Research Article

Virulence and Antimicrobial Resistance Profiles of *Enterococcus* spp. Isolated from Clinically Mastitic Versus Apparently Healthy Bovine Milk

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Abstract | *Enterococcus* spp. is one of the microorganisms responsible for environmental mastitis, a condition that is difficult to eradicate due to its resistance to commonly used antimicrobials. The current study aimed to evaluate the antimicrobial resistance profiles and virulence gene carriage of *Enterococcus* spp. isolated from clinical mastitic milk (CMM) and apparently healthy milk (AHM) in dairy cows. A total of 50 milk samples were analyzed, including 25 from cows with clinical mastitis and 25 from apparently healthy cows. Identification of *Enterococcus* spp. was performed using conventional microbiological methods and confirmed with the VITEK 2 Compact system. Antimicrobial susceptibility was assessed using the disc diffusion method, while the presence of virulence genes (*esp*, *asa1*, and *gelE*) was determined by PCR. The results showed no statistically significant difference in the isolation rates of *Enterococcus* spp. between CMM and AHM samples, though the detection rate was slightly higher in CMM (28%) compared to AHM (24%). In CMM, the predominant species identified were *E. faecium* and *E. faecalis*, while only *E. faecium* was detected in AHM samples. *Enterococcus* isolates from CMM exhibited high resistance rates to tetracycline, followed by erythromycin, ciprofloxacin, and penicillin. In contrast, AHM isolates showed high resistance to ciprofloxacin, erythromycin, penicillin, vancomycin, and tetracycline. Both CMM and AHM isolates demonstrated a high level of multidrug resistance (MDR) and elevated multiple antibiotic resistance (MAR) index values. PCR analysis revealed that *esp*, *asa1*, and *gelE* genes were present in CMM isolates at rates of 14.2%, 28.5%, and 28.5%, respectively. These virulence genes were not detected in AHM isolates. The presence of these virulence genes in mastitic milk isolates suggests a potential role in the pathogenesis of bovine mastitis. Additionally, the antimicrobial resistance observed in *Enterococcus* from apparently healthy milk raises public health concerns, as it may facilitate the transmission of resistance and virulence genes to humans through the food chain.

Keywords | *Enterococcus* spp., Antibiotic, *esp*, *asa*1, *gel*E, Mastitis

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INTRODUCTION

Mastitis is regarded as an endemic disease that affects dairy farming adversely which leads to physical and chemical changes in the milk and its compositional quality (Ruegg, 2017; Gonçalves *et al*., 2018). Pathogens causing mastitis in cows are classified as either contagious or environmental with common pathogens of contagious mastitis including *Streptococcus agalactiae*, *Mycoplasma* spp, and *Staphylococcus aureus* (Sheet, 2022).

E. faecalis and *E. faecium* which are consider the third and fourth utmost prevalent causes of nosocomial infections worldwide. The *Enterococcus* species is acknowledged as an substantial opportunistic pathogen, many isolates are associated with infections that are readily able to influence intestinal colonization, inclusive that of humans, where they are typically commensal microbes, and poison the food chain. When they are found in an environment, they serve as markers for water pollution caused by feces (Zhong et al., 2017; Torres *et al*., 2018). *Enterococcus* is frequently found in food manufacturing, mostly in dairy products, this is because of its strong tolerance for poor conditions, which enables it to persist in adverse climates (Różańska *et al*., 2019). The teat skin can get contaminated and colonized by numerous species in this genus, which can result in bovine mastitis, *E. faecalis*, *E. faecium* accounting for over 80% and 10-15% respectively of the infection (Dyson *et al*., 2022; de Oliveira *et al*., 2022). Additionally, there have been reports of these potentially zoonotic pathogens in animal-derived eating including meat, milk and their product and they have been connected to illnesses in humans (Abat *et al*., 2016; Hasan *et al*., 2018).

Extensive research has been conducted on the alarming rise of drug-resistant *Enterococcus* species and the horizontal transfer of virulence genes that enable them to evade the human immune system (Frieri *et al*., 2017; Selleck *et al*., 2019). *Enterococcus* spp. may serve as reservoirs for antimicrobial resistance (AMR) and virulence genes (Torres *et al*., 2018). These genes have the potential to spread horizontally to other bacterial species and genera, thereby raising significant public health concerns regarding enterococcal infections (Raza *et al*., 2018; Olopade *et al*., 2022). Normal strains lacking virulence genes can acquire them through spontaneous mutations and horizontal gene transfer, and virulence or antibiotic-resistance genes can be easily transport to other bacterial species through horizontal gene transfer (Raza *et al*., 2018; Kim *et al*., 2022). The virulence factors and genes that are important in the infestation and distribution of *Enterococcus* and influence in bovine mastitis are aggregation substance (*asa1*), collagen-binding protein (*ace*), virulence factor associated with infective endocarditis (*efa*A), enterococcal surface protein connected with biofilm production (*esp*), gelatinase (*gel*E), cytolysin (*cyl*A), and hyaluronidase (*hyl*) (Różańska *et al*., 2019; Kim *et al*., 2022; Aung *et al*., 2023). There is a significant public health concern regarding the transmission of *Enterococcus* spp. through food products, particularly milk. These pathogens can act as reservoirs for multidrug resistance (MDR), facilitating the transfer of resistance determinants to humans and potentially complicating treatment options (Różańska *et al*., 2019; Gao *et al*., 2019). In Iraq, limited studies have focused on *Enterococcus* spp. in mastitic milk, with most research emphasizing phenotypic identification and antimicrobial resistance patterns, while genotypic aspects remain underexplored. Therefore, the objectives of the present study were to assess the antimicrobial resistance profiles, MDR patterns, multiple antibiotic resistance (MAR) index, genotypic virulence markers, and phenotypic characteristics of *Enterococcus* spp. isolated from clinically mastitic and apparently healthy bovine milk samples.

MATERIALS AND METHODS

Collection of samples

A total of 50 bovine milk samples were collected from dairy farms located in various regions of Baghdad. The samples were divided equally, with 25 obtained from cows exhibiting clinical mastitis (CMM) and 25 from apparently healthy cows (AHM). The identification of clinical mastitis cases was carried out by the attending farm veterinarian based on visible clinical signs, including udder redness, swelling, tenderness, and the presence of flakes and/or clots in the milk. Milk samples were aseptically collected directly from the udder after thorough cleaning and disinfection of the teats using an iodine solution. Approximately 10 mL of milk from each cow was transferred into sterile containers and stored in cooler boxes for transportation. All samples were promptly delivered to the Zoonoses Research Unit, College of Veterinary Medicine, University of Baghdad, for further microbiological and molecular analysis.

Identification of *Enterococcus*

One milliliter of each milk sample was inoculated into 5 mL of HiChrome *Enterococcus* broth (HiMedia, India) and incubated at 37 °C for 24 hours. A color change in the broth to light green-blue was considered presumptive evidence of *Enterococcus* growth. From each positive broth culture, a loopful was streaked onto HiChrome *Enterococcus* agar and incubated at 37 °C for 18–24 hours. Colonies that appeared light green to blue in color were considered suspected *Enterococcus* spp.

These presumptive colonies were further subcultured onto esculin agar, where the appearance of black colonies due to esculin hydrolysis served as an additional confirmatory step. Preliminary identification was based on colony morphology, Gram staining (Gram-positive cocci), and catalase testing (catalase-negative). Final confirmation and species-level identification were performed using the VITEK® 2 Compact system (bioMérieux, France).

Detecting of antimicrobial resistance profile of *Enterococcus* spp.

Antimicrobial resistance was assessed using the disc diffusion method in accordance with the European Committee on Antimicrobial Susceptibility Testing

(EUCAST, 2024) guidelines. Six antibiotics commonly used in veterinary and human medicine were tested: vancomycin (30 µg), penicillin (10 µg), tetracycline (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), and ciprofloxacin (5 µg).

Bacterial suspensions were prepared by adjusting the turbidity to 0.5 McFarland standard (approximately 1 × 10^8 CFU/mL). A sterile cotton swab was immersed in the suspension and used to evenly inoculate the surface of Mueller-Hinton agar plates (HiMedia, India). After allowing the plates to dry for 15 minutes at room temperature, antibiotic discs were placed on the agar surface, and plates were incubated at 37 ˚C for 24 hours.

The diameter of the inhibition zones around each disc was measured, and isolates were classified as resistant, intermediate, or susceptible according to the Clinical and Laboratory Standards Institute (CLSI, 2022) guidelines. Multidrug resistance (MDR) was defined as resistance to at least one agent in three or more antimicrobial classes, following the criteria by Magiorakos *et al.* (2012). The Multiple Antibiotic Resistance (MAR) index was calculated as described by Sandhu *et al.* (2016), using the formula:

MAR = (number of antibiotics to which the isolate is resistant)/ (total number of antibiotics tested)

Phenotypic characteristic

Gelatinase production assay was done by stabbing nutrient agar containing 3% gelatin with heavy inoculum and incubating at 37 ºC, checked after 24 hrs intervals for one week was considered a positive result when gelatin liquefied (Lopes *et al.*, 2006). Hemolysis activity was estimated by streaking sheep blood agar 5% with pure colonies and incubated for 24 at 37 ˚C to distinguish type of hemolysis (Gaspar *et al.*, 2009).

Detecting virulence-associated genes

Three isolates each of *E. faecalis* and *E. faecium*, and one isolate of *E. gallinarum* from CMM, along with four *E. faecium* isolates from AHM, were screened for three virulence genes (*esp*, *asa1*, and *gelE*) using primers listed in Table 1. Genomic DNA was extracted from these isolates following the protocol of ABIOpur (USA). PCR reactions were performed in a total volume of 20 µL, containing 10 µL of master mix, 1 µL each of forward and reverse primers, 2 µL of DNA template, and 6 µL of nuclease-free water. The PCR program consisted of an initial denaturation at 95˚C for 5 minutes, followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 56˚C for 30 seconds, and extension at 72˚C for 30 seconds, with a final extension at 72˚C for 7 minutes.

Statistical analysis

Data were analyzed using SPSS software (version 20/ IBM Corp/ Armonk, NY, USA) to evaluate the presence of statistically significant differences among the studied variables. A p-value less than 0.05 was considered statistically significant.

RESULTS

Identification of *Enterococcus* spp.

A total of 13 out of 50 (26%) milk samples were positive for *Enterococcus* spp. Specifically, 7 of 25 (28%) samples from CMM and 6 of 25 (24%) from AHM tested positive. The difference between the two groups was not statistically significant (p > 0.05; p= 0.8, Chi-square= 0.06), as illustrated in Figure 1.

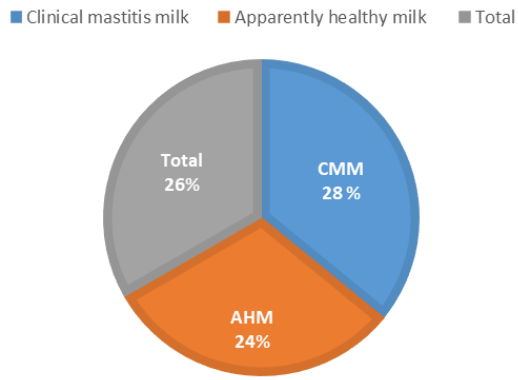

Figure 1: Percentage of *Enterococcus* spp. in milk samples. P value shown non-significant differences between CMM and AHM.

Table 1: Primers used for detection of virulence genes in *Enterococcus* spp.

Primer	Sequence 5`-3`	Size product (bp)	Reference
*asa*1	F-GCACGCTATTACGAACTATGA	375	(Vankerckhoven *et al.*, 2004)
	R-TAAGAAAGAACATCACCACGA		
esp	F-AGATTTCATCTTTGATTCTTGG		
	R-AATTGATTCTTTAGCATCTGG	510	
*gel*E	F-TATGACAATGCTTTTTGGGAT		
	R-AGATGCACCCGAAATAATATA	213	

Table 2: Antimicrobial resistance profile of *Enterococcus* spp.

Antimicrobial agents	% of antimicrobial resistance						
	CMM(n=7)				AHM(n=6)		
	E. faecalis	*E. faecium*	*E. gallinarum*	Total	*E. faecium*	*Enterococcus* spp.	Total
Vancomycin	0	0	0	0	0	50	16.67
Penicillin	66.67	66.67	0	57.14	50	50	50
Tetracycline	100	66.67	0	85.71	25	0	16.67
Erythromycin	100	33.33	100	71.43	75	50	66.67
Chloramphenicol	33.33	0	0	14.29	0	0	0
Ciprofloxacin	66.67	100	0	71.43	75	100	83.33
MDR	66.67	66.67	0	57.14	50	50	50
MAR index range	0.61	0.49	0.17	0.49	0.42	0.34	0.42

The analysis showed that *E. faecalis* and *E. faecium* each accounted for 3 out of 7 isolates (42.86%) from CMM, making them the most common species, followed by *E. gallinarum* (1/7, 14.29%). In AHM, *E. faecium* was identified in 4 out of 6 isolates (66.67%), while the remaining 2 isolates (33.33%) were identified only to the genus level as *Enterococcus* spp., as shown in Figure 2.

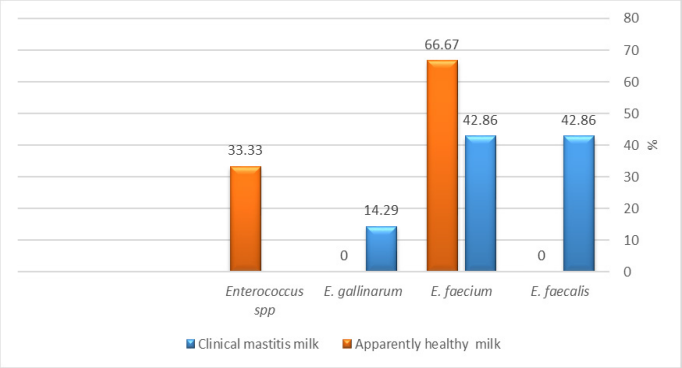

Figure 2: Distribution of *Enterococcus* spp. in cow milk

Antimicrobial resistance profile of *Enterococcus* spp.

The antimicrobial resistance profiles of *Enterococcus* isolates are summarized in Tables 2 and 3. Among the seven isolates from CMM, the highest resistance rates were observed against tetracycline (85.71%), followed by erythromycin and ciprofloxacin (each 71.43%), and penicillin (57.14%). Notably, none of the CMM isolates showed resistance to vancomycin. Specifically, *E. faecalis* isolates exhibited 100% resistance to erythromycin and tetracycline, 66.67% resistance to penicillin and ciprofloxacin, and 33.33% resistance to chloramphenicol. *E. faecium* isolates showed 100% resistance to ciprofloxacin, 66.67% resistance to penicillin and tetracycline, and 33.33% resistance to erythromycin. The single *E. gallinarum* isolate was 100% resistant to erythromycin. Overall, 57.14% of CMM isolates were MDR, with a MAR index averaging 0.49.

In contrast, isolates from AHM showed high resistance to ciprofloxacin (83.33%), erythromycin (66.67%), and penicillin (50%), while resistance to vancomycin and tetracycline was 16.67%. Among these AHM isolates, *E. faecium* strains were 75% resistant to erythromycin and ciprofloxacin, 50% resistant to penicillin, and 25% resistant to tetracycline. *Enterococcus* spp. isolates exhibited 100% resistance to ciprofloxacin and 50% resistance to vancomycin, penicillin, and erythromycin. Additionally, 50% of the AHM isolates were MDR, with a MAR index of 0.42.

Table 3: MDR and MAR index profile of *Enterococcus* spp.

Sample type	*Enterococcus* spp.	MAR index	MDR profile
CMM	*E. gallinarum*	1/6(0.17)	E
	E. faecalis	2/6(0.33)	E, TE
	E. faecalis	4/6(0.66)	P, E, TE, CIP
	E. faecalis	5/6(0.83)	P, E, TE, CIP, C
	E. faecium	2/6(0.33)	TE, CIP
	E. faecium	3/6(0.5)	P, TE, CIP
	E. faecium	4/6(0.66)	P, E, TE, CIP
AHM	*E. faecium*	1/6(0.17)	CIP
	E. faecium	1/6(0.17)	E
	E. faecium	3/6(0.5)	P, E, CIP
	E. faecium	4/6(0.66)	P, E, TE, CIP
	Enterococcus spp.	4/6(0.66)	VA, P, E, CIP,
	Enterococcus spp.	1/6(0.17)	CIP

E: Erythromycin; P:pencillin C: Chloramphencol; VA: Vancomycin; TE: Tetracyclin; CIP: Ciprofloxacin

Phenotypic and genotypic characteristics of *Enterococcus* isolates

The results for CMM isolates showed 28% hemolysis, with *E. faecalis* and *E. faecium* each exhibiting hemolysis at 33.33%, though this difference was not statistically significant ($p > 0.05$). Gelatinase production was observed in 24.2% of CMM isolates, occurring only in *E. faecalis* at 33.33%, also without significant difference ($p < 0.05$).

In contrast, *E. faecium* isolates from AHM demonstrated hemolysis and gelatinase production each at 25%.

Among the seven tested *Enterococcus* isolates from CMM, the virulence genes *esp*, *asa1*, and *gelE* were detected at rates of 14.2%, 28.5%, and 28.5%, respectively. Notably, only *E. faecalis* isolates tested positive for all three genes, with *esp* detected in 33.3% and both *asa1* and *gelE* in 66.6% of these isolates. None of the four *E. faecium* isolates from AHM carried any of the tested virulence genes (Table 4; Figures 3 and 4). There was no significant difference ($p > 0.05$) between CMM and AHM isolates regarding phenotypic and genotypic characteristics.

Table 4: Genotypic and phenotypic characterization of *Enterococcus* spp.

Sample type	*Enterococcus* spp. (No.)	Genotypic			Phenotypic	
		esp	*asa1*	*gelE*	*Hemolysis*	*Gelatinase*
CMM	*E. faecium* (3)	0	0	0	33.3	0
	E. faecalis (3)	33.3	66.6	66.6	33.3	33.33
	E. gallaniriium (1)	0	0	0	0	0
Total	7	14.2	28.5	28.5	28.5	14.2
AHM	*E. faecium* (4)	0	0	0	25	25
P-value		0.67^{NS}	0.5^{NS}	0.5^{NS}	1.0^{NS}	0.5^{NS}

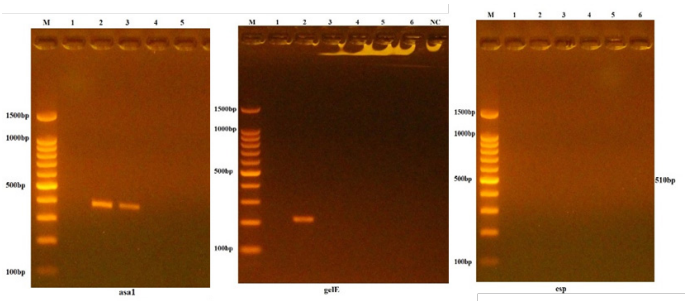

Figure 3: Amplification of *asa1* (375 bp), *gelE* (213 bp), and *esp* (510 bp) genes in *Enterococcus* spp. by PCR, analyzed by agarose gel electrophoresis stained with ethidium bromide. A 100 bp DNA ladder was used as a size marker. Lanes 1 and 4 represent *E. faecium*, lanes 2 and 3 represent *E. faecalis* isolates from clinically mastitic milk (CMM), and lanes 5 and 6 represent *E. faecium* isolates from apparently healthy milk (AHM). Electrophoresis was performed at 5 V/cm^2 in 1X TBE buffer for 1 hour and 30 minutes.

DISCUSSION

Several studies have shown that the composition and variation of the milk microbiome differ depending on the health status of the cattle. In cows with mastitis, the milk microbiome often shifts, with an increased presence of pathogenic bacteria, which can lead to reduced antimicrobial sensitivity (Selleck *et al.*, 2019; Kaczorowski *et al.*, 2022).

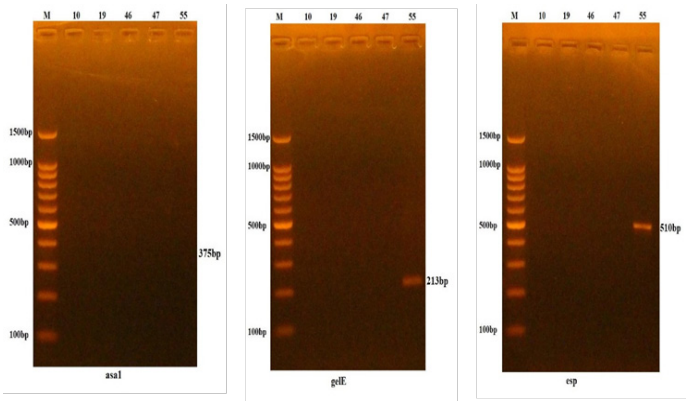

Figure 4: Amplification of *asa1* (375 bp), *gelE* (213 bp), and *esp* (510 bp) genes of *Enterococcus* spp. separated by agarose gel electrophoresis stained with ethidium bromide. M: 100 bp DNA ladder marker. Lanes 19 and 46 represent *E. faecium* isolates from apparently healthy milk (AHM), while lanes 10, 47, and 55 correspond to *E. faecium*, *E. faecalis*, and *E. gallinarum* isolates from clinically mastitic milk (CMM), respectively. Electrophoresis was run at 5 V/cm^2 in 1X TBE buffer for 1 hour and 30 minutes.

In this work, *Enterococcus* spp. was present in CMM higher than in AHMM. *E. faecalis* and *E. faecium* were the prominent isolates, followed by *E. gallinarum* from CMM. While only *E. faecium* was reported in AHM higher than in CMM, this indicates that *E. faecalis* may be more associated with clinical mastitis cases. In agreement with this study, *Enterococcus* spp. prevalence was higher in clinically mastitic milk (CMM) than in apparently healthy milk (AHM), with significant differences. In mastitis milk from Korea, *E. faecalis* accounted for 13.6% of isolates, which was lower than *E. faecium* at 86.4%. Conversely, in normal milk, *E. faecalis* and *E. faecium* were present at similar rates of 51.2% and 48.8%, respectively (Kim *et al.*, 2022). A study in Bangladesh reported that *E. faecalis* was found in 11.25% of mastitis milk samples, which was higher than its presence in normal milk at 4% (Bag *et al.*, 2022). Conversely, in Brazil, *E. faecalis* was detected more frequently in healthy milk (8.19%) than in mastitis milk (7.21%). Additionally, in mastitis milk from Brazil, *E. faecalis* accounted for 31.57%, exceeding *E. faecium* at 26.31% and *E. gallinarum* at 13.16%. In contrast, in normal milk, *E. faecium* was more prevalent at 25.42%, compared to *E. faecalis* at 15.25% and *E. gallinarum* at 8.47% (Paschoalini *et al.*, 2023). Furthermore, *Enterococcus* spp. isolated from bovine mastitis and bulk tank milk in Brazil were reported at rates of 16.66% and 83.22%, respectively (de Moraes *et al.*, 2023).

In comparison with studies conducted in Iraq, Hamzah and Kadim (2018) reported that *Enterococcus* spp. were recovered from 60% of cow mastitis milk samples. Another

study by Abdali *et al.* (2023) found *E. faecalis* at a higher prevalence of 8.3% compared to *E. faecium* at 3.3% in mastitis cow milk. More recently, Marwa and Abdulrazzaq (2025) identified 81 *Enterococcus* isolates from 300 mastitic cow milk samples, of which 42 isolates tested positive for *E. faecalis*.

Enterococcus as commensal bacteria commonly found in the intestinal flora of humans and animals, are considered opportunistic pathogens. For this reason, they can be isolated from milk samples even in the absence of mastitis. Due to their commensal nature, ability to thrive under various environmental conditions, and intrinsic resistance mechanisms, *Enterococcus* spp. are also frequently found in hygienic or non-infected samples (Lebreton *et al.*, 2014; Różańska *et al.*, 2019).

The degree of antibiotic resistance among *Enterococcus* species varies considerably. Several studies have documented antimicrobial resistance in *Enterococcus* strains isolated from milk and dairy products (Alkhafaje *et al.*, 2022; de Moraes *et al.*, 2023). Generally, isolates from mastitic milk tend to exhibit higher resistance rates than those from normal milk. In the present study, CMM isolates were susceptible to vancomycin, whereas some AHM isolates showed resistance. High resistance levels were observed in CMM isolates against tetracycline, erythromycin, ciprofloxacin, and penicillin. Similarly, AHM isolates exhibited high resistance to erythromycin, ciprofloxacin, and penicillin. Alarmingly, both groups displayed a high prevalence of MDR and elevated MAR index values. The presence of vancomycin-resistant isolates in apparently healthy milk is particularly concerning, indicating the circulation of MDR strains.

Supporting these findings, Kim *et al.* (2022) reported resistance rates among *E. faecalis* and *E. faecium* isolated from mastitis cases as follows: chloramphenicol (21.5%), ciprofloxacin (1.2%), tetracycline (59.3%), erythromycin (27.2%), and vancomycin (0%), with an MDR rate of 16%. In contrast, isolates from normal milk were resistant to tetracycline (17.1%) and erythromycin (34.1%) but remained fully sensitive to vancomycin, with an MDR rate of only 4.9%. Additionally, Abdali *et al.* (2023) found that *E. faecalis* and *E. faecium* isolated from mastitic milk had high levels of resistance to vancomycin, at 40% and 30% respectively. Bag *et al.* (2022) also reported high resistance rates of *Enterococcus* spp. from both normal and mastitis milk against tetracycline (20%) and erythromycin (18%), with lower resistance to vancomycin and ciprofloxacin (3%). *E. faecium* and *E. faecalis* showed increasing resistance to erythromycin and tetracycline, while *E. gallinarum* was resistant to tetracycline at a rate of 18.2%.

Furthermore, Marwa and Abdulrazzaq (2025) reported a high level of MDR *E. faecalis* contamination in mastitis milk, including 32% vancomycin-resistant strains. They also noted elevated resistance to azithromycin (80%) and cephalosporins (72%), highlighting the urgent need for monitoring and antimicrobial stewardship.

The MAR index is a practical, cost-effective tool used to track antibiotic-resistant bacteria, especially in surveillance of foodborne and zoonotic pathogens. A MAR index of ≥0.2 is considered indicative of high-risk contamination sources, suggesting frequent exposure of bacteria to multiple antibiotics (Sandhu *et al.*, 2016). The MAR index has previously been reported in *Enterococcus* spp. (Mohammed and Al-Gburi, 2023). In the present study, a high MAR index was observed in *Enterococcus* isolates from both clinically mastitic milk (CMM) and apparently healthy milk (AHM), indicating chronic antibiotic exposure in both diseased and healthy cattle. Similar findings were reported by Bouymajane *et al.* (2019), who found that *E. faecium* and *E. faecalis* exhibited MAR index values exceeding 0.5. Variations in antimicrobial resistance patterns across studies may be attributed to geographical differences, bacterial species, and evolutionary changes in resistance mechanisms (Różańska *et al.*, 2019).

Enterococcus spp. are also concerning due to their ability to harbor and transfer virulence genes via plasmids to otherwise non-virulent strains. This gene mobility increases the potential for enterococcal infections in both animals and humans (Eaton and Gasson, 2001). In our study, phenotypic characterization showed that CMM isolates exhibited a higher rate of hemolytic activity than AHM isolates, while gelatinase activity was slightly higher in AHM. *E. faecalis* from CMM showed both hemolysis and gelatinase activity, while *E. faecium* from CMM exhibited only hemolysis. Interestingly, *E. faecium* from AHM showed both activities, although not significantly. These findings are in line with Kim *et al.* (2022), who reported significantly higher rates of gelatinase production in *E. faecalis* and *E. faecium* from mastitic milk (22.2%) compared to normal milk (4.9%). Hemolysin activity was 2.5% in mastitis isolates and absent in healthy milk isolates. Nasiri and Hanifian (2022) also found hemolysin and gelatinase activity in *E. faecalis* from pasteurized milk at 34.4% and 11.3%, respectively, and in *E. faecium* at 4.3% and 9.5%.

In the current study, virulence genes (*esp*, *asa1*, and *gelE*) were absent in AHM isolates but present exclusively in *E. faecalis* from CMM samples. This suggests a strong association between these virulence genes and clinical mastitis. Among them, *gelE* and *asa1* were the most commonly detected. The *gelE* gene encodes for gelatinase, an enzyme that promotes biofilm formation and tissue invasion, while *asa1* is linked to the aggregation substance

responsible for enhanced adhesion to host tissues and transfer of plasmids carrying resistance genes (Kiruthiga *et al*., 2020).

Kim *et al*. (2022) also reported a higher prevalence of virulence genes in mastitis milk isolates than in normal milk: *E. faecalis* showed positivity rates of 85.7%, 71.4%, and 54.3% for *gelE*, *asa1*, and *esp*, respectively. Only one of eleven *E. faecium* isolates harbored both *asa1* and *gelE*. In contrast, *E. faecium* from healthy milk lacked all three genes, while *E. faecalis* harbored *asa1* (80%), *gelE* (60%), and *esp* (25%). These results align with Jahansepas *et al*. (2018), who reported high frequencies of *asa1* (88%) and *gelE* (74.4%) in *E. faecalis*, and with Song *et al*. (2019), who found *gelE* and *asa1* in 88% and 44% of isolates, respectively. Kiruthiga *et al*. (2020) also confirmed that *asa1* was the most common virulence gene in *E. faecalis*, followed by *gelE*. Conversely, Paschoalini *et al*. (2023) found no *esp* genes in any *Enterococcus* isolates from bovine milk (healthy or mastitic). However, *asa1* and *gelE* were found in 36% and 45% of *E. faecalis* isolates, respectively, while *E. faecium* lacked both genes. Notably, 9.1% of *E. gallinarum* isolates harbored the *asa1* gene.

CONCLUSION

The isolation of *Enterococcus* spp. from both clinically mastitic and apparently healthy milk, along with the detection of multidrug resistance and virulence genes exclusively in CMM isolates, underscores the pathogenic potential of these organisms. These findings highlight the need for implementing effective preventive measures to control their spread through the food chain. Continuous monitoring of antimicrobial resistance and the adoption of stringent hygienic farming practices are essential strategies to limit the dissemination of resistant and virulent *Enterococcus* strains to humans.

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NOVELTY STATEMENT

This study is the first to compare Enterococcus isolates from mastitic and apparently healthy milk, showing multidrug resistance in healthy milk and virulence genes exclusively in mastitic milk.

AUTHOR'S CONTRIBUTION

The first and second authors who completed the laboratory work and finished writing up this article.

Generative AI or AI-assisted Technology Statement

The authors declare that no Genrative AI was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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