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Identification of *Enterococcus spp*. and *Lactococcus spp*. Strains Isolated from Bovine Mastitis by MALDI-TOF MS and Evaluation of Antimicrobial Resistance Profiles

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Abstract

Mastitis is an important problem affecting animal health, welfare, and economy. Bacterial agents play a dominant role in the disease. The role of enterococcal and lactococcal species among environmental bacterial agents in mastitis has been underestimated due to inadequate identification. The aim of this study was to isolate and identify *Enterococcus* spp. and *Lactococcus spp*. from mastitic bovine milk and to evaluate the agents phenotypically in terms of antimicrobial resistance. A total of 108 milk samples from cattle with suspected mastitis were analyzed for enterococci and lactococci by standard microbiological techniques and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). By standard microbiological methods, 38 (35%) *Enterococcus* spp. and 13 (12%) *Lactococcus* spp. were isolated and identified from 51 milk samples. In species-level identification with MALDI-TOF MS, six different enterococci and three different lactococci were identified within acceptable score ranges. In antibiogram tests performed with the standard Kirby-Bauer method using 10 antimicrobials, 26% of the *Enterococcus* spp. and 46% of the *Lactococcus* spp. were resistant to at least 50% of the antimicrobials tested. In *Enterococcus* spp., the highest resistance rates were observed for enrofloxacin (79%) and ampicillin + cloxacillin (71%), while the best sensitivity (100%) was obtained for penicillin and ampicillin. In *Lactococcus* spp., the highest resistance rate was observed for enrofloxacin (85%) and amoxicillin + clavulanic acid (70%) and the best sensitivity (100%) was obtained for penicillin, ampicillin, and gentamicin.

As a result, it was concluded that the diversity and high rate of antimicrobial resistance of enterococcal and lactococcal species in mastitis isolates poses a serious potential threat to animal and public health.

Keywords: Bovine mastitis, *Enterococcus* spp., *Lactococcus* spp., Antimicrobial resistance, MALDI-TOF MS

Introduction

Mastitis, defined as mammary gland inflammation, is a major problem affecting the dairy industry worldwide. Bacterial agents constitute the primary source of the disease, which has many identified causative agents (1). Among the common bacterial agents classified into two classical groups as infectious and environmental agents, *Staphylococcus aureus*, *Streptococcus (S) agalactiae*, and *S. dysgalactiae* are classified as infectious pathogens, while *Escherichia coli*, *S. uberis*, and *Enterococcus* spp. are classified as environmental pathogens (1,2). Environmental agents from the genera *Enterococcus* and *Lactococcus* are closely related to bacteria in the genus *Streptococcus*, and information on their roles in mastitis remains limited, mostly due to a lack of identification (3,4). However, the increasing availability of proteomic and nucleic acid-based techniques, which are more specific and rapid identification methods than conventional microbiological identification, has made the diagnosis of mastitis agents more specific (5-7). Among the routine laboratory diagnostic methods, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a readily available and powerful

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proteomic technique, stands out for its rapid results and cost-effectiveness compared to classical phenotypic and genotypic identification. This technique plays a crucial role in the specific diagnosis of bacterial infections, such as those caused by enterococci and lactococci, which are difficult to identify at genus and species levels with traditional methods (6-8).

Mastitis is one of the animal husbandry problems requiring antibiotic treatment, and the frequent use of antimicrobials is one of the reasons for the development of antimicrobial resistance among bacteria. Therefore, specific identification of bacterial mastitis agents and the analysis of their antimicrobial susceptibilities play important roles in the fight against the disease.(9,10) Enterococcal and lactococcal species are resistant to multiple antimicrobials intrinsically or due to various reasons (11-13). In cases of mastitis in which these agents play a role, it is important to diagnose the primary agent and apply appropriate treatment to protect animal and public health and prevent the administration of unnecessary antimicrobials. The aim of this study was to isolate *Enterococcus* spp. and *Lactococcus* spp. from mastitic bovine milk and to phenotypically evaluate them in terms of species-level identification and antimicrobial resistance.

Materials and Methods

This study was conducted with milk samples taken from cattle suspected of clinical (n=55) or subclinical (n=53) mastitis brought to the laboratory of Harran University's Faculty of Veterinary Medicine, Department of Microbiology, in 2021 and 2022 upon a preliminary diagnosis of mastitis according to clinical signs or the California mastitis test (14). These samples were stored at -80 °C.

Microbiological Culture

Samples were subjected to standard culture methods for microbiological analysis in the laboratory. Briefly, 108 milk samples were inoculated on 5% sheep blood agar (Merck, Germany) and bile esculin agar (Merck, Germany), tryptic soy agar (Oxoid, United Kingdom) with 6.5% NaCl, and Mann Rogosa Sharpe (MRS) agar (Neogen, United Kingdom) incubated aerobically at 37 °C for 24-48 hours. The microorganisms grown were evaluated for *Enterococcus* spp. and *Lactococcus* spp. in terms of colony characteristics, Gram staining, catalase, indole, and other biochemical properties (14,15).

Identification by MALDI-TOF MS

Isolates presumed to be *Enterococcus* spp. and *Lactococcus* spp. based on conventional cultural and biochemical methods were identified to the species level with a MALDI-TOF MS device (Bruker Microflex LT, Germany) following the method reported by Mercanoğlu Taban and Numanoğlu Çevik in 2021 (16).

Alpha-cyano-4-hydroxycinnamic acid (HCCA; Bruker, Germany) was used as the matrix for MALDI-TOF MS. Acetonitrile (ACN, HPLC grade; Sigma-Aldrich, USA), trifluoroacetic acid (TFA; Sigma-Aldrich, USA), and DNA- and RNA-free 0.1-µm membrane-filtered ultrapure water (Sigma-Aldrich, USA) were utilized. For mass calibration, a Bruker bacterial test solution containing *E. coli* RNase and myoglobin protein profiles was also employed.

For microbial biomass analysis using MALDI-TOF MS, a single colony was collected with a sterile wooden stick and streaked onto a well of a 96-well special micro steel plate (MSP; Bruker Daltonics, Germany) in a film-like manner. After drying, 1 µL of HCCA matrix solution (12.5 mg/mL HCCA in a mixture of 50% ACN and 2.5% TFA) was added and allowed to dry completely at room temperature. The MALDI 96 MSP was placed into the MALDI-TOF MS device and operated using an optimized method in linear positive ion mode in the mass range of 2,000-20,000 Da for the identification of microorganisms. A nitrogen laser operating at 337 nm with a frequency of 60 Hz was used as the ion source. Laser pulses consisting of 240 bursts of 40 packets were applied for each column's measurement to obtain the spectra. Each sample was run in triplicate and the readings with the highest values were included in the analysis.

According to the explanatory table for Bruker Daltonics MALDI Biotyper scores, the isolates were evaluated according to the criteria of high probability of species identification within the score range of 2.300-3.000, reliable genus and possible species identification within the score range of 2.000-2.299, possible genus identification within the score range of 1.700-1.999, and unreliable identification within the score range of 0.000-1.699.

Antimicrobial Susceptibility Analysis

The susceptibility of *Enterococcus* spp. and *Lactococcus* spp. strains to ten antimicrobial agents was tested using the standard Kirby-Bauer method (17). Briefly, suspensions of 0.5 McFarland density were prepared in physiological saline from fresh cultures of the strains on blood agar and spread homogeneously on Mueller-Hinton agar with a swab.

Tylosin (30 mg, TY), oxytetracycline (30 mg, T), ampicillin

+ cloxacillin (30 mg, APX), penicillin (10 U, P), gentamicin (10 mg, CN), enrofloxacin (10 mg, ENR), ampicillin (10 mg, AM), cefquinome (10 mg, CEQ), spiramycin (100 mg, SP), and amoxicillin + clavulanic acid (30 mg, AMC) antimicrobials (all from Bioanalyse, Türkiye) were placed on medium with at least 2 cm between them and incubated at 37 °C for 24 hours. At the end of the incubation period, zone diameters were measured with calipers and values were interpreted according to the appropriate reference values from the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (17-20). In the absence of criteria for enterococci in the CLSI guidelines, guidelines for streptococci and the Bioanalyse internal quality control limits were used. In antibiogram tests, lactococcal isolates were evaluated according to the same criteria applied for enterococcal isolates. *Staphylococcus aureus* ATCC 25923 was used as a quality control strain.

Results

Thirty-eight (35%) *Enterococcus* spp. and 13 (12%) *Lactococcus* spp. isolates were collected from 108 cattle milk samples characterized by conventional microbiological methods in cows suffering from mastitis. Thirty-four enterococci were isolated from the milk samples of cattle diagnosed with clinical mastitis and four enterococci were isolated from milk samples of cattle diagnosed with subclinical mastitis. While 12 lactococci were isolated from cattle with subclinical mastitis, one strain was isolated from a clinical mastitis sample.

The enterococci (n=38) were identified as *E. faecalis* and *E. casseliflavus* (13 each), *E. faecium* (n=5), *E. hirae* (n=2), *E. mundtii* (n=3), *E. italicus* (n=1), and *E. devriesei* (n=1) by MALDI-TOF MS. The score value of the isolate identified as *E. devriesei* (1.97) was determined to reflect only possible genus-level identification (Table 1). Thirteen strains of lactococci were identified, including eight identified as *L. garvieae*, four as *L. lactis*, and one as *L. raffinolactis* (Table 2). Of the identified enterococci isolates, 31.58% showed catalase activity; all of those isolates were *E. faecalis* (n=12). Enterococci isolated from the milk of cattle with subclinical mastitis (n=4) were identified as *E. faecalis*, and the lactococcal strain isolated from the clinical mastitis samples (n=1) was identified as *L. lactis*.

According to the antibiogram tests performed while considering standard antimicrobial preparations for the treatment of mastitis in cattle, the highest resistance among the enterococci was seen for enrofloxacin, ampicillin + cloxacillin, and cefquinome at 78.95% (n=30), 71% (n=27), and 55.26% (n=21), respectively. In comparison, the best sensitivity was obtained for penicillin (100%) and ampicillin (100%) (Table 1). Among the 38 enterococci strains isolated, 7.9% (n=3) were susceptible to all antimicrobials. It was observed that 26.32% of the enterococci were resistant to 50% of the antibiotics used in the antibiogram tests (n=10). Enterococci isolated from milk samples with subclinical mastitis (n=4) were identified as *E. faecalis* and these strains were resistant to at least four of the 10 antimicrobial agents tested. *E. mundtii* (n=3), *E. hirae* (n=2), and *E. italicus* (n=1) isolates from milk with clinical mastitis were all resistant to more than one antimicrobial agent (Table 1).

Table 1 Antimicrobial resistance profile of *Enterococcus* spp. and species.

	Number/ratio of antimicrobial resistance (n/%)									
	TY	**T**	**APX**	**P**	**CN**	**ENR**	**AM**	**CEQ**	**SP**	**AMC**
***Enterococcus* spp. (n=38)**	**9/23.68**	**5/13.15**	**27/71**	**0**	**14/36.84**	**30/78.95**	**0**	**21/55.26**	**6/15.79**	**13/34.21**
E. faecalis (n=13)	6/46.15	2/15.38	9/69.23	0	5/36.46	12/92.3	0	8/61.54	5/38.46	2/15.38
E. faecium (n=5)	0	0	3/60	0	5/100	5/100	0	4/80	0	0
E. casseliflavus (n=13)	0	2/15.38	9/69.23	0	0	7/53.85	0	4/30.77	0	5/38.46
E. hirae (n=2)	2/100	0	2/100	0	2/100	2/100	0	2/100	1/50	2/100
E. mundtii (n=3)	1/33.33	0	3/100	0	2/66.66	3/100	0	3/100	0	3/100
E. italicus (n=1)	0	1/100	1/100	0	0	1/100	0	0	0	1/100

TY: Tylosin, 30 mg; T: Oxytetracycline, 30 mg; APX: Ampicillin + cloxacillin, 30 mg; P: Penicillin, 10 U; CN: Gentamicin, 10 mg; ENR: Enrofloxacin, 10 mg; AM: Ampicillin, 10 mg; CEQ: Cefquinome, 10 mg; SP: Spiramycin, 100 mg; AMC: Amoxicillin + clavulanic acid, 30 mg.

Table 2 Antimicrobial resistance profile of *Lactococcus spp*. and species.

	Number/ratio of antimicrobial resistance (n/%)									
	TY	**T**	**APX**	**P**	**CN**	**ENR**	**AM**	**CEQ**	**SP**	**AMC**
***Lactococcus* spp. (n=13)**	**8/61.54**	**1/7.7**	**8/61.54**	**0**	**0**	**11/84.61**	**0**	**4/30.77**	**8/61.54**	**9/69.23**
L. lactis (n=4)	1/25	1/25	2/50	0	0	3/75	0	0	3/75	2/50
L. garvieae (n=8)	7/87.5	0	6/75	0	0	8/100	0	4/50	5/62.5	7/87.5
L. raffinolactis (n=1)	0	0	0	0	0	0	0	0	0	0

TY: Tylosin, 30 mg; T: Oxytetracycline, 30 mg; APX: Ampicillin + cloxacillin, 30 mg; P: Penicillin, 10 U; CN: Gentamicin, 10 mg; ENR: Enrofloxacin, 10 mg; AM: Ampicillin, 10 mg; CEQ: Cefquinome, 10 mg; SP: Spiramycin, 100 mg; AMC: Amoxicillin + clavulanic acid, 30 mg.

It was further observed that 84.6% (n=11) of lactococci isolates showed a high level of resistance to enrofloxacin, followed by amoxicillin + clavulanic acid at 69.23% (n=9). Similarly to the enterococci, all lactococcal strains were susceptible to penicillin and ampicillin. All strains were also fully susceptible to gentamicin. Furthermore, 46.15% (n=6) of the lactococci were resistant to at least 50% of the antimicrobials tested. *L. garvieae* isolates were highly resistant to enrofloxacin (100%), tylosin (87.5%), and amoxicillin + clavulanic acid (87.5%) (Table 2). Among the ten antimicrobial agents tested, the enterococcal and lactococ-

cal strains showed a similar level of resistance to penicillin and ampicillin (0%), while differences were observed for resistance to other antimicrobial agents (Figure 1).

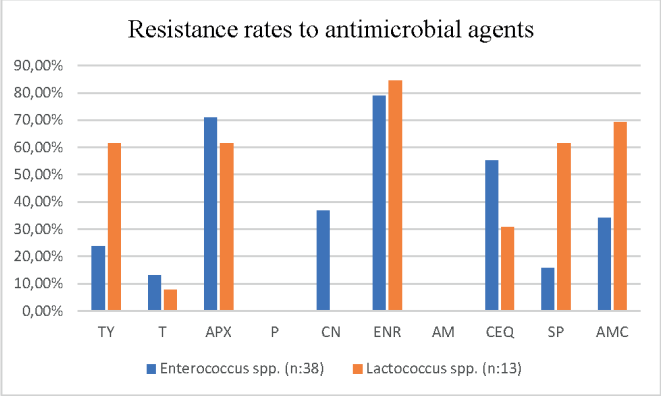

Figure 1 Antimicrobial resistance rates of *Enterococcus* spp. and *Lactococcus spp*. strains.

TY: Tylosin, 30 mg; T: Oxytetracycline, 30 mg; APX: Ampicillin + cloxacillin, 30 mg; P: Penicillin, 10 U; CN: Gentamicin, 10 mg; ENR: Enrofloxacin, 10 mg; AM: Ampicillin, 10 mg; CEQ: Cefquinome, 10 mg; SP: Spiramycin, 100 mg; AMC: Amoxicillin + clavulanic acid, 30 mg.

Discussion

Enterococci and lactococci, environmental mastitis agents, are Gram-positive cocci-like bacteria that are part of the host's flora. These microorganisms, which have the ability to survive outside the host, can cause mastitis in cattle at many stages of inadequate farm management, including milking or calving, the dry period, or times of abundance of infectious agents caused by humidity and temperature (21,22). Although there are many studies on bovine mastitis in many regions, including Türkiye, data on enterococcal and lactococcal diversity and the antimicrobial resistance profiles associated with mastitis are limited (4,23-28). In the present study, enterococci and lactococci isolated from the milk of cattle with clinical and subclinical mastitis were identified by MALDI-TOF MS and analyzed for antimicrobial resistance by disk diffusion method.

Jahan et al. compared the results of bacterial identification by MALDI-TOF MS with those of 16S rDNA sequencing, a highly reliable molecular method, and reported that MALDI-TOF MS was highly accurate in identifying common mastitis pathogens such as *Staphylococcus* spp., *Streptococcus* spp., *Lactococcus* spp., *Escherichia* spp., *Klebsiella* spp., and *Pseudomonas* spp (8). In the present study, species-level identification of 51 isolates of milk samples with mastitis caused by enterococci (n=38) and lactococci (n=13), which were identified at the genus level by traditional diagnostic methods, was performed by MALDI-TOF MS and it was determined that the species-level identifications of 50 isolates were within the range of reliable scores.

Enterococci are important environmental agents that play a role in mastitis (2). In the present study, enterococci were identified at a rate of 35.18% from among 108 mastitic bovine milk samples. In 2021, Ahmed et al. found the prevalence of enterococci isolated from mastitic milk to be 34.0% in their study conducted in Egypt (28). Different rates have been reported regarding the isolation of enterococci from mastitis in the studies conducted to date. This may be attributed to various factors, such as different identification techniques, geographical regions, and farm conditions (25,26,30-32). Previous studies showed that *E. faecalis* was the dominant enterococcal mastitis species (25,29,33,34). In the present study, *E. faecalis* (n=13), *E. casseliflavus* (n=13), *E. faecium* (n=5), *E. mundtii* (n=3), *E. hirae* (n=2), and *E. italicus* (n=1) were identified and *E. faecalis* and *E. casseliflavus* were the species with the highest rates (34.21%).

In this study, lactococcal species were isolated and identified at a rate of 12.03%, although it is not certain whether lactococci are primary factors in the development of mastitis. Although lactococci are also isolated from healthy milk, they have been reported to be isolated more often from cattle with mastitis (4). In 2016, Rodriques et al. found that lactococci were the primary pathogens in a mastitis outbreak (4) and *L. garvieae* was one mastitis isolate described as an emerging zoonotic pathogen (35). In 2023, Xie et al. reported that *L. garvieae* was isolated at a rate of 3.4% from 1441 clinical mastitis samples (12). In the present study, although *L. garvieae* was isolated from 108 mastitic milk samples with a rate of 7.4%, all of these isolates were obtained from the milk of cattle with subclinical mastitis (n=53). *L. lactis* was isolated from cattle milk samples with clinical and subclinical mastitis at a rate of 3.7%, while *L. raffinolactis* was identified from one subclinical mastitis sample (Table 2). The higher rate of isolation of lactococci from the milk of cattle with subclinical mastitis compared to those with clinical mastitis is consistent with the findings of Sorge et al (2). Further research is needed to reveal the relationship of these agents with mastitis and to understand their effects on public health (2,4,36,37).

In the current CLSI guidelines (VET01S, 6th Edition), the antimicrobial susceptibility criteria for environmental mastitis agents are very limited (18). In the present study, enterococci and lactococci were evaluated phenotypically for antimicrobial resistance by disk diffusion method according to the appropriate criteria for enterococci. Ten antimicrobials were tested and 78.95% of the enterococcal specimens were resistant to enrofloxacin, 71% to ampicillin + cloxacillin, 55.26% to cefquinome, 36.84% to

gentamicin, and 34.21% to amoxicillin + clavulanic acid. The rate of isolates susceptible to all antibiotics used (Table 1) was determined as 7.9%. Enterococci are known to be resistant to antimicrobials due to natural resistance or resistance acquired through various mechanisms (38). Resistance to multiple antimicrobials among enterococci has been demonstrated in many previous studies that support the results of the present study (30,33,38). Enterococcal isolates were found to be 100% susceptible to ampicillin in the present study. While this finding largely supports the previous studies conducted in Türkiye and other countries, significant differences were found in the susceptibility rates of the other tested antimicrobials (25,30,32,34,39). There may be many reasons for this, including the extent of antimicrobial exposure, the number of isolates analyzed, and laboratory techniques.

There is a serious gap in the literature regarding the diversity and antimicrobial susceptibility of lactococcal mastitis isolates. Plumed-Ferrer et al. did not detect resistance to penicillin, ampicillin, or amoxicillin among the lactococci evaluated in their study (40). Similarly, resistance to penicillin, ampicillin, or gentamicin was not detected among the isolates of the present study (Table 2). It was determined that 84.6% of lactococcal strains were resistant to enrofloxacin and 69.23% to amoxicillin + clavulanic acid. Werner et al. found that all of the lactococcal strains isolated from cattle with mastitis in their study were susceptible to enrofloxacin and amoxicillin + clavulanic acid (37). In China, Lin et al. found that *L. garvieae* strains isolated from cattle with clinical mastitis were fully susceptible to penicillin and ampicillin, similar to our findings, and full susceptibility to amoxicillin + clavulanic acid was also found (35). In contrast, we determined amoxicillin + clavulanic acid resistance among 87.5% of our isolates.

It has been scientifically proven that the widespread use of antimicrobials for diverse purposes in animal production leads to the emergence of resistant strains (41). The diversity and intensity of antimicrobial use varies among countries, and the high level of antimicrobial use in Türkiye is increasing the counts of resistant bacteria (42). The high level of resistance to multiple antimicrobials (Figure 1) among enterococci and lactococci in the present study is alarming and a larger prevalence study is needed.

Conclusions

In the present study, enterococci and lactococci, described as minor mastitis pathogens, were isolated at notably high rates from mastitic milk samples. The diversity observed at the species level among the isolates, the zoonotic nature of a considerable portion of these species, and the observation of resistance to multiple antimicrobials were found to be concerning for both animal and public health. It is important to identify these agents with advanced discriminative techniques, monitor their antimicrobial resistance, and conduct more studies on the subject.

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