Eurasian Journal
of Veterinary Sciences

www.eurasianjvetsci.org

RESEARCH ARTICLE

Evaluation of Lactic Acid Bacteria and Total Bacterial Load in Milk from Clinical Mastitis, Subclinical Mastitis and Healthy Cows

Semiha Yalcin*[1], Metehan Simsir[2], Eda Merve Kurtulus[3]

[1]Mugla Sitki Kocman University, Milas Faculty of Veterinary Medicine, Preclinical Sciences, Microbiology Department, 48000, Muğla, Türkiye

[2]Mugla Sitki Kocman University, Milas Faculty of Veterinary Medicine, 48000, Muğla, Türkiye

[3]Istanbul Gelisim University, Faculty of Health Sciences, Nutrition and Dietetics Department, 34310, İstanbul, Türkiye

Received: 10.03.2024 , Accepted: 28.11.2024

*semihayalcin@mu.edu.tr

Klinik Mastitisli, Subklinik Mastitisli ve Sağlıklı İnek Sütlerinde Laktik Asit Bakterileri ve Toplam Bakteri Yükünün Değerlendirilmesi

Eurasian J Vet Sci, 2024, 40, 4, 154-160
DOI: 10.15312/EurasianJVetSci.2024.440

Öz

Amaç: Sığır mastitis olgularında, alternatif önleyici ve tedavi edici yaklaşımlara yönelik çalışmalar, bakteriyel antibiyotik direnç nedeniyle veteriner hekimliği alanında da ivme kazanmıştır. Bu çalışmada, laktasyon dönemindeki ineklerden, klinik/subklinik mastitisli ve sağlıklı meme loblarından alınan sütlerde toplam bakteri sayıları ile laktik asit bakteri sayılarının belirlenerek bir tarama testi olan Kalifornia Mastitis Test sonuçları ile karşılaştırılması ve laktik flora yükünün olgulardaki değişkenliğinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Toplamda 15 farklı işletmede, laktasyon dönemindeki ineklerden aseptik koşullarda süt örnekleri toplandı. Örneklem yapılan meme lobları, sağlıklı (NM; n=35), klinik mastitisli (CM; n=30) ve subklinik mastitisli (SCM; n=31) olarak üç alt gruba ayrıldı. Süt örneklerinden MRS (De Man Ragosa Sharp Agar), M17 agar ve PCA (Plate Count Agar) besiyerlerine ekimler yapılarak toplam bakteri ve laktik asit bakteri sayımları gerçekleştirildi.

Bulgular: MRS ve M17 koloni sayılarının logaritmik ortalamaları açısından üç grup arasında istatistiksel olarak anlamlı bir fark yoktu. MRS ve M17 ortalamaları birlikte değerlendirildiğinde NM, SCM ve CM grupları arasında anlamlı fark saptanmadı (P=0.093). PCA, CMT, MRS ve M17 besiyerleri ortalamaları arasında pozitif korelasyon (P=0.001) mevcuttu.

Öneri: Sonuç olarak, sütteki toplam bakteri sayısı ve laktik asit bakteri yükü, yetiştirme ortamından ve çevre koşullarından etkilenmektedir. Sütün mikrobiyotası, sağlıklı meme loblarında ve klinik ve subklinik mastitis olgularında değişmektedir. Sığır mastitisli ve sağlıklı hayvanlarda sütün flora özelliklerinin anlaşılması, alternatif biyolojik tedavi kaynaklarının belirlenebilmesi için daha fazla araştırmaya ihtiyaç vardır.

Anahtar kelimeler: Laktik asit bakterileri, mastitis, süt, süt ineği, toplam bakteri

Abstract

Aim: Studies on alternative preventive and therapeutic approaches for bovine mastitis have gained momentum in the veterinary field due to bacterial antibiotic resistance. In this study; It was aimed to determine the total bacterial counts and lactic acid bacteria counts in milk taken from lactating cows, clinical/subclinical mastitis and healthy udder lobes, compare them with the results of the California Mastitis Test, used a screening test, and determine the variability of lactic flora load in the cases.

Materials and Methods: The milk samples were collected from lactating cows from fifteen farms. Udder quarters were categorized into three subgroups: non-mastitis (NM; n=35), clinical mastitis (CM; n=30), and subclinical mastitis (SCM; n=31). Total bacteria and lactic acid bacteria were counted by inoculating milk samples onto MRS (De Man Rogosa Sharpe Agar), M17 agar and PCA (Plate Count Agar) media.

Results: No significant difference between the three groups regarding the logarithmic averages of MRS and M17 colony numbers were found. When evaluated using MRS and M17, no significant difference existed among the NM, SCM, and CM groups (P=0.093). Positive correlations (P=0.001) existed between the mean of PCA, CMT, MRS and M17 media.

Conclusion: The total bacterial count in milk, as well as the LAB load, are affected by the growing environment and environmental conditions. The milk microbiota is altered in healthy udder quarters and clinical and subclinical mastitis cases. Further investigation is needed to understand the flora characteristics of milk in cases of bovine mastitis and healthy animals.

Keywords: Dairy cow, lactic acid bacteria, mastitis, milk, total bacteria

CITE THIS ARTICLE: *Yalcin S, Simsir M and Kurtulus E.M 2024. Evaluation of lactic acid bacteria and total bacterial load in milk from clinical mastitis, subclinical mastitis and healthy cows Eurasian J Vet Sci, 40,4, 154-160*

Introduction

Mastitis, characterized by the inflammation of mammary tissue, particularly during lactation, is a multifactorial condition caused by various bacterial agents affecting the mammary glands, milk ducts, and alveoli. In dairy farming, mastitis leads to significant economic losses due to reproductive complications, veterinary treatment costs, and challenges in milk quality evaluation caused by chemical, physical, and bacteriological changes (Tepeli and Zorba 2017; Algharib et al 2020; Berardo et al 2020).

Mastitis occurs in two main forms: clinical and subclinical, both of which can present acute or chronic courses. Clinical mastitis is readily diagnosed by observable inflammation of the mammary glands, indicated by redness, warmth, swelling, and other cardinal signs detected through inspection and palpation. In contrast, subclinical mastitis—responsible for approximately 70–80% of milk losses—is more common but harder to detect. It lacks visible changes in the milk that could be identified through organoleptic evaluation or palpation. Undetected contamination in subclinical cases can also compromise the quality of final dairy products (Çokal and Konuş 2012; Tepeli and Zorba 2017; Yu et al 2017).

An increase in somatic cell count (SCC) in milk is a key indicator for diagnosing clinical and especially subclinical mastitis. Somatic cells include erythrocytes, leukocytes, epithelial cells, and plasma cells (Özdemir and Kaymaz 2013). While SCC in normal milk typically does not exceed 200,000 cells/mL, this number significantly increases in diseased mammary lobes. Although the California Mastitis Test (CMT) does not provide an exact SCC value, it allows for diagnosis based on gradation. According to this test, diagnosis is made by scoring 300.000-500.000 cells in milk determined as +1 degree, 500.000-1.000.000 cells in milk defined as +2 degree, and more than 1.000.000 cells in milk +3 degree (Çokal and Konuş 2012).

There are many bacterial pathogens associated with mastitis infections. Among them, *Streptococcus uberis*, *Escherichia coli*, *Streptococcus dysgalactiae*, and coagulase-negative staphylococci often cause mastitis because they can form biofilms in vitro. A biofilm is a community of microorganisms irreversibly attached to a surface and surrounded by a matrix, and it has efficacy in protecting the host from the immune system. At the same time, it negatively affects treatment success by reducing the effectiveness of antibiotics (Wallis et al 2018). Bovine mastitis is a frequently occurring disease in dairy farming. Antibiotic treatments against the agents causing mastitis are not always successful due to bacterial resistance cases. Besides causing resistance problems, using antibiotics in animals brings some negative consequences, such as residues in milk. Therefore, new approaches are needed for the treatment of mastitis. One of the alternative treatment methods against infections is the administration of probiotics, defined as "live microorganisms that provide health physiological benefits to the host when administered in adequate amounts. Lactic Acid Bacteria (LAB) strains constitute a prominent category of prokaryotic organisms frequently employed for this objective and form an integral component of the microbial community residing in the mammary (Espeche et al 2012, Diepers et al 2017, Wallis et al 2018). LAB groups contain various strains and species, including rods, cocci, and coccobacilli. They are Gram-positive, immobile, non-spore-forming, catalase-negative, microaerophilic or anaerobic, acid-resistant, strongly fermentative, unable to reduce nitrate and require certain vitamins and amino acids. Many of these bacteria, called probiotics, have beneficial properties for human and animal health (Yörük and Güner 2011). In cattle health, probiotics are mainly applied to prevent gastrointestinal infections and for nutritional purposes (Rodriguez-Palacios et al 2009, Sun et al 2010). LAB strains have traditionally been used as a starter culture in the food industry, and many are considered harmless to consumers. Also, many LAB strains have been included in GRAS (generally recognized as safe) status by the FDA (Food and Drug Administration). These bacteria, isolated from various environmental sources and the natural flora of livings, are also found in the udder flora and form a natural barrier against infectious agents. This bacteria can prevent the development of other microorganisms and the formation of infection with their metabolic products, such as organic acids, hydrogen peroxide, diacetyl, bacteriocins, and antimicrobial compounds, and their effects, such as colonization on the epithelial surface, competition for nutrients, and modulation of the host's immune response (Diepers et al 2017). Researchers have recently conducted studies to detect suitable probiotic microorganisms to treat or prevent mammary infections (Diepers et al 2017, Berardo et al 2020). This study aimed to determine the Total Bacterial Count (TBC) and Lactic Acid Bacteria (LAB) count in milk from lactating cows with clinical/subclinical mastitis and without mastitis.

The term LAB in this study does not refer to probiotic lactic acid bacteria, but rather describes the group of lactic acid bacteria.

Material and Methods

Sampling

This study included 15 farms and lasted for a period of 7 months (from April 2022 to November 2022). A number of 96 milk samples from udder lobes of bovine breeds of 90 Holstein, 3 Simmental, 2 Montofon, and 1 Swiss were collected. Mammary quarters were grouped based on mastitis as follows: non-mastitis (NM; n=35), clinical mastitis (CM; n=30), and subclinical mastitis (SCM; n=31). Screening milk samples with CMT (Kerbl, Germany) determined mammary quarters as either of clinical mastitis, subclinical mastitis, or

non-mastitis. Cows with negative CMT results in all udder lobes were classified as non-mastitis. Before milking, CMT screenings were conducted on all udder lobes of each cow. The udders of cows from which milk samples would be collected were cleaned with paper towels and disinfected using 70% ethyl alcohol, as recommended by Espeche et al (2012). Following the evaporation of alcohol, the test procedure was carried out according to the manufacturer's guidelines.

The four nipples and lobes, named right anterior (A), right posterior (B), left anterior (C), and left posterior (D), are identified similarly to the CMT sampling cup. Wells that exhibit consistent thickness and a uniform appearance are considered negative and healthy samples, receiving a test score of "0". Mammary quarters without signs of inflammation in the mammary but giving +1, +2, and +3 scores according to CMT results, were evaluated as milk samples with subclinical mastitis (Kasikci et al 2012, Özenç 2019). Mammary quarters positive for CMT which also presented symptoms like tenderness, warmthness, and redness were evaluated as clinical mastitic udders (Yu et al 2017). Milk samples belonging to the selected mammary lobes were taken into sterile sample containers with an average of 50 ml and analyzed on the same day after they were delivered to the laboratory in heat-insulated containers containing ice packs under the cold chain.

Total aerobic bacteria and LAB counts in the milk samples

In order to calculate the number of colonies, 10-fold serial dilutions were prepared from milk samples with sterile physiological saline (FTS). By taking 0.1 ml from the dilutions, Plate Count Agar (PCA; Biolife, Italy) for total bacteria, M17 agar (Biolife, Italy) and De Man Ragosa Sharp Agar (MRS agar; Biolife, Italy) for LABs were seeded. PCA media were incubated at 37°C under aerobic conditions (Espeche et al 2012; Sobur et al 2019; Lianou et al 2021; Hassani et al 2022), while M17 and MRS media were incubated at 37°C in microaerophilic conditions for 48 hours (Espeche et al 2012, Taye et al 2021, Steinberg et al 2022) and bacterial counts were calculated in CFU/mL (Taye et al 2021).

Statistical Analysis

Statistical analyses were carried out by using SPSS 22. After counting the bacterial colonies, the values were multiplied according to their dilution factor. Values were recorded as repeated measurements. The logarithm, mean and standard deviation, range, minimum, and maximum values of the mean of these values are given in Table 2. The Kruskal-Wallis test was used to compare the mean logarithmic values between sub-groups of healthy, subclinical, and clinical mastitis since the normality distribution condition from parametric tests was not met. The correlation was confirmed with the Spearsman test (Table 3).

Results

In our study, mammary quarters were grouped based on mastitis as follows: non-mastitis (NM; n=35), clinical mastitis (CM; n=30), and subclinical mastitis (SCM; n=31). In the NM subgroup, the lowest lactation cow was 1, and the highest, 7; in the SCM subgroup, the lowest lactation cow was 1, the highest 8; and in the CM subgroup, the lowest lactation cow was 1, and the highest was 9. The mean age of cows with negative CMT results was significantly younger than those with clinical mastitis (P=0.013). The age, lactation period, and average daily milking number of the animals from which the samples were taken are given in Table 1.

It was found that the logarithmic means of colony counts on MRS media for the NM subgroup, SCM subgroup, and CM subgroup were 2.15±1.02 log CFU/mL, 2.17±0.70 log CFU/mL, and 3.03±1.49 log CFU/mL, respectively. The logarithmic mean of M17 media counts was determined as 3.12±;1.02 log CFU/mL in the NM subgroup, 3.75±0.83 log CFU/mL in the SCM subgroup, and 3.67 ±1.39 log CFU/mL in the CM subgroup. The mean logarithmic PCA in milk samples of the NM subgroup was 3.09±1.10 log CFU/mL, 3.88±0.81 log CFU/mL in samples from the SCM group and 4.09 ± 1.32 log CFU/mL in milk from the CM group (Table 2).

When the logarithmic means between the groups were compared, no difference was observed in the NM, SCM, and CM groups in terms of MRS and M17 media, while the PCA values in the NM group were significantly lower than the SCM (P=0.042) and CM (P=0.015) groups. There was no significant difference between the SCM and CM groups. When the MRS and M17 logarithmic count averages (MRS+M17) were taken and evaluated together, no significant difference was found between the NM, SCM, and CM groups (P=0.093).

Table 1. Comparison of healthy, clinical mastitis and subclinical mastitis milk sample groups in terms of age, lactation period and milking numbers

Group	Age in years	Lactation Period in years	Milking number (Times)
NM	3.50± 2.22	2.71± 1.67 (R:1-7)	2.71± 0.66
SCM	4.25± 2.11	3.03± 1,77 (R:1-8)	2.67± 0.54
CM	4.86± 1.71	3.16± 1.72 (R:1-9)	2.4± 0.49**

NM: Non-mastitis group, SCM: subclinical mastitis group and CM: clinical mastitis group, R: Range Comparison with CM group (**p= 0,013)

Table 2. Average of colony numbers of healthy, clinical mastitis and subclinical mastitis milk sample groups in MRS, PCA and M17 media in log CFU/ml

Media	NM (log CFU/mL)	SCM (log CFU/mL)	CM (log CFU/mL)
MRS+M17	2.88±1.037	3.47±0.82	3.5±1.42
M17	3.12±1.02	3.75±0.83	3.67±1.39
MRS	2.15±1.02	2.17±0.7	3.034±1.49
PCA	3.09±1.1	3.88±0.81*	4.09±1.32**

NM: Non-mastitis group, SCM: subclinical mastitis group, and CM: clinical mastitis group Comparison with NM * p=0,042 **p=0,015

It was observed that there was a negative (P=0.09) correlation between the number of milkings per day and CMT values and a negative (P=0.001) correlation between MRS counts (Table 3). It was determined that there were positive correlations between the lactation period and CMT and age (P=0.041 and P=0.001, respectively), and positive correlations were found between the age of animals and CMT values (P=0.004). Positive correlations (P=0.001; P<0.001; P=0.001 and P>0.001, respectively) were found between PCA mean values and CMT, MRS, M17, and MRS+M17 mean values. There were also positive correlations observed between MRS+M17 and CMT values and MRS+M17 and PCA counts (P=0.001 and P=0.001, respectively).

Discussion

Studies on alternative treatment methods have gained momentum today and attract attention in the veterinary field. When the types of studies on bovine mastitis cases are examined, many approaches including those on comparing CMT results and somatic cell counts, on mastitis diagnostic methods (Özdemir and Kaymaz 2013), on mastitis cases and total bacterial counts (Kasikci et al 2012, Qiao et al 2015) and lastly some studies that aim to determine in vitro properties of LAB strains from mastitic milk (Quigley et al 2013, Diepers et al 2017, Gagnon et al 2020) have been observed. However, to the best of our knowledge no study in the literature correlates total bacterial counts, lactic acid bacteria counts, and CMT data with mastitis cases in milk from healthy and mastitis cattle. A study on the samples collected from milk tanks determined a bacterial load of 332,000 CFU/mL when the geometric averages of the total bacterial numbers were taken (Van Schaik et al 2005). The total number of bacteria was between 6.76±0.039 and 6.83±0.032 log CFU/mL in the milk samples collected by Sobur et al (2019) from different farms. In our study, the average total bacterial load in milk in the NM group was 3.09±1.1 log CFU/mL. Although this value is lower than the studies mentioned above, this variability can be associated with seasonal, environmental, and maintenance conditions (Gagnon et al 2020, Toghdory et al 2022). In the study of Qiao et al (2015) grouping samples taken from mammary quarters with subclinical mastitis according to somatic cell numbers in 12 mild and 28 severe subclinical mastitic quarter milk samples, in the samples with severe subclinical mastitis, an average of 2.61±0.90 log CFU/mL and in the samples with mild mastitis an average of 2.01±0.58 log CFU/mL was detected. In another study, Considering the CMT results, clinical examinations, and somatic cell counts, milk samples from 386 mammary quarters with subclinical mastitis were examined for total bacterial counts. The samples were classified as +, ++, +++ according to the CMT results, and the total bacterial counts were found to be in the range of 3.4771 to 6.9395, from 3.4771 to 7.3617, and from 4.7782 to 7.5315 log CFU/mL, and the mean value was 6.4697 ± 0.5486 log CFU/mL (Kasikci et al 2012). In our current study, samples with subclinical mastitis were not grouped as mild or severe, and the mean total bacterial count in the SC group was found to be 3.88±0.81 log CFU/mL. The present study found that the total bacterial counts in the NM group were significantly lower than those in the SC and CM groups. Although there is no statistically significant difference between the SC and CM groups, when the numerical averages are examined, it is seen that the CM group has a higher load than the SCM group (6.4×10^5 and 3.4×10^4, respectively). A positive correlation between CMT data and total bacterial load was noted (Table 3). These results show that the total number of bacteria increases in parallel with the degree of intramammary infection (Lopes et al 2012, Qiao et al 2015). When the results of CMT were examined in the present study, it was determined clinical mastitis cases occur primarily in older animals with an increasing tendency towards the end of the lactation period. It has been reported that the increase in the prevalence of mastitis with increasing age and lactation stage can be due to pathogens penetrating the teat duct more easily (Kitila et al 2021).

The milk microbiota consists of a wide variety of microorganisms, including bacteria. LAB strains are also among the most common types of microorganisms in milk (Quigley et al 2013). The literature states that LAB strains colonize the mammary, form a protective biofilm that prevents the development of pathogens that cause infection, and prevent mastitis (Rainard and Foucras 2018, Wallis et al 2018). Similar studies were not found regarding LAB counts in milk taken from mammary lobes with clinical

Table 3. Correlation table

	Milking number	Lactation Period	CMT	logPCA	logMRS	logM17	Age (month)	logMRS+M17
Milking number								
Lactation Period	r=-.266** p=0.009							
CMT		r=.209* p=0.041						
logPCA			r=.339** p=0.00					
logMRS	r=-.403** p=0.001			p=.465** r=0.00				
logM17			r=.228* p=0.028	r=.880** p=0.00	r=.418** p=0.001			
Age (month)		r=.860** p=0.00	r=.292** p=0.004					
logMRS+M17			r=.221* p=0.034	r=.887** p=0.00	r=.507** p=0.000	r=.987** p=0.00		

CMT: California Mastitis Test, logPCA: Logarithmic mean of PCA colony numbers, logMRS: Logarithmic mean of MRS colony numbers, logM17: Logarithmic mean of M17 colony numbers, logMRS+M17: Logarithmic mean of MRS+M17 colony numbers

and subclinical mastitis in our studies. However, there are LAB count studies conducted with raw cow's milk taken by different methods. However, there are also various genetic-based studies on microbiological diversity in milk from mammary with mastitis (Oikonomou et al 2012, Catozzi et al 2017, Ronco et al 2018, Wang et al 2020). Taye et al (2021), detected in milk samples collected from farms, houses, and vending machines, an average of 4.5×10^7 CFU/mL Lactobacillus sp. to 1.12×10^7 CFU/mL *Lactococcus* sp. In general, the LAB numbers obtained from the NM group in our study were compatible with the rates previously reported for bovine milk (Quigley et al 2013, Gagnon et al 2020). In parallel with the literature, in our study, the number of cocci was higher in isolated LABs (Steinberg et al 2022).

Raw milk has high water activity and the suitability of its nutrient content allows the growth of microorganisms. LAB strains include genera such as *Lactococcus, Leuconostoc, Lactobacillus,* and *Pediococcus,* including *Streptococcus* genera. These bacteria are Gram-positive, catalase-negative, and commonly found in milk (Gagnon et al 2020). According to a study by Wang et al (2020) on the milk microbiome and metabolome, using 16S rDNA sequence analysis, it was found that the frequency of *Streptococcus* sp. was 2.21 and 1.67 times higher in the unhealthy group compared to the healthy group. Similarly, the frequency of *Staphylococcus* sp. was found to be increased by 4.25 and 2.35 times, respectively. The same study stated that these two bacterial species were found at the highest rate in milk with clinical mastitis. Milk microbiota varies in healthy, clinical, and subclinical mastitis cases (Wang et al 2020). Qiao et al (2015) conducted a genetic-based study concerning some pathogens in samples taken from cows with mild and severe subclinical mastitis. They examined the rates of *Lactobacillus* in these cases. In the aforementioned study, they found that while higher amounts of Lactobacilli and lower rates of the pathogen were observed in samples with mild subclinical mastitis, the opposite trend was observed in the group with severe subclinical mastitis. The researchers concluded that as the severity of mastitis cases increased, the amount of *Lactobacillus* decreased and that there could be a significant relationship between cattle udder health and the number of *Lactobacillus* in milk. In our study, according to the colony count results obtained from MRS media, an average bacterial load of $1.2x10^3$, $3.9x10^2$, $1.4x10^5$ CFU/mL was determined in the NM, SCM, and CM groups, respectively. In our study, no statistically significant difference was observed in LAB counts in the NM, SC, and CM groups according to the colony count results performed on MRS and M17 media. When the averages of MRS and M17 colony counts were examined together, it was determined as 5.3×10^3, 1.5×10^4, and 1.8×10^5 CFU/mL in the NM, SCM, and CM groups, respectively. When the average count results of the M17 medium were examined, it was seen that the NM group contained 9.5x103 CFU/mL; the SCM group contained $3.1x10^4$ CFU/mL and the CM group contained $2.1x10^5$ CFU/mL bacterial load. The low selectivity of the MRS medium may have allowed different types of bacteria to ferment carbohydrates and multiply, which could have led to the growth of pathogenic bacteria in CM and SCM samples, causing an increase in their numbers (Steinberg et al 2022). Steinberg et al (2022), examined the MRS medium in terms of logarithmic averages of colony numbers, it was stated that there was no statistically significant difference between animals from different farms, udder health, and breeds. Our findings are in agreement with these results.

As a result, the total bacterial count in milk, as well as the LAB load, are affected by the growing environment and environmental conditions. The milk microbiota is altered in healthy udder quarters and clinical and subclinical mastitis cases. In our study, LAB counts increased in direct proportion to the total amount of bacteria in subclinical and clinical mastitis cases. However, this might be related to a

complex microbial environment in which a number of lactic acid bacteria group are present and involved in udder's health.

Some studies have reported that various LAB strains can protect udder against mastitis when used through feed supplementation, teat dipping, or intramammary inoculation by their strong immunomodulatory activities (Klostermann et al 2008, Pellegrino et al 2017, Yu et al 2017, Rainard and Foucras 2018). Nipple immersion using a teat disinfectant containing probiotic bacteria was reported to be superior to commercial disinfectant in reducing somatic cell count (Yu et al 2017). Such environmentally friendly lactic acid bacteria preparations can replace commercially available chemical disinfectants. There are also studies with promising results with intramammary inoculation trials of selected strains among the LAB strains, whose inhibitory properties have been determined for prevention or treatment.

In this study, a general evaluation of the microbial load in milk in terms of LAB and total bacteria in cases of mastitis and in healthy conditions in cattle was made with classical methods. In order to prevent the spread of antimicrobial resistance, which poses a threat to the future, current alternative methods. The presence of probiotics has been highlighted. In addition, the bacteriological load in milk in health and disease has been evaluated in general. Our work on this subject continues, and we present the data obtained as a basis for further studies. In order to determine the bacterial load and diversity in milk in healthy and mastitis cases in dairy cows, we would like to emphasize that more comprehensive and advanced studies are needed.

Conclusion

New protection and treatment approaches on especially udder's health in cattle breeding by modulating udder's flora can contribute to reduce higher rates of antibiotic use and prevent the development of antimicrobial resistance in dairy cattle sector. Therefore, it is essential to investigate further the udder and milk microbiota characteristics in bovine mastitis cases and healthy animals to isolate beneficial bacteria adapted to the target species from udder flora, and to increase studies to identify qualified strains among them.

Conflict of Interest

The authors declared that there is no conflict of interest.

Acknowledgements

We would like to thank Gaye Keleş and Simge Çilenger for their support in milk collection and laboratory studies.

Funding

This study was supported by the Scientific and Technological Research Institution of Turkiye (TUBITAK) (Project Number of TUBITAK 2209-A, 1919B012101033).

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Author Contributions

Motivation/Concept: SY, MŞ; Design: SY, MS; Control/Supervision: SY, MS; Data Collection and Processing: SY, MS, EMK; Analysis and Interpretation: SY, MS, EMK; Literature Review: SY, MS, EMK; Writing the Article: SY, MS, EMK; Critical Review: SY, EMK

Ethical Approval

Animal Experiments Local Ethic Committee of Mugla Sitki Kocman University 2021/13 Number Ethics Committee Decision.