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Determination of the Ampicillin–Resistant Enterococcus strains, antibiotic resistance and virulence genes in raw milk and chicken meat samples from Sivas province, Türkiye

Determinación de cepas de Enterococcus resistentes a la ampicilina, resistencia a antibióticos y genes de virulencia en muestras de leche y carne de pollo de la provincia de Sivas, Turquía

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ABSTRACT

The object of this study was to determine the occurrence of ampicillin–resistant (Amp^R) *Enterococcus* spp. isolated from raw milk and chicken (*Gallus gallus domesticus*) meat samples focus on their antimicrobial resistance profiles and virulence genes from Sivas province, Türkiye. A total of 210 samples comprising raw milk (n = 150; cow (*Bos taurus*), sheep (*Ovis aries*) and buffalo (*Bubalus bubalis*) milk) and fresh chicken pieces (n = 60; thighs and wings with skin) were collected and analyzed. Antimicrobial susceptibility testing was performed using the disk diffusion method, while minimum inhibitory concentrations for Amp^R isolates were determined by broth microdilution. Polymerase chain reaction identified the isolates at the species level and screened for key virulence genes (*asa1, cylA, esp, gelE* and *hyl*). A total of 40 strains of Amp^R *Enterococcus* spp. were isolated from raw milk and chicken meat samples. Out of the isolates from raw milk and chicken meat, 67.5% were identified as *E. faecium*, 12.5% as *E. faecalis*, and 20% as other *Enterococcus* species. Among the Amp^R *Enterococcus* spp. isolates, minimum inhibitory concentration values ≥ 16 $\mu g \cdot mL^{-1}$ were detected in 25.0% of isolates from milk and 30.0% of isolates from chicken meat samples. Disk diffusion revealed varied resistance among isolates, with the highest against erythromycin (55.0%) and tetracycline (50.0%), followed by rifampin (42.5%), ciprofloxacin (20.0%), vancomycin (12.5%), gentamicin (10.0%), and chloramphenicol (7.5%). Amp^R *Enterococcus* spp. isolates exhibited a multidrug resistance rate of 67.5%. Virulence gene analysis indicated the presence of the asa1 gene in only one *E. faecalis* isolate (2.5%), while *cylA, esp, gelE* and *hyl* genes were not detected. Detection of antibiotic–resistant *Enterococcus* spp. in raw milk and chicken meat, and occurrence of vancomycin and gentamicin resistant enterococci are noteworthy for public health concerns. Monitoring the antimicrobial–resistant enterococci in animal–derived foods is crucial within the framework of the One Health concept.

Key words: Antimicrobial resistance; ampicillin resistant *Enterococcus* spp.; milk; chicken meat; one health

RESUMEN

El objetivo de este estudio fue analizar la presencia de *Enterococcus* spp. resistentes a la ampicilina aislados en muestras de leche cruda y carne de pollo (*Gallus gallus domesticus*), centrándose en sus perfiles de resistencia antimicrobiana (Amp^R) y genes de virulencia de la provincia de Sivas, Turquía. Se recolectaron y analizaron 210 muestras de leche cruda (n = 150; leche de vaca (*Bos taurus*), oveja (*Ovis aries*) y búfala (*Bubalus bubalis*)) y piezas frescas de pollo (n = 60; muslos y alas con piel). Se realizaron pruebas de susceptibilidad antimicrobiana mediante el método de difusión en disco, mientras que las concentraciones mínimas inhibitorias para los aislados de resistencia a la ampicilina se determinaron mediante microdilución en caldo. La reacción en cadena de la PCR identificó los aislados a nivel de especie y analizó los genes de virulencia clave (*asa1, cylA, esp, gelE* e *hyl*). Se aislaron 40 cepas *Enterococcus* spp. Amp^{R}a partir de muestras de leche cruda y carne de pollo. De los aislados de leche cruda y carne de pollo, el 67,5 % se identificó como *E. faecium*, el 12,5 % como *E. faecalis* y el 20 % como otras especies de *Enterococcus.* Entre los aislados de resistencia a la ampicilina *Enterococcus* spp., se detectaron valores de concentraciones mínimas inhibitorias ≥ 16 $\mu g \cdot mL^{-1}$ en el 25,0 % de los aislados de leche y el 30,0 % de los aislados de muestras de carne de pollo. La difusión en disco reveló resistencia variada entre los aislados, con las más altas, la eritromicina (55,0 %) y tetraciclina (50,0 %), seguida de rifampicina (42,5 %), ciprofloxacino (20,0 %), vancomicina (12,5 %), gentamicina (10,0 %) y cloranfenicol (7,5 %). Los aislados de Amp^R *Enterococcus* spp. exhibieron una tasa de resistencia a múltiples fármacos del 67,5 %. El análisis de genes de virulencia indicó la presencia del gen asa1 en un solo aislado de *E. faecalis* (2,5 %), mientras que no se detectaron los genes *cylA, esp, gelE* ni *hyl*. La detección de *Enterococcus* spp. resistentes a antibióticos en leche cruda y carne de pollo, así como la presencia de enterococos resistentes a la vancomicina y la gentamicina, son importantes para la salud pública. El monitoreo de enterococos resistentes a antimicrobianos en alimentos de origen animal es crucial en el marco del concepto “Una Salud”.

Palabras clave: Resistencia a los antimicrobianos; *Enterococcus* spp.; resistentes a la ampicilina; leche; carne de pollo; “Una Salud”

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INTRODUCTION

Enterococcus spp. is considered a typical flora component of the human and animal gastrointestinal systems, however in recent years it has come into being as one of the most prevalent causes of nosocomial and opportunistic infections in patients [1]. The rising relevance of *Enterococcus* spp. in hospital acquired infections is due to their capacity to acquire resistance to various classes of antimicrobials [2].

Recent directives from the European Commission Implementation Decision 2020/1729 indicate that *Enterococcus faecalis (E. faecalis)* and *Enterococcus faecium (E. faecium)* should also be monitored within the scope of reporting antimicrobial resistance [3].

Enterococcus spp. are significant contributors of nosocomial infections in humans and recognized for their intrinsic resistance to multiple antibiotics [4, 5]. Moreover, acquired resistance to β–lactam, aminoglycoside, tetracycline, erythromycin, fluoroquinolone, chloramphenicol, and glycopeptide antibiotics is frequently reported [6].

Severe invasive enterococcal infections are often treated with a cell–wall–active drug (e.g. ampicillin and vancomycin) and an aminoglycoside (gentamicin or streptomycin). Resistance to these antibiotics reduces the effectiveness of combination therapy [7]. *E. faecium*, especially among *Enterococcus* spp., has gained resistance to antibiotics such as aminoglycoside, ampicillin and vancomycin, making infection treatment more challenging [8, 9].

The prevalence of infections caused by Ampicillin–Resistant (Amp^R) *Enterococcus* spp. has risen during the 1980s. Currently, over eliminate 90% of *E. faecium* strains isolated from nosocomial infections have been found to be resistant to ampicillin. Furthermore, most nosocomial invasive *E. faecium* isolates in Europe exhibited resistance to ampicillin [10, 11].

Foodborne *Enterococcus* spp. are not typically considered pathogens, yet they can establish themselves in the gastrointestinal tract [12]. The prevalence of clinical infections caused by *Enterococcus* spp. has steadily increased during the 1970s [13, 14, 15]. The most common *Enterococcus* spp. encountered in humans are *E. faecalis* and *E. faecium,* which are responsible for a great deal of healthcare–associated infections [16].

Resistance to conventional medications, such as ampicillin, has made treating *E. faecalis* and *E. faecium* complicated. Concerns over public health risks have raised consumer awareness of the importance of consuming safe raw milk and retail chicken (*Gallus gallus domesticus*) meat. Raw milk and chicken meats are frequently contaminated with *Enterococcus* spp., and rising interest in the epidemiology of these pathogens continues globally [17].

Studies concerning the emergence of Amp^R *Enterococcus* spp. in foods are growing by the day, which is particularly crucial in terms of nosocomial infections worldwide [18]. The prevalence of *Enterococcus* spp. in different animal–derived foods in Türkiye has been studied [19, 20, 21, 22], however, no investigation has been conducted on the characterization of Amp^R *Enterococcus* isolates.

Accordingly, the purpose of this study was to determinate the presence of Amp^R *Enterococcus* spp. strains isolated from raw milk and chicken samples offered for consumption in Sivas, as well as their antimicrobial susceptibility profiles and virulence genes.

MATERIALS AND METHODS

Sampling of milk and chicken meat samples

A total of 210 samples comprising raw milk [(n = 150; cow (*Bos taurus*), sheep (*Ovis aries*) and buffalo (*Bubalus bubalis*) milk)] and fresh chicken pieces (n = 60; thighs and wings with skin) were collected from multiple supermarkets, butcher, and retail sale points located in the center of Sivas province, Türkiye, between 2022 and 2023. Raw milk samples (approximately 250–500 mL) offered for sale were collected into sterile bottles in a random manner from local sale points. Packaged fresh chicken pieces (between 500–1,000 g) from diverse companies from various supermarkets, butcher and local sales points were gathered on a regular basis in Sivas province.

The samples were promptly delivered to the laboratory under (Laboratory Cooler Box, 32 L, China) cold chain conditions (+ 4°C) and processed on the day of collection. In accordance with the study's objectives, sample collection was completed when the number of Amp^R *Enterococcus* spp. isolates reached n = 40.

Isolation and identification of *Enterococcus* spp.

To isolate the *Enterococcus* spp., 10 mL raw milk and 10 g of chicken meat samples were placed in sterile bags were diluted with sterile *Enterococcus* enrichment broth (Oxoid CM0984, United Kingdom) as 1:10 ratio under aseptic conditions. The prepared homogenate was enriched by incubation (Binder GmbH BD 115, Germany) at 35–37°C for 24 hours (h). A 0.1 mL aliquot of the homogenate was taken and spread on *Enterococcus* Agar (Oxoid CM0985, United Kingdom), then incubated for 48 h at 37°C under aerobic conditions. Following incubation, colonies of a black color, measuring 1 mm in diameter, suspected of *Enterococcus* spp. were selected and passed onto 5% Sheep Blood Columbia Agar (CBA, Oxoid CM0331, United Kingdom). Presumptive *Enterococcus* spp. isolates were kept at -20°C in (Bosch Deep Freezer GSN33VWE0N, Poland) Brain Heart Infusion Broth (BHI, Oxoid CM1135, United Kingdom) supplemented with 20% glycerol to ensure preservation for further analysis.

Extraction of total genomic DNA from bacterial isolates

Within the scope of the study, n = 40 Amp^R *Enterococcus* spp. strains were isolated from raw milk and chicken pieces. DNA extraction from these isolates was performed using the typical boiling method. To achieve this, pure colonies grown on blood agar were suspended in 200 µL of nuclease–free water and incubated at 95°C for 10 min. The samples were then centrifuged (Thermo Scientific Micro CL17 Microsantrifuge, Waltham, MA USA) at 13,000 g, and 120 µL of the supernatant was transferred to Eppendorf tubes and stored at -20°C (Bosch Deep Freezer GSN33VWE0N, Poland) for subsequent analysis [23].

PCR analysis for the confirmation of *Enterococcus* spp.

In this study, DNA samples obtained from *Enterococcus* strains isolated from milk and chicken meat were analyzed for species–

specific *tuf* genes using conventional polymerase chain reaction (PCR), (Turbo–Cycler Lite, Blue–Ray, Biotech, Taiwan) [24]. For species–level delineation of *Enterococcus* spp. PCR was performed using two pairs of primers unique to the *ddl* gene of *E. faecalis* and *E. faecium*. All primer sequences utilized in the investigation are included in the TABLE I. A negative control consisted of water devoid of Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA), whereas a positive control was DNA extracted from the *E. fecalis* ATCC 29212 strain.

TABLE I
Primer sequences used for the confirmation of *Enterococcus* spp. in this study

No	Target Gene	Primer Sequence (5'-3')	Base Pair (bp)	Reference
1	*Tuf*	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112	Ke *et al.* [24]
2	$ddl_{E.\ faecalis}$	ATCAAGTACAGTTAGTCTTATTAG ACGATTCAAAGCTAACTGAATCAGT	941	Kariyama *et al.* [25]
3	$ddl_{E.\ faecium}$	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	658	Cheng *et al.* [26]

For PCR amplification, a total of 25 µl of PCR reaction was prepared as follows: 12.5 µL of 2× master mix containing Taq DNA polymerase, dNTP mix, $MgCl_2$, and reaction buffer (FIREPol® Master Mix, Solis BioDyne, Tartu, Estonia), 0.5 µL of 10 pmol of each primer pair, 1 µL of target DNA (5–100 ng·µL^{-1}) and 10.5 µL of DNA/RNA–free water were added. The PCR procedure consisted of 35 cycles, beginning with a 3 min initial denaturation at 95°C, followed by 30 s of denaturation at 95°C, 30 s of annealing at 55°C, 1 min of extension at 72°C, and a final 7 min DNA synthesis at 72°C [25, 26].

From the obtained PCR products, 10 µL of DNA sample and 5 µL of loading dye were mixed and loaded onto the gel. Amplicons were separated via electrophoresis in a 1.5% agarose gel (Vivantis LE Grade Agarose Gel, Malaysia) at 90 volts and 500 mA electric current for 50 min. The presence of the amplicons was visualized under UV light by means of a transilluminator (Vilbert Loumart, ECX–F26.M, France).

Determination of virulence genes by multiplex PCR (mPCR)

For the detection of the presence of particular virulence genes (*asa1, cyl, esp, gelE,* and *hyl*) implicated in colonization, adhesion, and invasion in *Enterococcus* spp. isolates, mPCR was conducted with primers targeted to these genes (TABLE II). [27].

Determination of antibiotic susceptibility profiles

The antimicrobial susceptibility profiles of *Enterococcus* isolates were assessed by the Kirby–Bauer disk diffusion method [28, 29]. Bacterial suspensions were standardized to a 0.5 McFarland density with the aid of a densitometer (Biosan Den–1, Latvia) and subsequently spread onto Mueller Hinton Agar (MHA; Oxoid, CM0337, United Kingdom) by means of a sterile cotton swab. In order to define the antimicrobial susceptibility profiles of *Enterococcus* isolates, eight antibiotic disks were placed on to the MHA surface, including Ampicillin (Amp; 10 µg), Chloramphenicol

TABLE II
Primer sequences of virulence genes used in this study

No	Target Gene	Primer Sequence (5'-3')	Base Pair (bp)
1	*asa1*	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375
2	*gelE*	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	213
3	*cylA*	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688
4	*esp*	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510
5	*hyl*	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	276

(C; 30 µg), Ciprofloxacin (Cip; 5 µg), Erythromycin (E; 15 µg), Gentamicin (Cn; 120 µg), Rifampin (Rd; 5 µg), Tetracycline (Te; 30 µg), and Vancomycin (Van; 30 µg) (Oxoid Antibiotic Disks, United Kingdom) [28, 29]. *E. faecalis* ATCC 29212 served as a positive control throughout the research.

The MIC values of strains (n=40) previously defined to Amp^R (≤ 16 R) *Enterococcus* isolates by the disk diffusion method were evaluated using the broth microdilution technique [29]. Following the recommended 48-h incubation for enterococci, the growth in the wells of the plate was evaluated, the growth and sterility control wells were examined, and the MIC values of the reference organism (*E. faecalis* ATCC 29212) and isolates were obtained and recorded. The MIC values of the isolates were assessed in accordance with CLSI [29] guidelines. A loopful (10 µL) was taken from the growth control well and inoculated onto Blood Agar, and the colony morphologies were assessed on the subsequent day. For Enterococci, ampicillin MIC values of ≤ 8 µg·mL^{-1} were considered as susceptible and ≥ 16 µg·mL^{-1} as resistant [29].

RESULTS AND DISCUSSION

A total of 40 Amp^R *Enterococcus* spp. strains were recovered from raw milk and chicken meat samples in this study. Among the species isolated from these samples, 67.5% (n=27/40) were classified as *E. faecium*, 12.5% (n=5/40) as *E. faecalis*, and 20% (n=8/40) as other *Enterococcus* spp. The species–level distribution of Amp^R *Enterococcus* isolates revealed that 4 (20.0%) were *E. faecalis* and 10 (50.0%) were *E. faecium* in milk samples, on the other hand 1 (5.0%) was classified as *E. faecalis* and 17 (85.0%) as *E. faecium* in chicken meat samples (TABLE III).

TABLE III
Distribution of Ampicillin–Resistant *Enterococcus* spp. positive isolates and species in raw milk and chicken meat samples (%)

Sample	Number of Amp^{R1}	*E. faecalis*	*E. faecium*	Other *Enterococcus* spp.^{2}
Milk	20	4 (20.0)	10 (50.0)	6 (30)
Chicken meat	20	1 (5.0)	17 (85.0)	2 (10)
Total	40	5 (12.5)	27 (67.5)	8 (20.0)

[1]Amp^R: Phenotypically obtained Ampicillin–resistant isolates. [2] Other *Enterococcus* spp.: *Enterococcus* strains identified other than *E. faecalis* and *E. faecium*

Enterococcus spp. have gradually evolved from commensal bacteria due to their natural antimicrobial resistance abilities and virulence potential, inflicting to life threatening hospital–acquired infections worldwide. Although *Enterococcus* is commensal bacteria, it is the primary opportunistic pathogen in the digestive systems of humans and animals [30]. Multidrug resistance (MDR) enterococci represent a significant threat due to the possibility of spreading these resistant infections directly or indirectly from food producing animals to humans. According to results of the studies conducted globally, the prevalence of *Enterococcus* spp. is high in animal–derived foods [31, 32].

Studies on isolates of *Enterococcus* spp. have demonstrated that *E. faecalis* and *E. faecium* are the most commonly encountered species, followed by *E. durans, E. hirae, E. gallinarum*, and *E. casseliflavus* [21, 31, 33, 34, 35]. Indeed, in this study, of the Amp^R resistant strains observed in the milk and chicken meat samples, 12.5% were *E. faecalis* and 67.5% were *E. faecium*. The discrepancies obtained from all these investigations may be attributed to the origin, geographical differences, study period, sampling method, initial contamination of samples, farm, milking and personnel hygiene and methodological differences. A limitation of our study is that these factors were not controlled, which may have influenced our results.

In the 1980s, high–level resistance to ampicillin and vancomycin emerged in hospitals across the United States, leading to outbreaks and an increase in healthcare–associated infections caused by *E. faecium* strains resistant to both antibiotics. Ampicillin and vancomycin are the key antibiotics for managing enterococcal infections [36]. Clinical strains, on the other hand, frequently have distinct adaptation traits and are resistant to both antibiotics, and these strains are reported to be both metabolically and virulently enriched [37]. Indeed, ampicillin resistance has been reported to be high in adults in clinical isolates regardless of geographic origin [38].

In this study, disk diffusion and then microdilution methods were used to determine the ampicillin resistance of *Enterococcus* strains. Joste *et al.* [39] investigated phenotypic Amp^R *E. faecium* strains in their study, suggested that disk diffusion test results should be confirmed by microdilution method and reported that MIC results revealed more accurate results. Although investigation confirmed that all isolates exhibited ampicillin resistance phenotype (100%) by the disk diffusion technique, eleven of the isolates tested had MIC values more than or equal to 16 μg·mL^{-1}, which is considered as the cut–off value [29]. Six (35.3%) Amp^R *E. faecium* isolated from chicken meat samples and two (20%) isolates from milk samples had MIC values greater than 16 μg·mL^{-1}. Joste *et al.* [39] recommend using the broth microdilution method to determine the MIC values of *E. faecium* strains that exhibit to be resistant by the disk diffusion method, notably in severe infections to accurately detect ampicillin resistance.

Indeed, in line with the purpose of this study, a total of 40 phenotypically Amp^R resistant isolates were obtained from milk and chicken meat samples, genes specific to penicillin–binding proteins (such as PBP5) were not examined, as a limiting facet of this study, we didn't perform sequence analysis to elucidate the mechanisms responsible for ampicillin resistance due to financial constraints, however, previous studies have indicated that multiple mutations in the active site of the PBP5 was the common mechanism for high–level ampicillin resistance [40, 41]. Penicillin–binding proteins, like PBP5, play a vital role in the construction of the bacterial cell wall, also serve as targets for β–lactam antibiotics and therefore, it is significant to investigate them in *Enterococcus* spp. isolates. High–level resistance to β–lactams in *E. faecium* was found to be associated with the expression of PBP5 [42]. It is stated that ampicillin resistance in *E. faecium* has low–level resistance based on increased amounts of PBP5 with low affinity to beta–lactams (MIC 8 to 64 mg·L^{-1}) and high–level resistance based on mutation in PBP5 with high MICs of 16 mg·L^{-1} [39].

MIC values of all Amp^R isolates were determined using the broth microdilution method in the present study. The MIC profiles of the isolates were evaluated in accordance with the CLSI (2020) criteria. The results for isolates with ampicillin MIC values of 16 μg·L^{-1} or above are presented in TABLE IV. The MIC values of five Amp^R strains (two *E. faecium* and three other *Enterococcus* spp.) derived from milk samples, as well as six strains (*E. faecium*) collected from chicken meat samples, were revealed to be ≥ 16 μg·mL^{-1} (TABLE IV). Of the Amp^R *Enterococcus* spp isolates obtained from milk samples, 5 (25.0%) exhibited MIC values of ≥ 16 μg·mL^{-1}, 4 (20%) demonstrated MIC values of 4 μg·mL^{-1}, 8 (40%) indicated MIC values of 2 μg·mL^{-1}, and 3 (15%) had MIC values below 1 μg·mL^{-1}. Among the Amp^R *Enterococcus* spp isolates derived from chicken meat samples, 6 (30.0%) exhibited MIC values of ≥ 16 μg·mL^{-1}, 1 (5%) showed a MIC of 8 μg·mL^{-1}, 9 (45%) had MICs of 4 μg·mL^{-1}, 2 (10%) recorded MICs of 2 μg·mL^{-1}, and 2 (10%) presented MIC values below 1 μg·mL^{-1}. The Amp^R *Enterococcus* spp. isolates in this investigation had MIC values ranging from 32 μg·mL^{-1} to >512 μg·mL^{-1} (TABLE IV).

Amp^R *Enterococcus* spp. isolates derived from milk and chicken meat samples exhibited varying MIC values. The MIC range of Amp^R isolates obtained from milk samples (32 – >512 μg·mL^{-1}) was wider than the isolates obtained from chicken meat samples (32 – 256 μg·mL^{-1}). This phenomenon may indicate a higher level of ampicillin resistance in *Enterococcus* spp. isolates obtained from milk samples. In contrast to this study, Morandi *et al.* [32]. reported that the *Enterococcus* spp. strains isolated from milk and feces differed significantly, and all strains were susceptible to Amp as well as the antibiotics daptomycin, Cn, teicoplanin, and Van in MIC results.

Among Amp^R *Enterococcus* spp. isolates derived from raw milk and chicken meat samples, 80.0% of *E. faecalis* (n=5) isolates were resistant to rifampin, 40.0% to Cn, 20.0% to E and C. However, resistance to Cip, Te and Van were not detected in any *E. faecalis* isolates. Of the *E. faecium* isolates (n=27), 74.1% were resistant to E, 70.4% to Te, 7.4% to C, Cip, and Cn, and 3.7% to Rd and Van. In other Amp^R *Enterococcus* isolates (n=8), 62.5% resistance was detected to Rd, 50% to Van, 37.5% to Cip, 12.5% to E and Te. Furthermore, it was shown that the Amp^R *Enterococcus* spp. isolates (n=40) obtained from raw milk and chicken meat samples revealed a 67.5% (27/40) of MDR (a resistance pattern that encompasses at least one antibiotic, from three or more distinct antibiotic categories). Within the scope of the study, one strain obtained from chicken meat was phenotypically resistant to six antibiotics: Amp, E, C, Cn, Rd and Te (AmpECCnRdTe) (TABLE V).

TABLE IV
Minimum inhibitory concentrations of ampicillin–resistant *Enterococcus* spp. isolates collected from milk and chicken meat samples (μg·mL^{-1})

Species	Sample	No. (%) at MIC (μg·mL^{-1})										Ampicillin MIC Value (≥16 μg·mL^{-1})
		<1	2	4	8	16	32	64	128	256	>512	
E. faecalis (n= 5)	Milk (n= 4)	1 (25.0)	2 (50.0)	1 (25.0)	–	–	–	–	–	–	–	–
	Chicken meat (n= 1)	1 (1.0)	–	–	–	–	–	–	–	–	–	–
E. faecium (n= 27)	Milk (n= 10)	2 (20.0)	5 (50.0)	1 (10.0)	–	–	–	**1 (10.0)**	–	–	**1 (10.0)**	**2 (20.0)**
	Chicken meat (n= 17)	1 (5.9)	1 (5.9)	8 (47.1)	1 (5.9)	–	**2 (11.8)**	**2 (11.8)**	–	**2 (11.8)**	–	**6 (35.3)**
Other *Enterococcus* spp. (n= 8)	Milk (n= 6)	–	1 (16.7)	2 (33.3)	–	–	**1 (16.7)**	–	**1 (16.7)**	**1 (16.7)**	–	**3 (50.0)**
	Chicken meat (n= 2)	–	1 (50.0)	1 (50.0)	–	–	–	–	–	–	–	–
Total *Enterococcus* spp. (n= 40)		5 (12.5)	10 (25.0)	13 (32.5)	1 (2.5)	–	**3 (7.5)**	**3 (7.5)**	**1 (2.5)**	**3 (7.5)**	**1 (2.5)**	**11 (27.5)**

n: number of Amp^R isolates MIC values of ≤ 8 μg·mL^{-1} represents Ampicillin susceptible, ≥16 g·mL^{-1} indicates Ampicillin resistance according to CLSI [29]. MIC values highlighted in bold demonstrate ampicillin–resistant strains

TABLE V
Antibiotic Resistance Profiles of Ampicillin–Resistant *Enterococcus* spp. Strains Isolated from Raw Milk and Chicken Meat (%)

Antimicrobial Agent	*E. faecalis* (n=5)		*E. faecium* (n=27)		Other *Enterococcus* spp. (n=8)		Total (n=40)	
	S	R	S	R	S	R	S	R
Ampicillin (Amp, 10 μg)	–	5 (100)	–	27 (100)	–	8 (100)	–	40 (100)
Chloramphenicol (C, 30 μg)	4 (80.0)	1 (20.0)	25 (92.6)	2 (7.4)	8 (100)	–	37 (92.5)	3 (7.5)
Ciprofloxacin (Cip, 5 μg)	5 (100)	–	25 (92.6)	2 (7.4)	5 (62.5)	3 (37.5)	32 (80.0)	8 (20.0)
Erythromycin (E, 15 μg)	4 (80.0)	1 (20.0)	7 (25.9)	20 (74.1)	7 (87.5)	1 (12.5)	18 (45.0)	22 (55.0)
HLGR (Cn, 120 μg)	3 (60.0)	2 (40.0)	25 (92.6)	2 (7.4)	8 (100)	–	36 (90.0)	4 (10.0)
Rifampin (Rd, 5 μg)	1 (20.0)	4 (80.0)	26 (96.3)	1 (3.7)	3 (37.5)	5 (62.5)	23 (57.5)	17 (42.5)
Tetracycline (Te, 30 μg)	5 (100)	–	8 (29.6)	19 (70.4)	7 (87.5)	1 (12.5)	20 (50.0)	20 (50.0)
Vancomycin (Van, 30 μg)	5 (100)	–	26 (96.3)	1 (3.7)	4 (50.0)	4 (50.0)	35 (87.5)	5 (12.5)

HLGR: High level gentamicin resistance S: Susceptible R: Resistant

In the current study, resistance to vancomycin, a glycopeptide antibiotic, was detected in 12.5% (n=5/40) of Amp^R *Enterococcus* spp. isolates. In a study conducted in Egypt by Hammad *et al.* [43] vancomycin resistance in raw milk samples was reported as 91.6%. Results from previous studies conducted in Ireland, Türkiye, and Italy that either failed to delineate Vancomycin–Resistant Enterococcus (VRE) in raw milk and its products or identified VRE at low levels [20, 44, 45] contradict with these researchers' findings (91.6%). Similar to the current study, 5% of enterococcal isolates obtained from cheese in Egypt were discovered to be resistant to vancomycin, but vancomycin resistance genes were not detected in these isolates [46].

This dramatic wane can be attributed to the prohibition on antibiotic use in animal production since 2006, as the widespread use of avoparcin as a growth promoter in animal production facilities has contributed to the rise of VRE in humans, animals, and animal products [47]. VRE prevalence in European countries has markedly declined since 2006, when the use of avoparcin in animal production facilities was outlawed in the European Union [48, 49]. Similarly, studies conducted in Türkiye have also noted the presence of vancomycin–resistant enterococci in animal production [41, 50]. For instance, Aslantaş [41] reported ampicillin resistance in 3.3% (11 isolates) and vancomycin resistance in 1.5% (5 isolates) of *Enterococcus* spp. isolates derived from broilers. These findings accentuate the efficacy of antibiotic usage policies in mitigating the spread of resistant microorganisms.

Enterococci strains have been reported that they can develop intrinsic resistance to different classes of antibiotics, such as aminoglycosides, β–lactams, cephalosporins, lincosamides and trimethoprim–sulfamethoxazole at low or high levels [41, 51]. Numerous studies have designated the worldwide frequency of antibiotic–resistant enterococci in animal derived foods [32, 52, 53]. In this study, a substantial proportion of Amp^R *Enterococcus* spp. isolates exhibited resistance to erythromycin, tetracycline, and rifampin. Consistent with this study, high–level of tetracycline resistance in *E. faecium* isolates from milk and chicken meat has also been reported in countries such as Türkiye [52, 54], Korea [34], Italy [32], Iran [53] and Spain [55].

The results of this study indicated that Amp^R *E. faecalis* and *E. faecium* isolates exhibited significant resistance to the majority of the antibiotics tested. In this study, the majority of HighLevel Gentamicin Resistant (HLGR) and Amp^R enterococci isolated from raw milk and chicken meat samples were resistant to more than one antibiotic class. Infections caused by MDR *Enterococcus* are a significant global public health issue and it is critical to monitor

them within the scope of the one health concept. The widespread distribution of *Enterococcus* spp., along with contributing factors like genomic adaptability and extensive antibiotic usage, seems to have played a role in the recent emergence of *E. faecium* and *E. faecalis* as MDR pathogens [15]. A multifaceted one health concept is required to elucidate the relationship between antimicrobial use and antimicrobial resistance across the human, animal, and environmental settings, in order to conduct a comprehensive investigation into the current landscape of antimicrobial resistance.

HLGR was detected in 10.0% of Amp^R *Enterococcus* spp. isolates from raw milk and chicken meat samples. In this investigation, Amp^R *E. faecalis* strains were discovered to be 80% resistant to Rd, 40% to HLGR, and 20% to E and C among the antimicrobials tested. In *E. faecium* strains, resistance was found to be 74.1% to Erythromycin, 70.4% to Te, 7.4% to Cip and C, and 3.7% to Rd and Van, respectively. Nasiri and Hanifian [53] reported an HLGR rate of 26.1% in *E. faecalis* isolates using the disk diffusion technique. Their usage for the treatment of enterococcal infections is crucial due to the development of high–level resistance to gentamicin and streptomycin [56, 57]. HLGR resistance is critical for the success of therapeutic treatment efficacy, as gentamicin is utilized in combination with antibiotics that target the cell wall in the management of enterococcal infections. However, these combinations will not be effective in the treatment of HLGR enterococcal infections [58].

The development of resistance to other antibiotic classes, including erythromycin and tetracycline, has been reported as a common feature among enterococci isolated from animal derived foods in many previous studies [20, 21, 22, 35, 46, 59]. In the current study, 47.5-55.0% of enterococcal isolates were revealed to be resistant to E and Te, respectively. Additionally, low level of resistance to C (7.5%) was found in this study. The antibiotic resistance profile obtained from the investigation was largely similar with studies implemented on raw milk and chicken meat samples [21, 23, 35, 60]. The high resistance of enterococci isolated from animal–derived foods to Te has previously been attributed to the widespread use of this antibiotic in veterinary medicine [46].

Antibiotic susceptibility profiles according to species in Amp^R *Enterococcus* spp. strains (n=40) isolated from raw milk and chicken meat samples revealed that 80.0% were resistant to Rd, 40.0% to Cn, and 20% to E and C in *E. faecalis* isolates (n=5). However, resistance was not detected to Cip, Te and Van in any *E. faecalis* isolates. On the other hand, isolates of *E. faecium* (n=27) exhibited resistance to 74.1% for E and 66.7% for Te. Van resistance was detected in 3.7% of these isolates. The distribution of antibiotic resistance profiles exhibited by Amp^R *Enterococcus* spp. strains isolated from raw milk and chicken meat samples is presented in Table VI according to the respective species. In addition, the sample–based distribution of phenotypic resistance profiles of *E. faecalis, E. faecium*, and other *Enterococcus* spp. strains of Amp^R *Enterococcus* isolates recovered from raw milk and chicken meat samples is presented in Table VI.

E. faecalis isolates collected from milk samples were discovered to be resistant to Amp and Rd but sensitive to Cip, E, C, Cn, Van, and Te. *E. faecalis* isolates from chicken meat samples were found to be resistant to Amp, E, C, Cn, and Rd, but sensitive to Cip, Te, and Van. *E. faecium* isolates obtained from milk samples demonstrated resistance rates of 60% to Rd, 50% to E, 20% to Te, 10% to Van. Furthermore, it was established that these isolates were susceptible to Cip and C. *E. faecium* isolates derived from chicken meat samples exhibited 100% resistance to Te, 88.2% to E, and 11.8% to both Cip and C. This notwithstanding, these isolates were discovered to be susceptible to Van (TABLE VI). HLGR was detected in 4 out of 40 Amp^R *Enterococcus* isolates (40%). HLGR was identified in 1 *E. faecalis* isolate, 2 *E. faecium* isolates, and 1 other *Enterococcus* isolate. Besides, this investigation identified five vancomycin–resistant isolates. It was detected in 4 of the Amp^R *Enterococcus* isolates (20%) derived from milk samples and in 1 isolate (5%) obtained from chicken meat samples (TABLE VI).

In this study, 19 of *Enterococcus* spp. isolates were discovered to be phenotypically resistant to tetracycline. Resistance to E, Rd, and Te is frequently detected in both *E. faecalis* and *E. faecium* isolates. Resistance to E, Rd and Te is frequently detected in both *E. faecalis* and *E. faecium* isolates when other routinely used antibiotics are

TABLE VI
Sample based patterns of antibiotic resistance profiles in Ampicillin–Resistant *Enterococcus* spp. Strains (%)

Antimicrobial Agent	Species / susceptibility category															
	***E. faecalis* (n=5) (%)**				***E. faecium* (n=27) (%)**				**Other *Enterococcus* spp. (n=8) (%)**				**Total (n=40) (%)**			
	Milk (n=4)		**Chicken meat (n=1)**		**Milk (n=10)**		**Chicken meat (n=17)**		**Milk (n=6)**		**Chicken meat (n= 2)**		**Milk (n=20)**		**Chicken meat (n= 20)**	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
Ampicillin (Amp, 10 µg)	–	4 (100)	–	1 (100)	–	10 (100)	–	17 (100)	–	6 (100)	–	2 (100)	–	20 (100)	–	20 (100)
Chloramphenicol (C, 30 µg)	4 (100)	–	–	1 (100)	10 (100)	–	15 (88.2)	2 (11.8)	6 (100)	–	2 (100)	–	20 (100)	–	17 (85)	3 (15)
Ciprofloxacin (Cip, 5 µg)	4 (100)	–	1 (100)	–	10 (100)	–	15 (88.2)	2 (11.8)	2 (33.3)	4 (66.7)	–	2 (100)	16 (80)	4 (20)	16 (80)	4 (20)
Erythromycin (E, 15 µg)	4 (100)	–	–	1	5 (50)	5 (50)	2 (11.8)	15 (88.2)	6 (100)	–	1 (50)	1 (50)	15 (75)	5 (25)	3 (15)	17 (85)
HLGR (Cn, 120 µg)	4 (100)	–	–	1 (100)	9 (90)	1 (10)	16 (94.1)	1 (5.9)	5 (83.3)	1 (16.7)	2 (100)	–	18 (90)	2 (20)	18 (90)	2 (20)
Rifampin (Rd, 5 µg)	–	4 (100)	–	1 (100)	4 (40)	6 (60)	16 (94.1)	1 (5.9)	1 (16.7)	5 (83.3)	2 (100)	–	5 (25)	15 (75)	18 (90)	2 (20)
Tetracycline (Te, 30 µg)	4 (100)	–	1 (100)	–	8 (80)	2 (20)	–	17 (100)	6 (100)	–	1 (50)	1 (50)	18 (90)	2 (20)	2 (20)	18 (90)
Vancomycin (Van, 30 µg)	4 (100)	–	1 (100)	–	9 (90)	1 (10)	17 (100)	–	3 (50)	3 (50)	1 (50)	1 (50)	16 (80)	4 (20)	19 (95)	1 (5)

HLGR: High level gentamicin resistant, S: Susceptible, R: Resistant

considered. Particular antibiotics evaluated in this study (such as Rd) and classified by the World Health Organization (WHO) [61] are among the antibiotic classes that are critically important for the treatment of VRE infections. There is no data relative to the usage of these antibiotics in veterinary medicine in Türkiye. In this study, 25.0% of the isolates were found to be resistant to rifampin. It was observed that *E. feacalis* (80.0%) isolates exhibited higher resistance to rifampin than *E. faecium* (3.7%).

Within the scope of this study, Amp^R *Enterococcus* spp., *E. faecalis*, and *E. faecium* isolates were subjected to mPCR analysis in order to determine the presence of several key virulence genes, namely *asa1, cylA, esp, gelE* and *hyl*. In this study, the *asa1* gene was identified in a single *E. faecalis* isolate (1/40; 2.5%). Upon examination of the virulence genes, the *asa1* gene was identified only in an *E. faecalis* isolate derived from chicken meat. However, the *gelE, cylA, esp* and *hyl* genes were not identified in any of the Amp^R *Enterococcus* spp. isolates.

The present study investigated the presence of several key virulence genes *(asa1, gelE, cylA, esp,* and *hyl*) in *Enterococcus* spp. isolates. The presence of virulence factors does not necessarily indicate that strains isolated from animal–derived foods lead to diseases; however, it has been suggested that strains containing these virulence factors may have pathogenic potential and contribute to the severity of infection [62, 63].

Previous studies have reported that a number of virulence genes such as *gelE, esp, hyl,* and *asa1* were frequently detected in *Enteroccoccus* spp. strains isolated from raw milk samples [31, 33, 34, 43]. The presence of virulence genes in *E. faecalis* and *E. faecium* isolates, such as *esp* (enterococcal surface protein), *hyl* (hyaluronidase), and *gelE* (gelatinase), has been linked to intestinal colonization, host tissue invasion, and translocation through epithelial cells [43].

The aggregation substance encoded by *asa1* is an enterococcal surface protein that facilitates bacterial conjugation by promoting the formation of bacterial aggregates. It has been stated that *asa1* may increase the frequency of transfer of antimicrobial resistance and virulence genes to recipient cells [64]. In this study, *asa1*, one of the virulence genes, was only found in 2.5% of an *E. faecalis* strain isolated from chicken meat sample. *gelE, cylA, esp,* and*hyl* virulence genes were not found in any of the Amp^R *Enterococcus* spp. isolates studied. It has been reported that *cylA*, which is responsible for virulence properties associated with the production of active cytolysin, is detected in a small number of isolates [31]. It is known that the *esp* gene is associated with biofilm production, endocarditis and nosocomial infections [34, 65]. The results obtained from this study were also in accordance with previous studies [34, 43]. Indeed, a study comparing the virulence characteristics of enterococcal strains of diverse origins revealed that those associated with food–borne strains had the lowest incidence of virulence and pathogenicity [66].

CONCLUSIONS

According to the findings of this investigation, the presence of multidrug resistant *Enterococcus* spp. strains in raw milk and chicken meat samples suggests a potential source of contamination for humans. The occurrence of *Enterococcus* spp. strains exhibiting resistance to multiple antibiotic classes in raw milk and chicken meat samples warrants monitoring due to their potential health risks to people.

The presence of multidrug–resistant *Enterococcus* spp. in animal–derived foods represents an important public health risk, as these bacteria can act as reservoirs for antibiotic resistance genes and potentially spread them to human infections. This study highlights the urgent need for improved surveillance programs to monitor the prevalence and resistance trends of *Enterococcus* spp. throughout the food chain.

Given the potential consequences for food safety and public health, preventive measures – such as improved hygiene practices, decreased use of antimicrobials in animal husbandry, and strengthened regulatory frameworks – are essential to limit the spread of resistant bacteria. Continuous surveillance, combined with targeted intervention techniques, will be essential to reduce the risk of transmission and to ensure the microbiological safety of food of animal origin.

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Conflict of interest

The authors declare no potential conflicts of interest.

Ethics approval

Ethical approval is not required for this study.

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