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Research Article

EVALUATION OF VIRULENCE AND ANTIMICROBIAL RESISTANCE GENES OF *ENTEROCOCCUS* SPECIES ISOLATED FROM SHEEP MILK WITH SUBCLINICAL MASTITIS

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ABSTRACT. This study aimed to investigate the formation of antimicrobial resistance genes associated with vancomycin and gentamicin, as well as the capacity to develop biofilms, in *Enterococcus* sp. strains isolated from subclinical mastitis in sheep. In total, *Enterococcus faecalis* (22/26, 84.62%) was found to be the most prevalent species among the samples of mastitic milk collected from sheep. In general, *E. faecalis* strains exhibited higher resistance compared to *Enterococcus faecium*. After isolation and identification using polymerase chain reaction, a total of twenty-six enterococci (22 *E. faecalis*, 4 *E. faecium*) were submitted to susceptibility tests against eight antimicrobial agents. The isolates indicated multidrug resistance to amoxicillin-clavulanic acid (100%), kanamycin (100%), and trimethoprim-sulfamethoxazole (100%). All isolates were susceptible to ampicillin (100%), and florfenicol (100%) and vancomycin susceptibility was observed in 53.85% of the isolates. The dominant antimicrobial resistance genes detected in our isolates were *vanB* (19.23%), *aph(2")-Ia1* (61.54%), *aph(2")-Id1* (26.92%), and *esp* (65.38%) as the most common virulence gene. This research indicated a significant occurrence of antimicrobial resistance in *E. faecalis* and *E. faecium* strains obtained from subclinical sheep mastitis. The increasing resistance of enterococci to antibiotics poses a growing challenge to both human well-being and the environment.

Keywords: *Antimicrobial resistance, Enterococcus faecalis, Enterococcus faecium, sheep mastitis, virulence genes.*

INTRODUCTION

Mastitis is a common condition that affects both sheep and cattle. The categorization of diseases is determined based on the absence or presence of clinical or subclinical symptoms. It leads to reduced milk yield and quality, increased veterinary expenses, and the need to cull severely affected animals [1]. Many pathogens, such as coliforms (*Escherichia coli*, *Enterobacter aerogenes*), environmental Streptococci (*Streptococcus dysgalactiae*, *S. uberis*), and Enterococci (*Enterococcus faecium*, *E. faecalis*), can cause environmental mastitis. *Enterococcus* species are Gram-positive and catalase-negative cocci which cannot be decisively differentiated from similar bacteria through biochemical tests because of the presence of strains that share high phenotypic and biochemical

similarities and possess abnormal phenotypes [2]. *Enterococcus* demonstrates salt tolerance and bile resistance, allowing it to thrive even in the presence of sodium azide. The bacterium can produce of producing acids from various sugars but cannot produce acids from certain other sugars [3]. Enterococci are frequently present in the intestinal tracts and faecal matter of animals [4]. These opportunistic bacteria from the gut flora are causing significant infections in humans, especially in hospitals lately. *E. faecalis* and *E. faecium* are responsible for 80.0% and 10.0% to 15.0% of these infections roughly [5].

The widespread use of antimicrobials in the treatment of mastitis leads to the emergence of antimicrobial resistance. The *Enterococcus* genus inherently demonstrates resilience to various antimicrobial agents, including aminoglycosides and β-lactam antibiotics. Additionally, it has acquired resistance to ciprofloxacin, erythromycin, linezolid tetracycline, and vancomycin [6]. While Enterococci are typically susceptible to vancomycin, there has been an observed increase in strains carrying associated with resistance that possesses the capacity to be transmitted. Strains are known as vancomycin-resistant enterococci (VRE) and are classified as super bacteria based on their genotypic and phenotypic characteristics. Also, the existence of vancomycin-resistant enterococci in cases of subclinical mastitis can result in human infection through the consumption of milk contaminated with these bacteria. This poses a significant public health concern as vancomycin-resistant enterococci can act as a foodborne pathogen [7]. Recent reports have indicated an alarming rise in resistance among enterococci species, particularly against high-level aminoglycosides, beta-lactams, and glycopeptides [8].

In clinical strains of *Enterococcus* species, several virulence genes have been identified, such as *ace*, *cylA*, *es*p, *gelE*, and *hyl*, which contribute to enhancing the pathogenicity of these bacteria [9]. The primers designed to target the *tuf* gene (*EF-Tu*), known as the elongation factor, in enterococci serve to detect and identify bacteria at the genus level. Besides, primers designed explicitly for the D-alanine/D-ligase gene (*ddl*) are utilized to identify bacteria at the species level [10].

Pathogenic contaminants that are commonly present in milk and dairy products, such as *Enterococcus* sp. have the potential to cause various public health issues within the dairy industry. These problems may include biofilm formation, food spoilage, and outbreaks of foodborne illnesses [11].

This study aimed to reveal the presence of enterococci causing subclinical mastitis in Küçük Menderes Basin in Türkiye sheep and also to investigate the antimicrobial resistance genes and virulence genes in these isolates.

MATERIALS AND METHODS

Sample Collection

A total of 100 mastitic milk samples were collected from sheep of various breeds and ages in dairy farms located in Küçük Menderes Basin (Bayındır-BA, Beydağı-BYD, Kiraz-K, Ödemiş-Ö, and Tire-T) between November and February 2022. Teats were cleaned and disinfected with 75% ethanol before collecting milk samples. Milk samples were taken from udders with CMT results of ++ and +++. The first foremilk was discarded, and 10-15 ml was collected aseptically into sterile tubes. The samples were kept under cold chain conditions and transported to the molecular laboratories of Aydin Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology for laboratory analysis.

Isolation and Identification of Enterococci

In the laboratory, milk samples from cows with mastitis were spread on sheep blood agar (Oxoid, USA) plates. Subsequently, the Petri dishes were incubated in aerobic conditions at 37 °C and observed every 24 hours for three days. A catalase test was performed on samples containing Gram-positive cocci. Those with a negative catalase test were classified as *Streptococcus* sp. and inoculated onto Kanamycin Aesculin Azide agar (Oxoid, USA) to identify enterococci. Following incubation at 37 °C for 24 hours, the agar plates underwent careful selection of colonies (black), which were subsequently transferred onto agar (brain heart infusion). To determine the genus-level identification of the *Enterococcus* isolates, a set of tests including the oxidase test, PYR test, and the 6.5% NaCl reproduction test were performed. The isolated bacteria were confirmed as *Enterococcus* spp. and subsequently kept in a deep freeze at -20 °C for PCR analyses [12].

DNA extractions

The *Enterococcus* sp. were subjected to DNA isolations using genomic DNA extraction units (Fermentas®) following the recommended procedure. The extracted DNA samples were subsequently stored and preserved at -20 °C.

Primers

The utilized primer pairs to identify *E. faecium* - *E. faecalis* and detect to *esp* gene, vancomycin and aminoglycoside resistance genes are presented below in Table 1.

Table 1. *The used specific primer pairs and the expected amplicon sizes to correspond*

	Target gene	**Primer sequences (5'-3')**	**Amplicon size (bp)**	**References**
***Enterococcus* sp.**	*tuf*	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112	[13]
E. faecium	*ddl* $_{E.faecium}$	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC	550	[14]
E. faecalis	*ddl* $_{E.faecalis}$	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	941	[14]
Vancomycin resistance genes	*vanA*	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	[15]
	vanB	ACCTACCCTGTCTTTGTGAA AATGTCTGCTGGAACGATA	300	[16]
Gentamicin resistance genes	*aph(2")-Ia1*	GAGCAATAAGGGCATACCAAAAATC CCGTGCATTTGTCTTAAAAAACTGG	369	[17]
	aph(2")-Ib1	TATGGATCCATGGTTAACTTGGACGCTGG ATTAAGCTTCCTGCTAAAATATAAACATC TCTG	867	[17]
	aph(2")-Ic1	TGACTCAGTTCCCAGAT AGCACTGTTCGCACCAAA	444	[17]
	aph(2")-Id1	GGTGGTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC	641	[17]
Esp gene	*esp*	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGAA	933	[18]

Positive Control

In our study, *E. faecalis* ATCC 29212 and *E. faecium* ATCC 700221 strains were utilized as positive controls.

Polymerase Chain Reaction (PCR)

The detection of *E. faecalis* and *E. faecium* was accomplished through a PCR test utilizing primer pairs described by [19] for the detection of *ddl E. faecalis* and *E. faecium*. The PCR amplifications were conducted following the established method using a thermal cycler (Eppendorf® Mastercycler Personal).

The cycling parameters for PCR consisted of an initial denaturation step at 94 °C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 54 °C for 1 minute, extension at 72 °C for 1 minute, and a final extension step at 72 °C for 10 minutes [19]. The PCR assay used to identify the presence of gentamicin resistance genes followed specific conditions: an initial denaturation at 94 °C for 20 seconds, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds, and extension at 72 °C for 30 seconds. Subsequently, a final elongation step at 72 °C for 30 seconds was performed [17]. For the detection of the *esp* gene, PCR amplifications were carried out using the previously documented primer pair *esp*, as described in the study by [20]. The amplification process was conducted under the following conditions: an initial denaturation step at 94 °C for 1 minute, followed by 30 cycles consisting of denaturation at 94 °C for 45 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 1 minute.

Detection of the Amplification Product

The PCR amplicons (10 µl) were analysed in 2% agarose gel electrophoresis using ethidium bromide staining (0.5 µg/ml) and at 80 volts for 40 minutes. PCR analysis was used to identify *Enterococcus* sp., with *E. faecalis* species yielding a 941 bp product and *E. faecium* species yielding a 550 bp product. To determine the presence of vancomycin and gentamicin resistance genes, as well as the *esp* gene related to biofilm formation, the following amplicon sizes were analysed: 369 bp for *aph(2'')-Ia*, 867 bp for *-Ib*, 444 bp for *-Ic*, 641 bp for *-Id*, and 732 bp for *vanA*, 300 bp for *vanB*, and 933 bp for *esp*.

Determination of Antimicrobial Susceptibility of Enterococcus Isolates

The antibiotic susceptibility of the enterococci isolates was assessed using the disc diffusion method. The bacteria were inoculated onto Mueller-Hinton agar and exposed to various antimicrobials, including amoxicillin-clavulanic acid (30 µg), ampicillin (10 µg), erythromycin (15 µg), gentamicin (10 µg), florfenicol (30 µg), kanamycin (30 µg), and trimethoprim-sulfamethoxazole (25 µg) and vancomycin (30 µg) (Oxoid, USA). *Enterococcus faecalis* ATCC 29212 was used as a standard strain in antimicrobial susceptibility tests. The test results were evaluated based on the Clinical and Laboratory Standards Institute [21].

RESULTS AND DISCUSSION

Phenotypic and genotypic identification of Enterococcus isolates

In our study, 100 mastitic milk samples collected from sheep were analysed for the description of *Enterococcus* species. A total of 26 *Enterococci* sp. (26%) were detected using PCR. Out of the isolates tested, 22 (84.61%) *E. faecalis* were detected, while 4 (15.39%) were classified as *E. faecium* using specific primer pairs targeting *ddl* genes for *E. faecalis* and *E. faecium*. The gel image of the isolates is given in Figure 1.

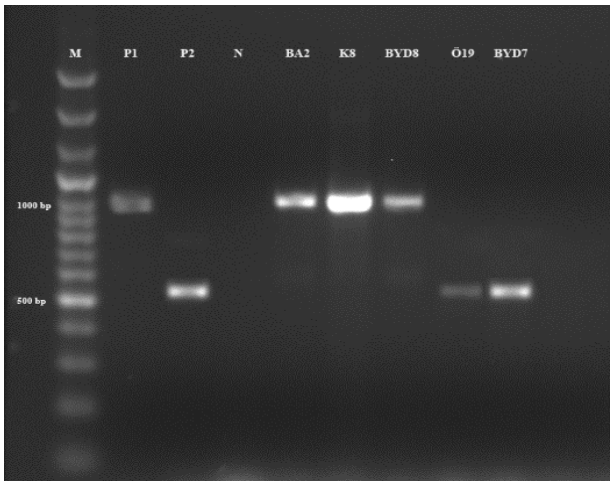

Fig. 1. *Multiplex PCR results for E. faecalis and E. faecium.* ***M:*** *Marker 100 bp (Hibrigen®);* ***P1:*** *E. faecalis ATCC 29212,* ***P2:*** *E. faecium ATCC 700221,* ***N:*** *Negative control,* ***BA2, K8, BYD8****: Enterococcus faecalis positive isolates,* ***Ö19, BYD7****: Enterococcus faecium positive isolates*

Only one isolate (*E. faecium*) was detected in Ödemiş, while the remaining isolates exhibited a comparable distribution in Kiraz (7 *E. faecalis*, 1 *E. faecium*), Bayındır (8 *E. faecalis*, 1 *E. faecium*), and Beydağı (7 *E. faecalis*, 1 *E. faecium*). There was no evidence of reproduction observed in the Tire district. The identification rates of the isolated *Enterococcus* species by districts are given in Figure 2.

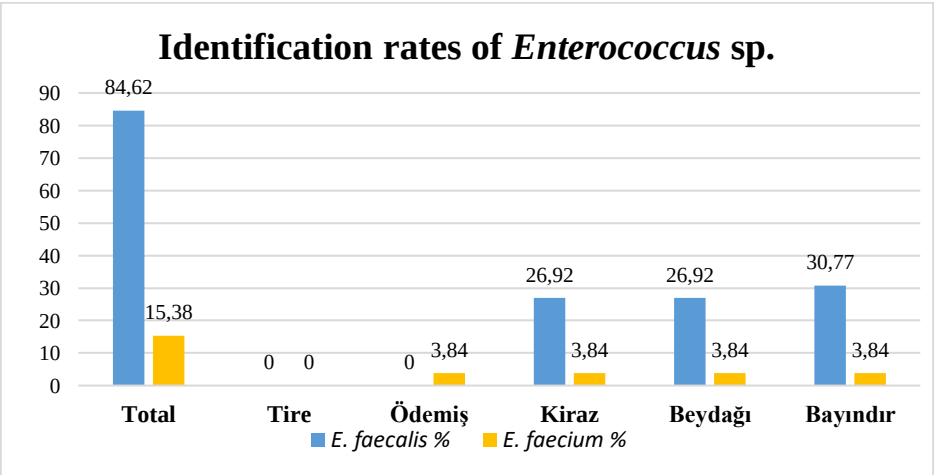

Fig. 2. *Identification rates of Enterococcus spp.*

Detection of esp gene

The presence of a biofilm matrix plays a crucial role in promoting the proximity of bacterial communities, thereby facilitating the transfer (horizontal) of resistance genes between enterococci. In *E. faecalis*, the enterococci surface protein (esp) has been identified as a crucial factor in biofilm formation, enabling attachment to and colonization of abiotic surfaces [22]. In our study, out of the 26 biofilm-producing strains, only 17 strains were found to possess the esp gene, while the remaining biofilm-producing strains lacked it. Furthermore, remarkably the *esp* gene was described with high prevalence among the isolates of *E. faecalis*. Furthermore, the esp resistance gene presence was identified in 13 *E. faecalis* (50%) and 4 *E. faecium* (15.38%). The results for the *Enterococcus* sp. *esp* resistant genes were given in Figure 3.

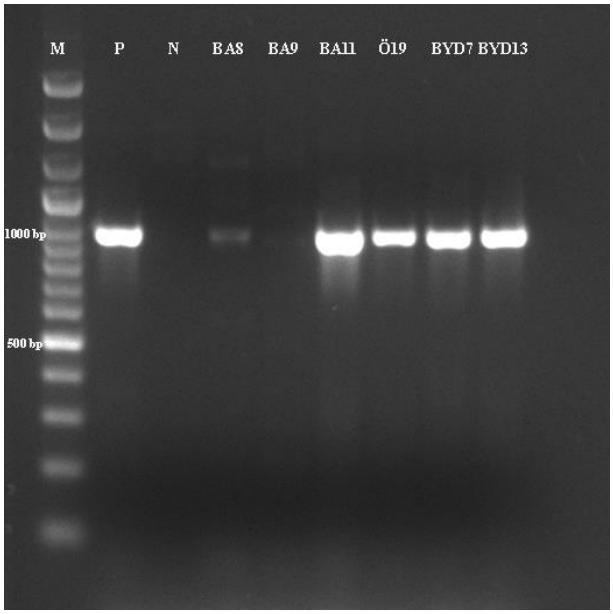

Fig. 3. *Enterococcus sp. esp gene PCR results.* ***M:*** *Marker 100 bp (Hibrigen®);* ***P1:*** *E. faecalis ATCC 29212,* ***N:*** *Negative control,* ***BA8, BA1, Ö19, BYD7, BYD13****: esp gene positive samples,* ***BA9****: esp gene negative samples*

Antimicrobial susceptibility of Enterococcus isolates

All of the 26 *Enterococcus* sp. isolates were found to be 100% resistant to amoxicillin-clavulanic acid, kanamycin, sulfamethoxazole-trimethoprim and to 96.15% gentamicin, and 100% susceptible to ampicillin and florfenicol, and 53.84% to vancomycin. Additionally, we observed that out of the 14 vancomycin-susceptible Enterococcus isolates; 10 were identified as *E. faecalis* (71.42%) and 4 (28.58%) as *E. faecium*.

Detection of virulence genes of Enterococcus isolates

In our study, strains exhibited phosphorylation enzyme activity, indicating aminoglycoside resistance. To detect the resistance, we used specific primer pairs (*aph(2'')-Ia, aph(2'')-Ib, aph(2'')-Ic, and aph(2'')-Id*) in the PCR analysis. The presence of the *aph(2'')-Ia* gene was determined in 16 (61.54%) *E. faecalis* and 1 (3.85%) *E. faecium*. In addition, 1 (3.85%) *E. faecalis* tested positive for the *aph(2'')-Ib1* gene, while

3 (11.54%) *E. faecalis* showed positivity for the *aph(2'')-Ic1* gene. Furthermore, 7 (26.92%) *E. faecalis* was detected positive for the *aph(2'')-Id1*.

Among the isolates tested, 2 *E. faecalis* (7.69%) harboured the *vanA* resistance gene, while 5 *E. faecalis* (19.23%) isolates carried the vanB resistance gene. Interestingly, both resistance genes were detected in only one isolate. The vancomycin-resistance gene wasn't detected in *E. faecium*. Besides, all of the phenotypic isolation, virulence genes, antimicrobial resistance genes and antibiogram results obtained in the study are given in the heatmap (Figure 4).

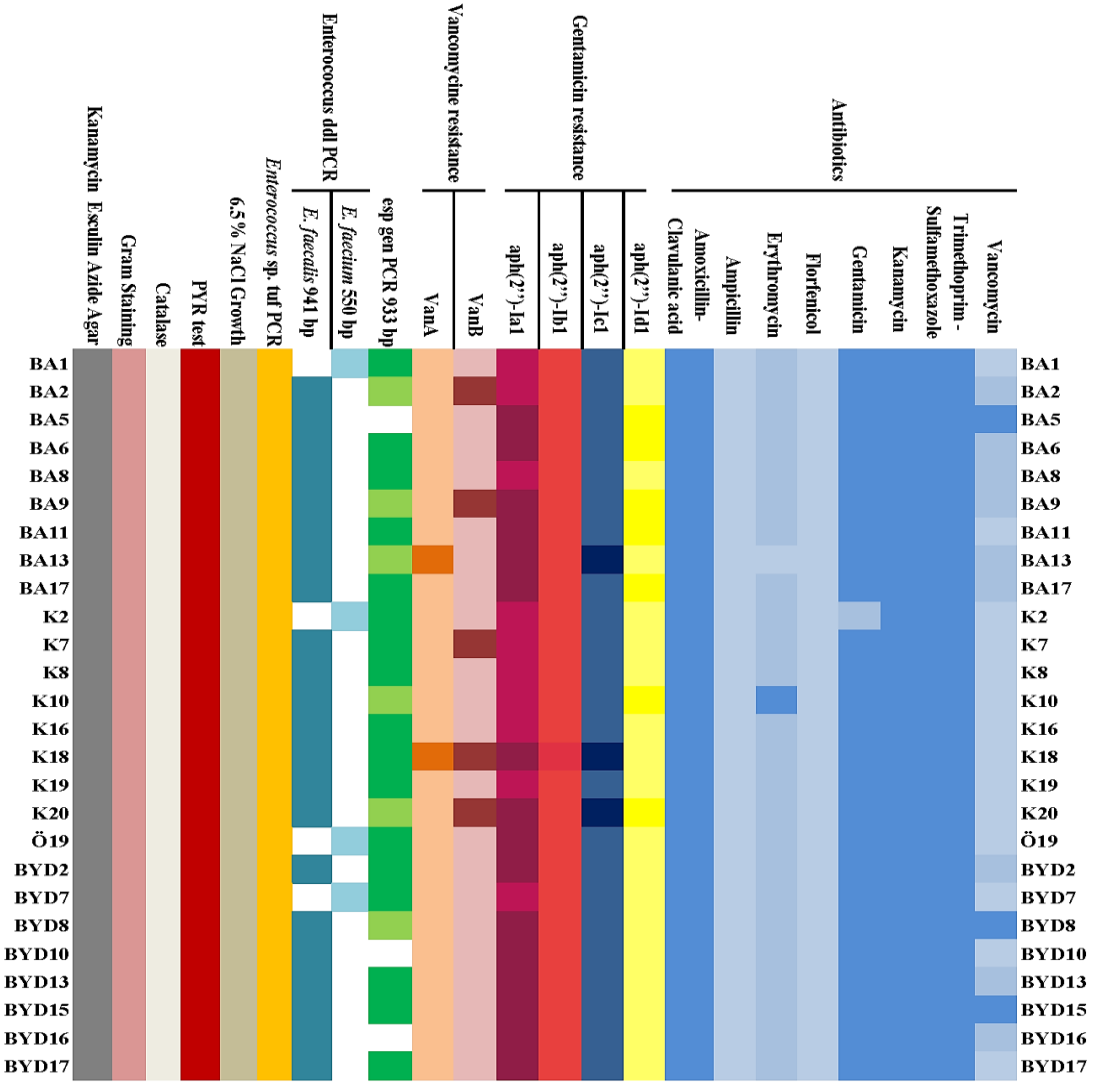

Fig. 4. *Heatmap; all results for Enterococcus faecalis and E. faecium isolated from a sample of milk mastitis (n=26). (In the resistance genes parameters, dark colours represent positive values for the, and light colours represent the negative values. The dark blue colour represents resistant values, the medium blue colour represents intermediate values and the light blue colour represents susceptible values for antimicrobial activity. BA: Bayındır; K: Kiraz; Ö:Ödemiş; BYD: Beydağı).*

Mastitis, a widespread and costly disease affecting dairy animals worldwide, presents a significant challenge. In addition, the poor quality of milk resulting from mastitis has economic consequences. The primary strategy for managing active mastitis infections is through antibiotic treatment, although the widespread use of antibiotics raises concerns regarding the emergence of resistant pathogens [23]. Enterococci, which are Gram-positive cocci and inherently resistant, are frequently present in the gastrointestinal tracts of various in land animals, including humans. They also serve as one of the environmental agents responsible for causing mastitis, an inflammatory condition [24]. In clinical infections, *Enterococcus faecalis* and *E. faecium*, both frequently isolated from humans, make up approximately 85-95% and 5-10% of the total enterococci strains, respectively [25]. In our study, we inoculated 100 clinical mastitis milk samples, taken in equal numbers from five sites, resulting in the isolation of 60 black colonies and Gram-positive cocci. Among these 60 isolates, 12 (20%) isolates showed negative PYR results. Out of the remaining 48 samples, 22 tested negative for the *tuf* gene PCR. The remaining 26 samples yielded positive results for *tuf* PCR, and the subsequent steps of the study were conducted on these 26 isolates.

It has been reported in many studies that different virulence factors in enterococci could potentially contribute to the severity or progression of diseases in both humans and animals. Mobile genetic elements can carry antimicrobial resistance and virulence genes. Consequently, enterococci in milk may pose a risk of transmitting potentially pathogenic strains that are resistant to antimicrobials through food, thereby affecting human health [26]. *Enterococcus* sp. has been identified as an environmental pathogen associated with mastitis. The prevalence of different types of mastitis caused by *Enterococcus* sp. varies among countries, with the incidence of mastitis cases attributed to *Enterococcus* sp. generally ranging from 10% to 67% [27]. In our study, we found a prevalence rate of 26% for *Enterococcus* sp. This prevalence can be attributed to the classification of *Enterococcus* sp. as an environmental pathogen known to cause mastitis [2] and the most commonly encountered species are *E. faecium* and *E. faecalis* [28]. In our study, we found that *E. faecalis* was the dominant species, which is interesting because previous research [4] reported *E. faecium* as the dominant species. We observed a low isolation rate of *E. faecium* in our study. The prevalence of *Enterococcus* species can vary among different studies. Several studies conducted in Türkiye have examined enterococci in milk samples from bovine subclinical mastitis, with reported prevalence rates as follows: 0.7% (3/421) in Samsun [29], 10.9% in Afyon (43/392) [30], and 16% (96/600) in Aydın [31]. In Canada, Cameron et al. [32] detected enterococci in 15.25% of the samples, while in the Czech Republic, Cervinkova et al. [33] isolated these bacteria in 16.1% of the samples. Similarly, Kateete et al. [34] identified enterococci in 19.5% of the samples collected in Uganda. In Poland, Rozanska et al. [5] isolated *Enterococcus* sp. bacteria from 21.3% of mastitic milk samples, and in Egypt, Ahmed et al. [1] found *Enterococcus* sp. bacteria in 34% of mastitic milk. Kim et al. [27] reported the isolation *of Enterococcus* sp. bacteria from 66.4% of milk samples in South Korea, and Hamzah et al. [35] isolated *Enterococcus* sp. bacteria from 67.7% of mastitic milk in Iraq. Consistent with these studies, our findings identified *E. faecalis* as the predominant species, while the proportion *of E. faecium* was the lowest. The lower incidence of *Enterococcus* sp. infections may be attributed to the application of appropriate management practices, such as proper manure disposal, regular farm cleaning, effective veterinary control, and the use of sanitation measures. Enterococci naturally possess resistance to diverse antibiotics, including aminoglycosides, kanamycin, and β-lactam antibiotics. This resistance is due

to the limited permeability of the enterococci cell wall, resulting in moderate resistance specifically to aminoglycosides. It is worth noting that this resistance is more frequently observed in *E. faecium* compared *to E. faecalis* [36]. Moreover, Enterococci demonstrate a significant degree of resistance to the majority of cephalosporin and all semi-synthetic penicillin. Among the clinically significant species, *E. faecalis* is intrinsically resistant to most β-lactams, except for a restricted group of penicillin, including ampicillin and penicillin [37]. In our study, we observed that all isolates showed 100% resistance to amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole, and kanamycin. Interestingly, we also found a 100% high sensitivity rate for ampicillin and florfenicol, which aligns with the aforementioned study results. Ahmed et al. [1], Amini et al. [38], and Moussa et al. [39] reported higher resistance rates for erythromycin, trimethoprim/sulfamethoxazole, and ampicillin among *E. faecalis* isolates. Additionally, our study findings confirmed the resistance of both *E. faecalis* and *E. faecium* to trimethoprim-sulfamethoxazole. Notably, we observed a high prevalence of gentamicin resistance among *E. faecalis* isolates.

Moreover, we observed that both *E. faecalis* and *E. faecium* strains were devoid of the *aph(2'')-Ib* and *aph(2'')-Ic* genes [40]. Our study revealed a high prevalence of gentamicin resistance among both *E. faecalis* and *E. faecium* isolates, with a rate of 96.15%. Notably, a significant occurrence of gentamicin resistance was specifically observed among the *E. faecalis* isolates. However, by the provided information, the presence of the *aph(2'')-Ib* and *aph(2'')-Ic* genes was detected at very low frequencies. Interestingly, no instances of resistance to erythromycin and ampicillin were identified in our study. Vancomycin resistance encompasses various phenotypes, including *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*. Among these, strains bearing the *vanA* and *vanB* genes hold the highest clinical significance. *VanA* gene-bearing strains exhibit the highest level of resistance to both vancomycin and teicoplanin, while *vanB* gene-bearing strains display resistance exclusively to vancomycin [41]. In our study, 3 *E. faecalis* strains were found to be resistant to vancomycin. The prevalence of the *vanA* resistance gene must be relatively low among isolated *Enterococcus* strains, and reports indicate its absence or low prevalence in *vanA*-mediated vancomycin-resistant *Enterococcus* (VRE) strains [36, 42]. However, we detected the *vanA* resistance gene in 2 *E. faecalis* strains and the *vanB* vancomycin resistance gene in 5 *E. faecalis* strains. The increase in vancomycin tolerance may be attributed to biofilm formation. *E. faecium* and *E. faecalis* isolates lacking the *vanA* and *vanB* genes exhibit phenotypical resistance, which suggests the involvement of factors other than genetic agents. *Enterococcus* sp. can horizontally transmit antimicrobial resistance or virulence genes, making them potential facilitators of gene spread. Consequently, their presence in milk can contribute to the emergence of multidrug-resistant (MDR) strains, which in turn affects the selection of appropriate medications [4]. In line with this, Cui et al. [36] reported a nearly identical occurrence of MDR among both *E. faecalis* and *E. faecium* isolates, which aligns with the pattern observed in our study regarding resistance phenotypes of both species. Usually, the standard approach for managing enterococcus infections typically involves a combination therapy method consisting of aminoglycosides, such as gentamicin and streptomycin, in conjunction with cell wall-active inhibitors like beta-lactams or glycopeptides. This therapeutic approach has demonstrated efficacy in the treatment of enterococcus infections. However, the emergence of high-level aminoglycoside resistant (HLAR) enterococci presents a significant challenge in the management of these infections. The effective management of *Enterococcus* infections is a major challenge. This is due to the

presence of high-level aminoglycoside resistant (HLAR) strains. This resistance is primarily attributed to the production of aminoglycoside-modifying enzymes (AME), which render aminoglycosides ineffective. The acquisition of AME genes (*aph(2")-Ia*, *-Ib*, *-Ic*, and *-Id*), by enterococci has greatly impacted the treatment outcomes of enterococcal infections [39]. In our study, the use of PCR analysis consistently detected the presence of the aminoglycoside resistance genes *aph(2")-Ia* and *aph(2")-Id* [38]. Our comprehensive analysis revealed that these resistance genes played a significant role in the observed aminoglycoside resistance among both *E. faecalis* and *E. faecium* strains. While some studies did not find the *aph (2")-Id* gene in *E. faecalis* and *E. faecium* strains [38, 43], our findings demonstrated a noteworthy detection rate of this gene, in contrast to the study by Li et al. [44] who reported a prevalence rate of 1.3%. The biofilm matrix plays a critical role in facilitating the proximity of bacterial communities, thereby promoting the horizontal transfer of antimicrobial resistance genes in Enterococci. In the case of *E. faecalis*, the enterococcus surface protein (*esp*) has been identified as a key factor in biofilm formation, enabling attachment to and colonization of abiotic surfaces [22]. In our study, out of the 26 biofilm-producing strains, only 17 strains were found to possess the *esp* gene, while the remaining biofilm-producing strains lacked it. Furthermore, we observed a high prevalence of the *esp* gene among *E. faecalis* isolates.

CONCLUSION

In conclusion, *E. faecalis* was found to be the dominant species of subclinical enterococci causing sheep mastitis. There is a possibility of genetic transfer of virulence and antibiotic resistance genes from *Enterococcus* spp. to other pathogenic bacteria. The presence of aminoglycoside-modifying enzyme genes on plasmids and transposons is also associated with a higher incidence of gentamicin resistance in enterococci. The prevalence of gentamicin resistance was high in both *E. faecalis* and *E. faecium* isolates in the study. The relationship between the presence of the *esp* gene and the ability of *E. faecalis* to form biofilms was also established in this study, as it is known that the *esp* gene promotes primary attachment and biofilm formation of *E. faecalis* on abiotic surfaces. The persistence of the mastitis problem increases the importance of studying the virulence and resistance factors of field isolates, despite hygienic practices. Thus, the transfer of these genes from *Enterococcus* species to other pathogens and the ability of pathogens to use these genes should also be studied.

Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: S.K.,V.Ö., Design: H.T.Y.D.,S.K.,V.Ö., Data Collection or Processing: S.K.,V.Ö.,Y.S Analysis or Interpretation: S.K.,H.T.Y.D., V.Ö,Y.S., Literature Search: H.T.Y.D., S.K,V.Ö., Writing: S.K.,V.Ö.

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