIME (http://www.drive5.com/usearch/manual/uchime_algo.html) algorithm. The CHIMERA (http://www.drive5.com/usearch/manual/chimera_formation.html) sequences were then extracted. Finally, effective tags were obtained. Additionally, a quality filter was applied to the combined read results, and those with an expected error rate (p-value) above 0.05 were eliminated. 16S rRNA gene sequences were divided into OTU clusters with a 97% similarity cut-off using the UPRASE (Uparse v7.0.1001 http://drive5.com/uparse/) algorithm. To determine taxonomic classes, OTUs were mapped using the optimized version of the SILVA database (http://www.arb-silva.de/), which specifically contains the 16S V3–V4 region. Densities were obtained by mapping demultiplexed reads using UPARSE OTUs. To obtain the phylogenetic relationships among representative sequences of all OTUs, MUSCLE (Version 3.8.31 http://www.drive5.com/ muscle/) was used, as it is capable of comparing large numbers of sequences. Alpha-diversity and beta-diversity analyses were subsequently conducted using the OTU tables created in the preceding two steps [25].

2.8. Statistical analysis

The investigation of whether the percentages (%) of metagenome bacterial phyla and genera differed in healthy and subclinical mastitis milk samples was conducted using two independent tests. First, preliminary analyses were performed to check whether the data met the parametric test assumptions, including normality and variance homogeneity tests. The test results revealed that only the percentages of bacterial phyla called "others" within the metagenome branch met the parametric test assumptions. Subsequently, the Student t-test was applied solely to the "others" category, while the Mann-Whitney U test was utilized for all remaining metagenome phyla and genera. The chi-square test was performed to test whether there were differences in the presence of bacteria and yeast (whether present or absent) between healthy and subclinical mastitis milk samples. In chi-square tests, Fisher's exact test was applied in cases where the expected numbers in 2 × 2 cross tables were below 5. As a result of the analysis, the chi-square test was performed only in the presence of total bacteria, while Fisher's exact test was applied in the presence of all remaining bacteria. Spearman rank correlations (rho) were calculated between bacteria and yeast in all milk samples. All analyses were performed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corporation, Armonk, NY, USA).

3. Results

This project investigated the diversity and bacterial rates in the udder tissues of both healthy and subclinical mastitic cows. The study focused on animals that did not exhibit clinical mastitis symptoms and were from dairy farms in the İzmir Region.

3.1. CMT results

Eighteen samples were detected as healthy, while another 18 were found to have subclinical mastitis.

3.2. SCC results

Twenty-one samples were ≤400,000/mL, indicating healthy; whereas 15 were detected as <400,000/mL, indicated subclinical mastitis.

3.3. ACC results

After incubating at 30 ^{0}C for 48 h, the colonies formed in the medium were counted, and the results were evaluated. Sixteen samples were determined to be healthy because ≤100,000 cfu/mL was detected, while 20 samples were determined to have subclinical mastitis because >100,000 cfu/mL was detected.

Samples found to be mastitis positive in any of the CMT, SCC, and ACC analyses at 30 ^{0}C applied to raw milk

were considered to have subclinical mastitis, and samples found to be negative and below the limits were considered healthy. Accordingly, 7 of the raw milk samples were considered healthy, while 29 were found to be subclinically mastitic. CMT, SCC, and ACC results are presented in Table 1.

3.4. Conventional bacteriological isolation and identification results

Bacteria and yeast isolated and identified from healthy raw milk were as follows: *Staphylococcus* (5/7), yeast (4/7), *Bacillus* (3/7), *E. coli* (2/7), *Streptococcus* (1/7), *Proteus* (1/7), *Pseudomonas* (1/7). Similarly, in raw milk with subclinical mastitis, the isolation and identification of bacteria and yeast yielded the following results: *Bacillus* (12/29), *Staphylococcus* (11/29), yeast (11/29), *E. coli (10/29) Acinetobacter (10/29), Streptococcus* (9/29), *Klebsiella (3/29), Enterobacter (3/29)*, *Pseudomonas* (2/29), *Serratia* (1/29), *Pasteurella* (1/29), *Shigella* (1/29), and *Arcanobacter* (1/29). Notably, in bacteriological cultivations from raw milk, no growth of *Brucella* and *Mycoplasma* was detected.

Table 1. CMT, SCC, and ACC analysis results for healthy and subclinical mastitis raw milk at 30 °C.

Samples	SCC	CMT	ACC	Healthy (H)/ subclinical mastitis (SM)	Samples	SCC	CMT	ACC	Healthy (H)/ subclinical mastitis (SM)
1.1	381,282	2	1.8×10^6	SM	**2.1**	225,303	3	1.5×10^7	SM
1.2	190,641	Trace	0	H	**2.2**	173,310	0	1.3×10^8	SM
1.3	1,057,191	0	0	SM	**2.3**	450,606	3	8.7×10^8	SM
1.4	675,909	0	0	SM	**2.4**	693,240	3	2.3×10^7	SM
1.5	1,802,424	3	6.2×10^5	SM	**2.5**	433,275	Trace	9.3×10^7	SM
1.6	277,296	0	0	H	**2.6**	207,972	0	6.4×10^8	SM
1.7	762,564	Trace	0	SM	**2.7**	398,613	İz	1.6×10^9	SM
1.8	363,951	0	0	H	**2.8**	190,641	0	2.1×10^8	SM
1.9	155,979	Trace	0	H	**2.9**	34,662	Trace	$9,5 \times 10^7$	SM
1.10	0	0	1.2×10^4	H	**2.10**	329,289	0	7.2×10^8	SM
1.11	69,324	2	9.3×10^3	SM	**2.11**	86,655	0	5.2×10^8	SM
1.12	1,646,445	3	1.9×10^4	SM	**2.12**	1,351,818	Trace	3×10^8	SM
1.13	571,923	3	0	SM	**2.13**	745,233	0	1.2×10^8	SM
1.14	381,282	3	0	SM	**2.14**	2,651,643	0	2.9×10^9	SM
1.15	0	3	3.7×10^3	SM	**2.15**	5,528,589	Trace	2.5×10^9	SM
1.16	138,648	Trace	0	H	**2.16**	121,317	0	6.7×10^8	SM
1.17	1,161,177	3	0	SM	**2.19**	970,536	3	7.7×10^7	SM
1.18	346,620	Trace	0	H	**2.20**	0	0	1×10^9	SM

As a result of conventional bacteriological isolation and identification of raw milk from animals with subclinical mastitis in the İzmir Region, various bacteria, including *Escherichia-Shigella, Acinetobacter, Enterobacteriaceae, Vibrio, Streptococcus, Pseudomonas* and *Lactococcus,* as well as *Glutamicibacter* and *Bacillus,* were frequently detected.

3.5. Metagenome analysis results

Phyla with metagenome analysis results detected from healthy raw milk were as follows: *Firmucutes* (7/7), *Proteobacteria* (7/7), *Bacteroidota* (6/7), *Actinobacteriota* (4/7); Genera: *Macrococcus* (4/7), *Pseudomonas* (4/7), *Acinetobacter* (3/7), *Enhydrobacter* (3/7), *Escherichia-Shigella* (2/7), *Rothia* (2/7), *Prevotella_7* (2/7), *Paenibacillus* (2/7), *Vibrio* (2/7), *Chryseobacterium* (2/7), *Staphylococcus* (1/7), *Streptecoccus* (1/7), *Aerococcus* (1/7), *Sphingobacterium* (1/7), and *Psychrobacter* (1/7).

Phyla identified by metagenome analysis results from raw milk with subclinical mastitis were *Firmucutes (27/29), Proteobacteria (27/29), Actinobacteriota (11/29), Bacteroidota (10/29);* Genera: *Escherichia-Shigella (22/29), Acinetobacter (20/29), Vibrio (13/29), Streptococcus (12/29), Lactococcus (11/29), Pseudomonas (11/29), Glutamicibacter (7/29), Bacillus (6/29), Chryseobacterium (4/29),* and *Staphylococcus (3/29).*

The percentages of phyla determined through metagenome analysis of raw milk samples, obtained from both healthy glands and those with subclinical mastitis, are presented in Table 2. In Table 3, the genera and percentages resulting from metagenome analysis of raw milk samples with healthy glands are detailed. Additionally, Table 4 displays the genera and percentages determined through metagenome analysis of raw milk samples exhibiting positive subclinical mastitis.

The phyla and their numbers are given in raw milk that were mastitis-negative and subclinical mastitis were determined through metagenome analysis in Table 5. The genera and their numbers are given in raw milk that were mastitis-negative and subclinical mastitis were determined through metagenome analysis in Table 6.

3.6. Statistical analysis results

While the percentage of occurrence of *Bacteriodata* and branches called "others" in healthy milk samples was found to be significantly higher than the percentage of occurrence in milk samples with subclinical mastitis ($U = 37.50$, $z = -2.81$, $p = 0.005$; $t = 3.06$, $p = 0.004$, respectively), there was no significant difference in the incidence percentages of the remaining phyla between milk samples with healthy and subclinical mastitis ($p > 0.3$) (Table 7).

The percentages of *Enhydrobacter* ($U = 63$, $z = -2.82$, $p = 0.005$), *Enterobacteriaceae* Unknown *1* ($U = 87$, $z = -2.04$, $p = 0.042$), *Paenibacillus* ($U = 75$, $z = -2.21$, $p = 0.027$), *Macrococcus* ($U = 47.50$, $z = -3.33$, $p = 0.001$), *Spingobacterium* ($U = 87$, $z = -2.20$, $p = 0.028$), and "Others" ($U = 33$, $z = -2.74$, $p = 0.006$) in healthy milk samples are significantly higher than the percentages of milk samples with subclinical mastitis. The only exception is *Escherichia-Shigella* ($U = 47.50$, $z = -2.82$, $p = 0.005$), which showed an opposite situtation in the percentage of the genus. No significant difference was found between the percentages of occurrence in healthy and subclinical mastitis milk ($p > 0.1$) (Table 8).

In terms of the presence of bacteria and yeast (whether present or absent), there was no significant difference in healthy milk samples with subclinical mastitis ($p > 0.07$). Similarly, regarding the presence of all bacteria and yeast, no significant differences were found in both milk samples ($p > 0.4$) (Table 9).

Bacillus with *E. coli* (rho = 0.45, $p = 0.006$), *Staphylococcus* with *Streptococcus* (rho = 0.38, $p = 0.023$), *Pseudomonas* with *Shigella* (rho = 0.56, $p < 0.001$), *Pseudomonas* with *Arcanobacter* (rho = 0.37, $p = 0.028$), *Klebsiella* with *Serratia* (rho = 0.56, $p < 0.001$), *Pasteurella* with *Arcanobacter* (rho = 0.47, $p = 0.004$) showed a moderate positive correlation. There was a strong positive correlation between *Pasteurella* with *Actinobacillus* (rho = 0.70, $p < 0.001$) and *Arcanobacter* with *Actinobacillus* (rho = 0.70, $p < 0.001$). In other words, the presence of one bacterium has increased along with the presence of another bacterium. On the other hand, there was a moderately negative correlation between *Staphylococcus* and *Acinetobacter* (rho = –0.35, $p = 0.036$). In other words, the increase or decrease of one of these two bacteria caused an increase or decrease in the other bacteria (Table 10).

4. Discussion

Malinowski et al. [26] detected *Streptococcus* and gram-negative bacilli in samples with more than 2 million cells per milliliter in their study. The highest number of SCCs (≥10 million cells per milliliter) was detected in milk samples associated with intramammary infections caused by *Arcanobacterium pyogenes* (95.5%), *S. agalactiae* (57.6%), and gram-negative bacteria. SCC numbers was detected very high (≥5 million cells per milliliter) due to *Prototheca spp.* (64.5%), yeast-like fungi (60.2%) and *Streptococcus spp*. (55.1%). SCC numbers was detected <5 million cells per milliliter due to *S. aureus* (76.2%), coagulase-negative *Staphylococcus* (84.2%), gram-positive bacilli (72.4%) and *Corynebacterium* (83.2%). In our study, *Escherichia-Shigella*, *Pseudomonas* and *Streptococcus* genera were found to be higher in raw milk with subclinical mastitis and high SCC.

In America [27], milk samples were taken from a total of 106 dairy cows and subjected to 16S rRNA microbiota analysis. One hundred and forty-two OTUs were detected and *Staphylococcus*, *Knoellia*, *Aerococcaceae,*

Table 2. Percentage of phylum determined through metagenome analysis in raw milk samples from healthy glands and subclinical mastitis.

Phylum	Percentage of phylum detected in raw milk from healthy animals						
	1.2	**1.6**	**1.8**	**1.9**	**1.10**	**1.16**	**1.18***
Firmucutes	5.7	26.1	23.2	60.8	46.3	75.3	37.9
Bacteroidota	0	5	26.1	8.9	9.8	6.5	14.9
Proteobacteria	89.9	66.3	41.4	10.5	18.5	7.8	44.3
Actinobacteriota	0	0	7.5	3	2.3	6.7	0
Unassigned; Unknown_1	0	0	0	9.1	11.8	0	0
Eukaryota; Unknown_1	0	0	0	0	0	0	0
Eukaryota; Unknown_2	0	0	0	4.7	8.2	0	0
Others	4.5	2.6	1.8	2.9	3.1	3.9	3

Phylum	**Percentage of phylum detected in raw milk from the animals with subclinical mastitis**																												
	1.1	**1.3**	**1.4**	**1.5**	**1.7**	**1.11**	**1.12**	**1.13**	**1.14**	**1.15**	**1.17**	**2.1**	**2.2**	**2.3**	**2.4**	**2.5**	**2.6**	**2.7**	**2.8**	**2.9**	**2.10**	**2.11**	**2.12**	**2.13**	**2.14**	**2.15**	**2.16**	**2.19**	**2.20***
	40.7	**12.5**			**24**		**45.1**	**14**		**5**	**16.6**		**10.4**		**29.1**	**32.2**	**4**				**23.9**	**39.5**	**31.7**				**19.3**	**38.4**	**31.4**
Bacteroidota	**33.9**	**2.6**	**3**	**11.9**	**8.7**	**11.5**		**6.9**	**2.9**	**3**																		**2.5**	
	21.8		**3**	**16.9**		**11**	**44.4**					**43.4**	**41**		**6.7**			**30.9**		**34**									
Actinobacteriota		**11.6**		**4.9**		**4.4**				**6.3**		**16**				**8.4**				**2.9**	**9.9**	**3**			**2.2**				
Unassigned; Unknown_1		**4**	**2.6**	**9.6**	**9**	**14.5**	**6.3**	**4.5**	**14.7**	**16.5**	**8.8**	**17.3**																**3.6**	
Eukaryota; Unknown_1				**6.6**		**7.8**	**2.3**	**2.5**	**8.1**	**9.3**	**4**				**26.3**													**3.5**	
Eukaryota; Unknown_2	**3.6**	**2.8**	**2**	**1.9**	**2.6**	**3.8**	**1.9**	**2**	**2.5**	**0.8**	**3**	**13.2**			**34.3**									**0.4**	**0.2**				
Others	**0.9**	**0.9**	**2.3**	**2.6**	**2.7**	**2.7**	**1.8**	**2.9**	**3.2**	**3.8**	**2.9**	**1.4**	**0**	**1.8**	**3.5**	**2.7**	**0.8**	**1.1**	**0.4**	**0.1**	**0.9**	**2.4**	**3**	**0.4**	**0.2**	**0**	**0.2**	**2**	**3.1**

*Numbers indicate sample numbers.

Table 3. Percentage of genera in metagenome analysis of healthy raw milk samples.

Genera	Percentage of genera detected in healthy raw milk						
	1.2	**1.6**	**1.8**	**1.9**	**1.10**	**1.16**	**1.18***
Chryseobacterium			18.1				14.2
Streptococcus			3.8				
Enhydrobacter			13.8		6.8		13
Exiguobacterium							
Aerococcus			2.4				
Pseudomonas	58.2		25.9	5.2	5.2		
Escherichia-Shigella	31.1						20.6
Rothia			6.2			6.2	
Enterococcus							
Prevotella_7				3.7	3.8		
Prevotella_9							
Bacteroides							
Faecalibacterium							
Acinetobacter		58.9			2.5		10.2
Glutamicibacter							
Lactococcus							
Staphylococcus					36.3		
Vagococcus							
Macrococcus			5.6	54.7		71.8	25.7
Sphingobacterium			3.6				
Paenibacillus			2.2				5.5
Veillonella							
Psychrobacter						2.3	
Bacillus							5.1
Serratia		4.9					
Vibrio				9.1	11.8		
***Enterobacteriaceae*; Unknown_13,48**				4.7	8.2		
Unknown_2		15.7	18.4	22.5	25.5	19.7	5.7

*Numbers indicate sample numbers.

Table 4. Percentage of genera in metagenomic analyses of subclinical mastitic milk samples.

Genera	Percentage of genera																												
	1.1	1.3	1.4	1.5	1.7	1.11	1.12	1.13	1.14	1.15	1.17	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	2.10	2.11	2.12	2.13	2.14	2.15	2.16	2.19	2.20*
Chryseobacterium	40.6		2.9		2.7																							2.1	
Streptococcus	33.9	3.3	19.9	35.6		32.1	2		65.4		5.9						3.5			59.4							17.8	32.2	
Enhydrobacter	21.9																												
Exiguobacterium	2.4																												
Aerococcus			21.9								4.4										5.1	3.9							
Pseudomonas			6.9	7.2	21	2	36.2	5.6			58.5	3				26.4			53.9	2.2									
Escherichia-Shige		63	37.2	4.4	33.8		7.4					25.2	39.7	88.4	4.5	15	64.9	30.6	22.5	19.6	7.6	51.3	61.4	31.4	48.3		23.1	34.7	35.8
Rothia		8.5								5.2																			
Enterococcus		4.8																				2.7							
Prevotella_7					2.6	4																							
Prevotella_9				3.8		2.2		3.5																					
Bacteroides				3.6																									
Faecalibacterium				2.8																									
Acinetobacter				2.6	2.6			63.2		57.6	5	8.8		9.6		14.6	29.3		8.8	7.2	56.9	2.2	2.4	11.6	46.5		57.2	14.4	25.8
Glutamicibacter				2.3								12.6	48.1			6.5				2.9	9.6				2.2				
Lactococcus					2.3										15.6	29.2		67.7	8.7		11.6	3.8	29.2	54.4		98.8			27.7
Staphylococcus					14.4			5.6							81														
Vagococcus					2.2																		2.2						
Macrococcus									4.9				8.5																
Sphingobacterium																													
Paenibacillus																						2							
Veillonella						2.8																							
Psychrobacter											3.5																		
Bacillus											5	2	2								4.6	21.8						4.5	
Serratia																				2,6									
Vibrio		4	2.6	9.6	2.9	14.5	6.3	4.5	14.7	16.5	8.8	4.7	4.7																3.5
***Enterobacteriaceae*; Unknown_13,48**		2.8	2	6.6	2.6	7.8	2.3	2.5	8.2	9.3	4	13.2	13.2		26.3														7.5
Unknown_2	11.8	13.7	6.6	21.7	13	34.5	5.6	15.1	6.8	11.4	4.8	17.3	17.3		34.3													3.6	

*Numbers indicate sample numbers.

Table 5. The phyla and the numbers of healthy and subclinical mastitis-positive isolates determined through metagenome analysis.

Phyla	Healthy (7)	Subclinical mastitis (29)
Firmucutes	7/7	26/29
Bacteriodata	6/7	9/29
Proteobacteria	7/7	26/29
Actinobacteriota	4/7	8/29
Unassigned; Unknown_*1*	2/7	12/29
Eukaryota_1	0/7	10/29
Eukaryota_2	3/7	13/29

Table 6. The genera and the numbers of healthy and subclinical mastitis-positive isolates determined through metagenome analysis.

Genera	Healthy (7)	Subclinical mastitis (29)
Chryseobacterium	2	4
Streptococcus	1	12
Enhydrobacter	3	1
Exiguobacterium	0	1
Aerococcus	1	4
Pseudomonas	4	11
Escherichia-Shigella	2	22
Rothia	2	2
Enterococcus	0	2
Prevotella_7	2	2
Prevotella_9	0	3
Bacteroides	0	1
Faecalibacterium	0	1
Acinetobacter	3	19
Glutamicibacter	0	7
Lactococcus	0	11
Staphylococcus	1	3
Vagococcus	0	2
Macrococcus	4	2
Sphingobacterium	1	0
Paenibacillus	2	1
Veillonella	0	1
Psychrobacter	1	1
Bacillus	1	6
Serratia	1	1
Vibrio	2	13
Enterobacteriaceae**; Unknown_13,48**	2	14
Unknown_2	6	15

Table 7. Percentages of the presence of metagenome phyla in milk samples with healthy and subclinical mastitis (%).

	Healthy (n = 7)					Subclinical mastitis (n = 29)								
Phylum	Average	Standard error	Median	Interquartile distribution	Average rank	Average	Standard error	Median	Interquartile distribution	Average rank	Mann-Whitney U statistics	Z score	t statistics	p value
Firmicutes	39.29	8.96	37.87	37.60	21.14	32.36	4.44	31.41	33.26	17.86	83.00	-0.74		0.460
Bacteriodata	10.16	3.17	8.93	9.87	**27.64**	3.00	1.28	0.00	3.00	**16.29**	37.50	-2.81		**0.005**
Proteobacteria	39.82	11.51	41.44	55.80	15.29	50.85	5.16	55.06	34.07	19.28	79.00	-0.90		0.368
Actinobacteriota	2.77	1.20	2.24	6.64	20.29	4.09	1.77	0.00	4.67	18.07	89.00	-0.56		0.577
Unknown	2.98	1.95	26.52	9.13	16.43	4.81	1.48	0.00	7.56	19.00	87.00	-0.65		0.517
Eukaryota 1	0.00	0.00	0.00	0.00	17.00	1.48	1.00	0.00	0.00	18.86	91.00	-0.88		0.381
Eukaryota 2	1.85	1.25	0.00	4.70	18.00	1.78	0.53	0.00	2.71	18.62	98.00	-0.16		0.871
Others	3.12	0.32	2.99	1.24	28.14	1.61	0.23	1.75	2.24	16.17			3,.6	**0,004**

Table 8. Percentages of presence of metagenome genera in milk samples with healthy and subclinical mastitis (%).

	Healthy (n = 7)					Subclinical mastitis (n = 29)							
Genera	Average	Standard error	Median	Interquartile distribution	Average rank	Average	Standard error	Median	Interquartile distribution	Average rank	Mann-Whitney U statistics	Z score	t statistics
Staphylococcus	5.18	5.18	0.00	0.00	19.29	0.97	0.58	0.00	0.00	18.31	96.00	–0.40	0.687
Streptococcus	0.54	0.54	0.00	0.00	14.00	12.01	3.59	0.00	25.46	19.59	70.00	–1.46	0.143
Chryseobacterium	4.62	3.01	0.00	14.22	20.93	1.42	1.16	0.00	0.00	17.91	84.50	–1.05	0.295
Enhydrobacter	4.80	2.42	0.00	20.58	**24.00**	0.73	0.73	0.00	0.00	**17.17**	63.00	–2.82	**0.005**
Enterobacteriaceae Unknown 1	0.69	0.69	0.00	0.00	**20.57**	0.00	0.00	0.00	0.00	**18.00**	87.00	–2.04	**0.042**
Escherichia-Shigella	7.38	4.90	0.00	0.00	**10.79**	25.86	4.48	23.12	36.26	**20.36**	47.50	–2.20	**0.028**
Acinetobacter	10.24	8.24	0.00	10.15	15.29	14.69	3.87	5.09	20.05	19.28	79.00	–0.93	0.354
Lactococcus	2.93	2.93	0.00	0.00	15.00	12.03	4.44	0.00	13.57	19.34	77.00	–1.17	0.243
Faecalibacterium	0.00	0.00	0.00	0.00	18.00	0.09	0.09	0.00	0.00	18.62	98.00	–0.49	0.623
Vagococcus	0.00	0.00	0.00	0.00	17.50	0.15	0.10	0.00	0.00	18.74	94.50	–0.71	0.481
Bacteroides	0.00	0.00	0.00	0.00	18.00	0.12	0.12	0.00	0.00	18.62	98.00	–0.49	0.623
Provotella 7	1.07	0.69	0.00	3.74	21.64	0.23	0.16	0.00	0.00	17.74	79.50	–1.61	0.107
Prevotella 9	0.00	0.00	0.00	0.00	17.00	0.33	0.19	0.00	0.00	18.86	91.00	–0.88	0.381
Pseudomonas	13.51	8.22	5.19	25.98	21.43	7.69	2.98	0.00	6.27	17.79	81.00	–0.92	0.360
Paenibacillus	1.09	0.79	0.00	2.18	**22.29**	0.07	0.07	0.00	0.00	**17.59**	75.00	–2.21	**0.027**
Macrococcus	22.55	11.22	5.60	54.73	**26.21**	0.46	0.33	0.00	0.00	**16.64**	47.50	–3.33	**0.001**
Spingobacterium	0.51	0.51	0.00	0.00	**20.57**	0.00	0.00	0.00	0.00	**18.00**	87.00	–2.04	**0.042**
Aerococcus	0.34	0.34	0.00	0.00	18.29	1.22	0.78	0.00	0.00	18.55	100.00	–0.10	0.921
Enterococcus	0.00	0.00	0.00	0.00	17.50	0.26	0.19	0.00	0.00	18.74	94.50	–0.71	0.481
Glutamicibacter	0.00	0.00	0.00	0.00	15.00	2.90	1.71	0.00	1.11	19.34	77.00	–1.42	0.156
Veillonella	0.00	0.00	0.00	0.00	18.00	0.10	0.10	0.00	0.00	18.62	98.00	–0.49	0.623
Pschrobacter	0.33	0.33	0.00	0.00	20.00	0.12	0.12	0.00	0.00	18.14	91.00	–1.06	0.291
Bacillus	0.73	0.73	0.00	0.00	18.29	1.31	0.78	0.00	0.00	18.55	100.00	–0.09	0.926
Vibrio	0.00	0.00	0.00	0.00	17.50	0.29	0.20	0.00	0.00	18.74	94.50	–0.71	0.481
Rothia	1.77	1.14	0.00	6.17	21.64	0.47	0.34	0.00	0.00	17.74	79.50	–1.61	0.107
Exiguobacterium	0.00	0.00	0.00	0.00	18.00	0.08	0.08	0.00	0.00	18.62	98.00	–0.49	0.623
Serratia	0.00	0.00	0.00	0.00	18.00	0.09	0.09	0.00	0.00	18.62	98.00	–0.49	0.623
Unknown 1	0.00	0.00	0.00	0.00	17.00	1.48	1.00	0.00	0.00	18.86	91.00	–0.88	0.381
Unknown 2	2.98	1.95	0.00	9.13	16.43	4.81	1.48	0.00	7.56	19.00	87.00	–0.65	0.517
Unknown 3	1.85	1.25	0.00	0.00	18.36	1.66	0.52	0.00	2.56	18.53	100.50	–0.05	0.962
Unknown 7	0.00	0.00	0.00	0.00	18.00	0.08	0.08	0.00	0.00	18.62	98.00	–0.49	0.623
Others	16.89	2.58	18.41	11.71	**28.29**	8.27	1.32	6.07	8.74	**16.14**	33.00	–2.74	**0.006**

Table 9. Comparison of the presence of bacteria and yeast in healthy and mastitis milk samples.

		Number			χ^2	p value	Fisher's exact Test p value
	Group	-	+	+ (Row%)			
Bacillus	Healthy	3	4	57.1			0.684
	Subclinical mastitis	16	13	44.8			
E. coli	Healthy	5	2	28.6			1.000
	Subclinical mastitis	18	11	37.9			
Staphylococcus	Healthy	2	5	71.4			0.204
	Subclinical mastitis	18	11	37.9			
Streptococcus	Healthy	6	1	14.3			0.400
	Subclinical mastitis	19	10	34.5			
Pseudomonas	Healthy	6	1	14.3			0.488
	Subclinical mastitis	27	2	6.9			
Proteus	Healthy	6	1	14.3			0.194
	Subclinical mastitis	29	0	0			
Acinetobacter	Healthy	7	0	0			0.076
	Subclinical mastitis	18	11	37.9			
Klebsiella	Healthy	7	0	0			1.000
	Subclinical mastitis	26	3	10.3			
Enterobacter	Healthy	7	0	0			-
	Subclinical mastitis	29	0	0			
Serratia spp.	Healthy	7	0	0			1.000
	Subclinical mastitis	28	1	3.4			
Pasteurella	Healthy	7	0	0			1.000
	Subclinical mastitis	27	2	6.9			
Shigella	Healthy	7	0	0			1.000
	Subclinical mastitis	28	1	3.4			
Arcanobacter	Healthy	7	0	0			1.000
	Subclinical mastitis	27	2	6.9			
Actinobacillus	Healthy	7	0	0			1.000
	Subclinical mastitis	28	1	3.4			
Yeast	Healthy	3	4	57.1			0.418
	Subclinical mastitis	18	11	37.9			
Total bacteria	Healthy	84	14	14.3			
	Subclinical mastitis	338	68	16.7	0.352	0.553	

Table 10. Spearman sequence correlations between bacteria and yeast in all milk samples (rho).

	Bacillus spp.	*E. coli*	*Staphylococcus* spp.	*Streptecoccus* spp.	*Psedomonas* spp.	*Proteus* spp.	*Acinetobacter* spp.	*Klebsiella* spp.	*Enterobacter* spp.	*Serratia* spp.	*Pasteurella* spp.	*Shigella* spp.	*Arcanobacter* spp.	*Actinobacillus* spp.	Yeast
Bacillus	1	**0.447****	−0.174	−0.265	−0.084	−0.160	−0.023	−0.084		−0.160	0.013	−0.160	0.013	0.179	−0.122
p value		**0.006**	0.310	0.118	0.627	0.352	0.892	0.627		0.352	0.938	0.352	0.938	0.297	0.477
E. coli		1.000	−0.207	−0.248	−0.017	−0.127	0.003	0.192		−0.127	0.070	−0.127	0.070	0.225	−0.166
p value			0.226	0.145	0.920	0.460	0.984	0.262		0.460	0.684	0.460	0.684	0.187	0.333
Staphylococcus			1.000	**0.378***	−0.270	0.189	**−0.351***	−0.067		−0.151	−0.217	−0.151	−0.217	−0.151	−0.076
p value				**0.023**	0.112	0.270	**0.036**	0.696		0.379	0.204	0.379	0.204	0.379	0.661
Streptococcus				1.000	−0.200	−0.112	−0.309	−0.200		−0.112	−0.161	−0.112	−0.161	−0.112	−0.194
p value					0.242	0.515	0.067	0.242		0.515	0.349	0.515	0.349	0.515	0.258
Pseudomonas					1.000	−0.051	0.018	−0.091		−0.051	−0.073	**0.561****	**0.366***	−0.051	0.153
p value						0.768	0.916	0.598		0.768	0.672	**<0.001**	**0.028**	0.768	0.373
Proteus						1.000	−0.112	−0.051		−0.029	−0.041	−0.029	−0.041	−0.029	0.200
p value							0.515	0.768		0.869	0.812	0.869	0.812	0.869	0.242
Acinetobacter							1.000	0.236		0.255	0.102	0.255	0.102	0.255	−0.071
p value								0.165		0.134	0.552	0.134	0.552	0.134	0.679
Klebsiella								1.000		**0.561****	−0.073	−0.051	−0.073	−0.051	−0.255
p value										**<0.001**	0.672	0.768	0.672	0.768	0.134
Enterobacter p value															
Serratia										1.000	−0.041	−0.029	−0.041	−0.029	−0.143
p value											0.812	0.869	0.812	0.869	0.406
Pasteurella											1.000	−0.041	**0.471****	**0.697****	0.041
p value												0.812	**0.004**	**0.000**	0.812
Shigella												1.000	−0.041	−0.029	0.200
p value													0.812	0.869	0.242
Arcanobacter													1.000	**0.697****	−0.205
p value														**0.000**	0.230
Actinobacillus														1.000	−0.143
p value															0.406
Yeast															1.000
p value															

*p < 0.05; **p < 0.01 at significance level.

and *Coxiella* were the most common herbs. These OTUs have been *Bacteroidetes* and *Enhydrobacter*. In our study, *Pseudomonas*, *Macrococcus,* and *Acinetobacter* were the genera detected in healthy raw milk; *Escherichia-Shigella*, *Acinetobacter*, *Vibrio*, *Streptococcus*, *Pseudomonas*, *Lactococcus*, *Glutamicibacter,* and *Bacillus* genera were found in raw milk with subclinical mastitis. In the positive group where bacterial reproduction was detected and somatic cell count was ≥150,000 cells/mL, the *Staphylococcus* genus had the highest prevalence, accounting for 16% of the sequences. In our study, the highest prevalence, 22%, was observed in the *Escherichia-Shigella* genus, while the *Staphylococcus* genus had a prevalence of 3%. In contrast, only 0.75% of the healthy samples was found to belong to the *Staphylococcus* genus. In the study on *Coxiella spp.*, the prevalence of the most common OTU found to be higher in the newly detected areas of mastitis included *Bacteroidetes*, *Enhydrobacter*, *Acinetobacter*, *Staphylococcus*, *Janthinobacterium*, *Ellin6075*, *Fibrobacter*, *Knoellia*, *Cupriavidus*, *Pantoea*, *Arthrobacter*, *Aerococcaceae*, *Aerococcus*, *Coxiella*, *Rhodocyclaceae*, *Solibacteriales*, *Brevundimonas*, *Psychrobacter*, *Burkholderia*. Due to differences in geographical conditions, different genera have been determined in our study.

In a study conducted in China [28], 36 animals from each of two farms were selected and these animals consisted of 16 healthy and 16 with subclinical mastitis. Milk samples were taken from a total of 72 animals and divided into 8 groups. The microbiota of these samples were analyzed using the pyrosequencing method. The 10 most common branches are *Proteobacteria, Firmicutes, Bacterioidetes, Actinobacteria, Tenericutes, Spirochaetae, Fusobacteria, Chloroflexi, Deinococcus-Thermus, Planctomycetes*. In our study, the most frequently detected pyla were *Firmucutes* and *Proteobacteria*, followed by *Bacteriodata* and *Actinobacteriota* at the same rate. In the study, it was reported that the prevalence of *Proteobacteria*, the main phylum, varied between 39.96% and 48.30% for each group. This was followed by *Firmicutes* (30.25%–40.28%), *Bacteroidetes* (8.38%–12.21%), and *Actinobacteria* (5.17%–11.29%) [28]. They reported that a total of 32 dominant genera were observed. Notably, the common genera differ across different groups.

Another study [29] was conducted on a dairy farm at the University of Illinois-Urbana. Using next-generation sequencing and quantitative real-time PCR (RT-PCR), cows that were found to be mastitis-negative during the dry period were randomly selected. They were either given antibiotics (intrauterine ceftiofur hydrochloride) and nipple sealant (36 cows) or only nipple sealant (36 cows). The five most abundant genera are *Corynebacterium*, *Acinetobacter, Arthrobacter, Staphylococcus,* and *Psychrobacter*. Bacteria from the genera *Acinetobacter* and *Staphylococcus* have also been detected in our study; however, there are differences in other genera.

In New York [30], in the study, milk samples from 35 cows were subjected to DNA extraction. *Firmicutes* and *Proteobacteria* were abundant in healthy milk samples. It is reported that in mastitis samples caused by *E. coli* and *Klebsiella, Proteobacteria* are seen in approximately 98%, while in mastitis samples caused by *Streptococcus*, the majority consist of *Firmicutes* and *Proteobacteria.* It is reported that *Firmicutes* and *Proteobacteria* constitute the majority of mastitis samples. In our study, *Firmicutes* and *Proteobacteria* phyla were frequently detected in both healthy and mastitis raw milk.

Milk samples were collected from 10 farms in Shanghai, China, for 12 months and 16S rRNAs were studied using high-throughput DNA sequencing methods. *Firmicutes* (40%), *Proteobacteria* (39%), and *Actinobacteria* (9.4%) were the most abundant phyla, showing a mutually balanced relationship. *Pseudomonas* (19.6%), *Bacillus* (13.8%), *Lactococcus* (11.7%), and *Acinetobacter* (10.2%) were found to be the most common genera in accordance with our study. However, in our study, *Firmicutes* and *Proteobacteria* were detected at higher rates; subsequently, *Bacteriodata* and *Actinobacteriodata* were detected [31].

In Russia, in 2021, as a result of comparative microbiome analyses of milk associated with bovine mastitis on two farms, some genera were found to be dominant, including *Staphylococcus aureus* (*S. aureus*), *Aerococcus,* and *Streptococcus*. It has been reported that the dominant phyla are *Firmicutes*, especially *Bacillus*, *Proteobacteria,* and *Actinobacteria*. Additionally, bacteria such as *Enterobacter, Macrococcus, Corynebacterium, Acinetobacter, Psychrobacter, Ignavigranum,* and *Atopostipes* have also been detected. The dominant *Staphylococcus* and *Streptococcus*, and *Acinetobacter* were mostly observed in milk samples exhibiting both subclinical and clinical mastitis; In milk samples with subclinical mastitis, *Streptococcus* (93.95%), *Enterobacter* (59.32%), and *Macrococcus* (60.26%) were prevalent; in healthy milk, *Aerococcus* (44%) was detected. They also emphasized that *S. aureus*, along with *E. coli* and *S. uberis*, are important intramammary pathogens. In our study, the *Staphylococcus* genus was detected at lower rates, while *Escherichia-Shigella, Streptococcus, Pseudomonas,* and *Acinetobacter* were found at higher rates. Although there were some differences between the two farms, they stated that no significant difference was observed between the farm with healthy conditions and the one affected by mastitis. In the study, the genera found to be high in all groups were *Staphylococcus* and *Streptococcus. Acinetobacter* was detected in 82% of subclinical mastitis and 74% of mastitis milk samples in the same study [32].

In our study, *Acinetobacter* was found at a rate of 42.8% in healthy raw milk and 65.5% in raw milk with subclinical mastitis.

In another study conducted in Russia in 2018, microbiome changes were examined in the milk of healthy, clinical, and subclinical mastitic cows. In addition to *Streptococcaceae, Staphylococcoceae,* and *Bacillaceae*, primarily *Pseudomonadales* and *Burkholderiales* OTUs were detected in animals with mastitis. On the other hand, a decrease in *Planococcaceae* OTU rates was detected. In all three groups—healthy, subclinical mastitis, and mastitis—*Proteobacteria* (63.8%–87.2%) was the highest, and *Firmicutes* (11.6%–35.2%) was in second place. They reported that the *Streptococcus* genus is dominant in healthy cows. Additionally, as one moves from healthy cows to cows with mastitis, the number of *Actinomycetales* increases, and the number of *Burkholderiales* decreases [33]. In our study, *Proteobacteria* (100%) and *Firmicutes* (89.6%) were detected at the same rate in both healthy and subclinical mastitis raw milk. However, the *Acinetobacter* rate was 42.8% in healthy raw milk, increasing to 65.5% in raw milk with subclinical mastitis. Furthermore, it was found that the rate of *Streptococcus* increased in those with subclinical mastitis, rising from 15.8% to 41.3%.In Russia, Gryaznovs et al. [34], in their study, associated the increase in *Cutibacterium, Blautia, Clostridium sensu stricto 2, Staphylococcus, Streptococcus* and *Microbacterium* genera with breast pathology, and the increase in *Staphylococcus* and *Streptecoccus* genera with subclinical mastitis.

The most frequently isolated pathogens are *S. aureus, E. coli, Klebsiella, Streptococcus, Mycoplasma, Enterobacter, Bacillus,* and *Corynebacterium.* As a result of the study, the most common phyla were determined to be *Actinobacteriota, Firmicutes, Proteobacteria,* and *Bacteriodota* [34]. In our study, the rate of *Streptococcus* increased in raw milk with subclinical mastitis, but there was no significant increase in the rate of *Staphylococcus; Firmicutes* and *Proteobacteria* were detected at higher rates.

In the study conducted in Italy in 2022, the most dominant microorganisms were *Lactococcus, Lactobacillus, Pseudomonas, Micrococcus, Staphylococcus,* and yeast species. Other notable findings included *Leuconostoc, Enterococcus, Streptococcus, Bacillus, Clostridium, Listeria,* and *Enterobacteriaceae.* Additionally, gram-negative bacteria also included *Acinetobacter, Alcaligenes, Flavobacterium,* and *Aeromonas* species. Consistent with our study, *Proteobacteria* and *Firmucutes* were generally the most dominant phyla, with a rate of 32%. Following them, *Actinobacteria* was detected in 29% and *Bacteroidetes* in 6%. *Streptococcus, Escherichia, Staphylococcus,* and *Enterococcus*, along with the *Corynebacterium* genus, were the genera most associated with mastitis on the farm [35]. *Streptococcus* and *Escherichia* species were found at high rates in raw milk with subclinical mastitis in our study.

As a result of metagenome studies conducted on raw milk from healthy and mastitic animals, significant differences have been detected in some phyla and genera. Our study has also determined that changes in microbiota play a crucial role in mastitis cases. The findings of this study will shed light on the studies on mastitis treatment by improving the microbiota.

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