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Original Research

Enterococcus faecalis in Traditional Cheeses: Prevalence and Antibiotic Resistance Profile

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Abstract

Background: This study aimed to determine the prevalence and phenotypic and genotypic characteristics of *Enterococcus faecalis* strains in traditionally produced cheeses in Ankara, Türkiye. **Methods:** Enterococcal isolates from cheese samples were identified using phenotypic and molecular methods. Given the public health relevance of *E. faecalis*, antimicrobial resistance testing was conducted on 33 isolates using a panel of commonly used antibiotics representing a broad range of clinically relevant classes. Antimicrobial resistance of *E. faecalis* isolates was evaluated using the disk diffusion method following standard guidelines. **Results:** A total of 98 probable enterococcal isolates were obtained from 108 cheese samples and identified phenotypically and molecularly. *E. faecium* was the most prevalent species (63.27%), followed by *E. faecalis* (33.67%) and *E. lactis* (3.06%), with the predominance of *E. faecium* being statistically significant ($p < 0.05$). High resistance rates were observed to rifampicin and kanamycin (100%), followed by ciprofloxacin (90.91%), enrofloxacin (84.85%), trimethoprim (78.79%), and ampicillin (60.61%). Moderate resistance was detected to several β-lactams and other antibiotic classes, whereas lower resistance frequencies were observed for erythromycin, chloramphenicol, and gentamicin. Notably, all *E. faecalis* isolates were fully susceptible to vancomycin. Multidrug resistance was common among *E. faecalis* isolates, with 33.33% of isolates resistant to six or more antibiotics. Resistance patterns differed significantly among antibiotic classes ($p < 0.05$), indicating a heterogeneous resistance distribution. The Multiple Antibiotic Resistance (MAR) index ranged from 0.166 to 0.333, with a mean of 0.27 ± 0.06, exceeding the threshold for high-risk contamination sources. **Conclusions:** The high prevalence of multidrug-resistant *E. faecalis* in traditional cheeses highlights the need for improved hygienic practices during artisanal production, rational antimicrobial use in animal husbandry, and ongoing resistance surveillance to mitigate potential risks to food safety and public health.

Keywords: *Enterococcus faecalis*; traditional cheese; antibiotic resistance; prevalence

1. Introduction

Enterococci are Lactic Acid Bacteria (LAB) widely distributed in natural environments and commonly present in the gastrointestinal tracts of humans and animals [1,2]. Members of the genus *Enterococcus* are Gram-positive, non-spore-forming microbiota that lack oxidase and catalase activity and exhibit a facultative anaerobic metabolism. These bacteria display considerable ecological versatility, as they are capable of growth over a broad temperature range of 10–45 °C and can tolerate high salt concentrations of up to 6.5% NaCl [3]. According to the List of Prokaryotic names with Standing in Nomenclature, the genus currently comprises 89 validly described species and three subspecies [4]. Certain enterococcal strains have been investigated for their probiotic potential due to their reported roles in maintaining intestinal microbial balance and alleviating gastrointestinal inflammation [5,6].

In the dairy industry, enterococci play a significant role, primarily as Non-Starter Lactic Acid Bacteria (NSLAB) in a wide variety of cheeses. Cheese is one of the most widely consumed fermented dairy products worldwide and constitutes an important component of the human diet. It is characterized by a highly complex microbiota shaped by multiple factors, including milk origin, processing conditions, starter culture composition, ripening environment, and hygiene practices [7]. Within this ecosystem, enterococci are particularly prevalent in traditional and artisanal cheeses produced in southern Europe and other regions, especially those manufactured from raw or pasteurized milk, as well as in natural milk- or whey-based starter cultures [8]. During cheese ripening, enterococci may contribute to the development of characteristic sensory properties through their proteolytic and lipolytic activities, citrate utilization, and the formation of aroma-related volatile compounds [9]. Among the species most frequently associated with cheese ecosystems are *Enterococcus faecalis* and *Enterococcus faecium*, followed by *Enterococcus durans* [10,11]. However, despite their technological relevance, elevated enterococcal loads in dairy products are often interpreted as indicators of inadequate hygiene during milk production and processing. Potential sources of contamination include human or animal fecal material, contaminated water, the external surfaces of animals, milking equipment, and bulk storage tanks [10,12].

The ecological success of enterococci in dairy environments is further supported by their psychotropic behavior, thermal tolerance, and metabolic adaptability, which allow them to proliferate during refrigerated storage of milk and to survive pasteurization processes. Consequently, enterococci are frequently detected as components of the indigenous microbiota of both raw and pasteurized milk [11]. For many years, these microbiotas were regarded as harmless commensals with limited relevance to human health. Nevertheless, accumulating evidence has demonstrated that enterococci can act as opportunistic pathogens, particularly in hospital settings, where they are associated with a broad spectrum of infections, including urinary tract infections, bacteremia, sepsis, infective endocarditis, and wound infections [13,14,15].

Among the members of this genus, *E. faecalis* is the species most frequently implicated in clinical infections and is of particular concern due to its pronounced ability to acquire and disseminate antimicrobial resistance [15]. The frequent detection of *E. faecalis* in both industrially produced and traditional cheeses—especially those manufactured from raw milk—therefore raises important food safety concerns. While certain strains may exert beneficial effects on flavor development during cheese ripening, the presence of potentially pathogenic and antibiotic-resistant *E. faecalis* strains in cheese is undesirable and represents a potential risk to consumer health [11,16]. In line with these concerns, *Enterococcus* species have not been granted Generally Recognized as Safe (GRAS) status and are not included in the Qualified Presumption of Safety (QPS) list, underscoring the need for careful evaluation of their occurrence in food-related applications [17].

Despite the growing body of literature addressing the occurrence of enterococci in dairy products [8,18,19,20,21,22,23,24,25], significant gaps remain in the comprehensive evaluation of their technological relevance in relation to food safety. Many studies have focused either on the beneficial roles of enterococci in cheese ripening or on their clinical significance as opportunistic pathogens [8,11,26]; however, integrated assessments that simultaneously consider their ecological distribution, functional properties, and antimicrobial resistance potential within cheese matrices are still limited [16]. Data on the prevalence and resistance profiles of *E. faecalis* strains isolated from different cheese types, production systems, and processing conditions remain fragmented and highly variable [8]. Given the widespread consumption of cheese and the frequent detection of enterococci throughout the dairy chain, a clearer understanding of the balance between their technological contributions and associated health risks is essential. Such knowledge is crucial not only for improving hygienic practices and risk management strategies in cheese production but also for informing regulatory decisions regarding the use or control of enterococci in fermented dairy products [27].

Antimicrobial resistance has emerged as one of the most pressing challenges to global public health, prompting coordinated actions at both national and international levels. In response to this escalating threat, numerous initiatives have been launched through partnerships among global health authorities and regulatory bodies. Central to these efforts is the One Health framework, which recognizes the interconnectedness of human, animal, and environmental health in the emergence and dissemination of antimicrobial-resistant bacteria [28,29,30]. Within this framework, food-producing animals and related food production systems play a significant role in the transmission dynamics of antimicrobial-resistant bacteria. Dairy production, particularly traditional or small-scale cheese manufacturing, represents a relevant interface for the persistence and dissemination of resistant bacteria, where variability in hygienic practices and production conditions may facilitate microbial survival and adaptation. Raw milk and dairy products can harbor enterococci, which are natural members of the animal gastrointestinal microbiota and are able to persist in food matrices while maintaining or acquiring antimicrobial resistance determinants. Among these, *E. faecalis* is of particular concern due to its clinical relevance in human infections and its capacity to act as a reservoir of antimicrobial resistance within the food chain [30]. It has been estimated that nearly three-quarters of emerging infectious diseases in humans are of zoonotic origin, underscoring the importance of animal reservoirs in the epidemiology of antimicrobial resistance. The emergence and spread of resistant bacteria are driven by multiple factors; however, the inappropriate and excessive use of antibiotics in both human medicine and animal husbandry remains the primary driver of this global public health problem [31].

Although various *Enterococcus* species can be detected in dairy products, *E. faecalis* was chosen for in-depth antimicrobial resistance analysis due to its established importance as an opportunistic pathogen, its frequent association with clinically relevant human infections, and its recognized capacity to act as a reservoir and vector for antimicrobial resistance determinants. In addition, traditional dairy products may serve as potential vehicles for the dissemination of antimicrobial-resistant bacteria through the food chain, particularly under variable hygienic and production conditions [11,15]. Therefore, from this broader public health perspective, the present study aimed not only to determine the prevalence of *E. faecalis* in traditionally produced cheeses but also to characterize the antimicrobial resistance profiles of the recovered isolates, based on the hypothesis that these products may harbor resistant and potentially multidrug-resistant strains of *E. faecalis*.

2. Materials and Methods

2.1 Sampling and Bacterial Strains Cultivation

A total of 108 traditional cheese samples were randomly collected from different local bazaars and retail mar-

kets in the Ankara region (Türkiye). Sampling was conducted throughout 2025 to account for potential seasonal variation. The sampled products represented a range of ripened cheese types, including hard, semi-soft, and soft varieties, namely White (n = 24), Tulum (n = 24), Ezine (n = 20), Lor (n = 20), Örgü (n = 9), and Civil (n = 11). All samples were transported to the laboratory on the same day of purchase under refrigerated conditions using insulated ice containers and processed without delay.

Enterococcal isolates obtained in the present study, together with the reference strains, were routinely cultured in Tryptone Soy Broth (TSB) (Merck, Darmstadt, Germany) and Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany). Cultures were incubated at 37 °C for 24 h before further analysis. For long-term preservation, primary isolates were stored at −20 °C in 20% (v/v) glycerol (Merck, Darmstadt, Germany). The reference strains used for comparative purposes included *Enterococcus faecalis* ATCC 29212, *Escherichia coli* LMG 3083 (enterotoxigenic *Escherichia coli*, [ETEC]), and *Staphylococcus aureus* ATCC 6538.

2.2 Isolation of Enterococci and Phenotypic Characterization

For microbiological analysis, 10 g of each cheese sample was aseptically transferred into sterile stomacher bags and homogenized with 90 mL of sterile ¼ Ringer's solution (Merck, Darmstadt, Germany). The homogenates were allowed to stand at room temperature for approximately 10 min to facilitate thorough dispersion. Thereafter, tenfold serial dilutions were prepared up to 10^{-5} using sterile 0.85% (w/v) NaCl solution (Merck, Darmstadt, Germany). Aliquots (100 µL) from appropriate dilutions were surface plated onto Kanamycin Aesculin Azide (KAA) agar (Merck, Darmstadt, Germany). The inoculated plates were incubated aerobically at 37 °C for 18–48 h. Following incubation, colonies exhibiting typical enterococcal morphology were randomly selected, subcultured to obtain pure isolates, and preserved for subsequent characterization.

Presumptive *Enterococcus* isolates were phenotypically characterized using conventional biochemical and physiological assays commonly employed for enterococcal identification. The applied tests included Gram staining and catalase activity, as well as the ability to grow in TSB supplemented with 6.5% (w/v) NaCl, and at an alkaline pH of 9.6. Esculin hydrolysis was evaluated on Bile Esculin Azide agar (Merck, Darmstadt, Germany). In addition, temperature tolerance was assessed by incubating the isolates at 10 °C and 45 °C. The combined results of these assays were used to confirm the isolates as members of the genus *Enterococcus*. These phenotypic tests were performed in accordance with previously described methodology [32].

2.3 Genotypic Characterization

Genomic DNA was extracted from presumptive enterococcal isolates according to the protocol described by Cancilla et al. [33]. Purified DNA samples were stored at −20 °C until use. Polymerase Chain Reaction (PCR) assays were performed following previously established procedures [34]. Amplification of the 16S Ribosomal Ribonucleic Acid (16S rRNA) gene (900 bp region) was carried out using the universal primer pair 27f (AGAGTTTGATCMTGGCTCAG) and 907r (CCGTCAATTCMTTTRAGTTT), as described by Beasley and Saris [35]. PCR reactions were conducted in a total volume of 50 µL, containing 3 µL of template DNA, 34.75 µL of Ribonuclease (RNase)/Deoxyribonuclease (DNase)-free water (Thermo Scientific, Waltham, USA), 0.25 µL of Taq DNA polymerase (Thermo Scientific, Waltham, USA) with reaction buffer, 1 µL of each deoxyribonucleoside triphosphate (dNTP) mix (2 mM, Thermo Scientific, Waltham, USA), 4 µL of $MgCl_2$ (25 mM), 1 µL of each primer, and 5 µL of PCR buffer. Amplifications were performed using a thermal cycler (Techne TC–512, Staffordshire, UK) under the following conditions: an initial denaturation at 95 °C for 2 min, with the next step including 30 cycles (denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 2 min). The final extension step was performed at 72 °C for 7 min. The amplified PCR products were purified using the GeneJET PCR Purification Kit (Thermo Scientific, Waltham, USA) and analyzed by electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide (0.1 µg/mL). DNA bands were visualized under ultraviolet illumination. Fragment sizes were determined by comparison with an O'GeneRuler™ DNA ladder (Thermo Scientific, Waltham, USA) used as a molecular weight marker. Purified PCR amplicons were subjected to Sanger sequencing by a commercial sequencing service. The obtained nucleotide sequences were then analyzed and aligned against reference data in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) tool to confirm species-level identification.

2.4 Determination of Hemolytic Activity

Hemolytic activity of the isolates was assessed on Tryptic Soy Agar (TSA) (Merck, Darmstadt, Germany) plates supplemented with 5% (v/v) defibrinated sheep blood. The inoculated plates were incubated at 37 °C for 24–48 h under anaerobic conditions. Following incubation, hemolytic reactions were evaluated based on the appearance of zones surrounding the colonies. The presence of clear zones was interpreted as β-hemolysis, greenish discoloration as α-hemolysis, and the absence of any visible zone as γ-hemolysis [36]. All assays were performed in duplicate. *Escherichia coli* LMG 3083 (ETEC) and *Staphylococcus aureus* ATCC 6538 were included as reference control strains.

2.5 Antimicrobial Resistance of Enterococcus faecalis

Owing to its frequent association with human infections and its role as an important reservoir and potential disseminator of antimicrobial resistance determinants, a detailed evaluation of the antimicrobial resistance profiles was performed on all *E. faecalis* isolates recovered in this study. Antimicrobial resistance profiles of *E. faecalis* isolates were determined using the disk diffusion method on Mueller–Hinton agar (Merck, Darmstadt, Germany), in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [37,38]. The antimicrobial agents tested in this study represented a broad spectrum of clinically relevant antibiotic classes. These included: (i) β-lactams: Ampicillin (10 µg/disc), methicillin (5 µg/disc), penicillin G (10 µg/disc), cefazolin (30 µg/disc), cefoxitin (30 µg/disc), (ii) Glycopeptides: Vancomycin (30 µg/disc), (iii) Aminoglycosides: Gentamicin (10 µg/disc), kanamycin (30 µg/disc), streptomycin (10 µg/disc), (iv) Phenicols: Chloramphenicol (30 µg/disc), (v) Lincosamides: Clindamycin (2 µg/disc), (vi) Macrolides: Erythromycin (15 µg/disc), (vii) Tetracyclines: Tetracycline (30 µg/disc), (viii) Fluoroquinolones: Enrofloxacin (5 µg/disc), ciprofloxacin (5 µg/disc), norfloxacin (10 µg/disc), (ix) Rifamycins: Rifampicin (5 µg/disc), and (x) Trimethoprim: Trimethoprim (5 µg/disc). *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 6538 were included as quality control strains. After incubation, inhibition zone diameters were measured and interpreted as susceptible, intermediate, or resistant according to the Clinical and Laboratory Standards Institute [37,38]. Isolates displaying resistance to at least three distinct classes of antimicrobial agents were classified as Multi-drug Resistant (MDR). In addition, the Multiple Antibiotic Resistance (MAR) index was determined for each strain in accordance with the method proposed by Krumperman [39], using the following equation:

MAR index = (number of antibiotics to which the strain exhibited resistance) / (total number of antibiotics tested).

2.6 Statistical Analysis

Statistical analyses were performed using the SPSS software package (version 16.0; SPSS Inc., Chicago, IL, USA). Antimicrobial resistance frequencies among the tested antibiotics were compared using the chi-square (χ^2) test to determine statistically significant differences in resistance distributions. The level of statistical significance was set at $p < 0.05$. Descriptive statistics for MAR values were expressed as mean ± standard deviation (SD). The proportions of MDR isolates were calculated and reported as percentages.

3. Results and Discussion

From the 108 traditional cheese samples analyzed in this study, 98 (90.74%) yielded presumptive enterococcal isolates, demonstrating an exceptionally high prevalence of *Enterococcus* spp. in these products (data not shown). This high isolation frequency is consistent with several recent reports showing that *Enterococcus* spp. are common constituents of the microbiota in a wide range of dairy products. For instance, a study on traditional cheeses in northwestern Iran reported enterococci in 96% (48/50) of samples, underscoring pervasive contamination or natural colonization by these bacteria in artisanal cheeses [40]. In other geographic contexts, enterococcal prevalence varies by product type and processing; for example, French raw-milk cheeses have detected *Enterococcus* in up to >90% of samples, particularly in unpasteurized or thermized cheeses [41]. Similarly, artisanal cheeses from Brazil exhibited prevalence values between ~23% and ~75% across different regional varieties, illustrating that prevalence can be influenced by local practices and milk sources [42]. Previous studies in Türkiye [25,43,44,45,46] similarly reported a high prevalence of enterococci in traditional cheeses, with reported isolation rates ranging broadly from 62% to near 100% depending on region and cheese type. Taken together, these data indicate that traditional cheeses—particularly those produced from raw or minimally processed milk under artisanal conditions—frequently harbor *Enterococcus* spp. at high prevalence, reflecting both their ecological niche in dairy fermentation and the impact of production hygiene and processing variables on microbial composition.

Morphological and cultural characterization was performed on a total of 98 presumptive enterococcal isolates. All isolates demonstrated the ability to grow under selective conditions characteristic of the genus, including tolerance to alkaline pH (9.6), elevated salinity (6.5% NaCl), and a broad temperature range (10–45 °C). In addition, all isolates exhibited phenotypic traits consistent with enterococci, namely Gram-positive staining, absence of catalase activity, and positive esculin hydrolysis. In the present study, all 98 enterococcal isolates were successfully identified to the species level using 16S rRNA gene sequence analysis, whereas Fig. 1 presents representative results from 20 isolates. The species-level identification revealed that 62 isolates (63.26%) were assigned to *E. faecium*, whereas 33 isolates (33.67%) were identified as *E. faecalis*. The detailed characteristics of the 33 *E. faecalis* isolates are presented in Table 1. In addition, a minor proportion of the isolates was classified as *E. lactis* (3/98; 3.06%). The predominance of *E. faecium* over *E. faecalis* was statistically significant ($p < 0.05$), indicating a non-random distribution of enterococcal species among the analyzed cheese samples. Based on the hemolytic activity assays, 19 of the *E. faecalis* strains exhibited β-hemolytic activity, whereas 8 isolates were classified as γ-hemolytic and 6 as α-hemolytic (Table 1). The hemolytic activity profiles observed among the *E. faecalis* isolates in the present study warrant attention from both food safety and public health perspective. In addition, the presence of β-hemolytic *E. faecalis* in tra-

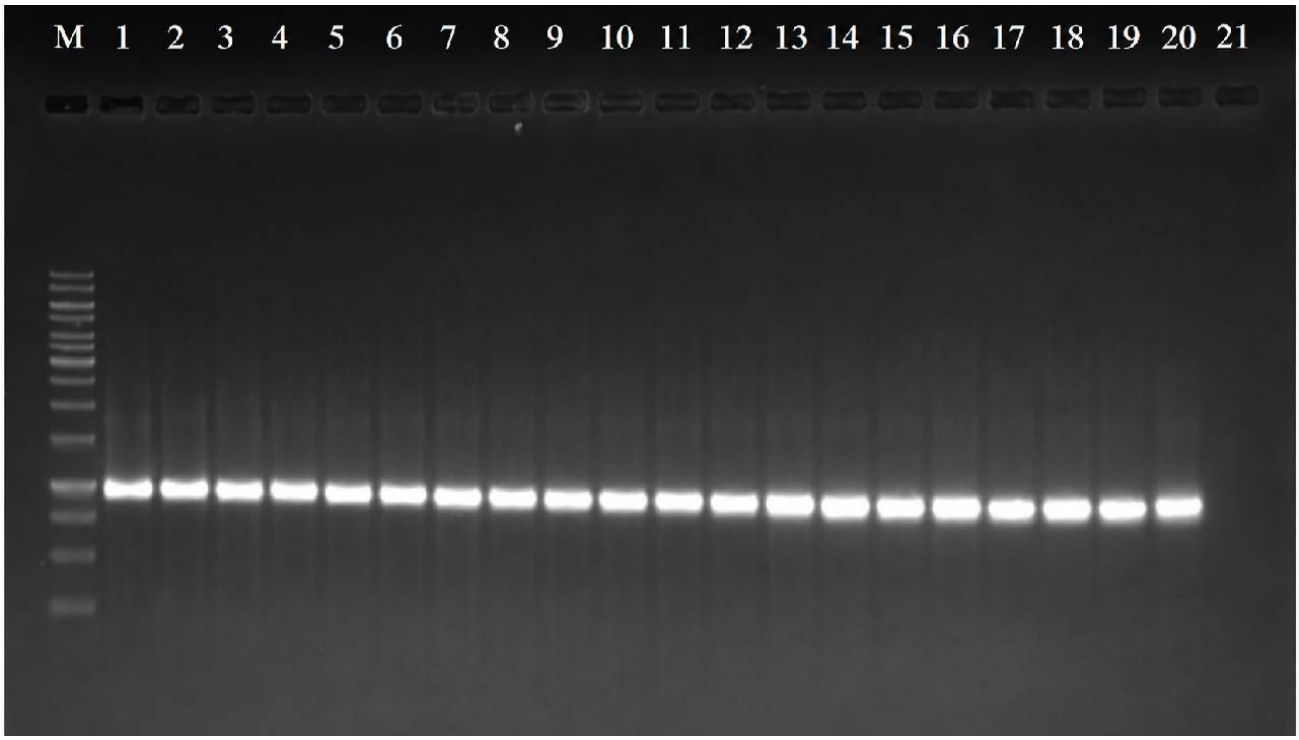

Fig. 1. Polymerase Chain Reaction (PCR) amplification of 16S rRNA gene fragments from *Enterococcus* strains. M: O'GeneRuler DNA marker; 1–20: E1, E4, E10, E14, E15, E17, E20, E30, E33, E35, E36, E38, E40, E45, E47, E49, E52, E55, E56, E60; 21: Negative control.

ditional cheese samples suggests that certain artisanal production conditions may facilitate the persistence of strains with enhanced virulence-associated traits.

Both *E. faecalis* and *E. faecium* are widely recognized as the most frequently encountered enterococcal species in dairy products, particularly in traditional and artisanal cheeses [47,48,49,50]. In agreement with our findings, several previous studies have reported *E. faecium* as the dominant species isolated from cheese matrices, including investigations conducted by Buyukyoruk et al. [3], Hanzelová et al. [8], Kırmacı et al. [44], Furlaneto-Maia et al. [51], Pesavento et al. [52], Bulajić et al. [53], Hammad et al. [54], and Bastião Rocha et al. [55]. In contrast to the findings of the present study, several previous investigations have reported a significantly higher prevalence of *E. faecalis* in cheese samples, including the studies conducted by González et al. [2], Jahansepas et al. [40], Çıtak et al. [56], Nieto-Arribas et al. [57], Kürekci et al. [58], and Gajewska et al. [59]. Conversely, in agreement with our results, Kırmacı et al. [44] detected *E. lactis* in cheese samples at a comparable prevalence rate (5.71%), indicating that this species, although less frequently encountered, may constitute a consistent component of the cheese-associated enterococcal population. Comparative analysis with previous studies reveals both similarities and regional variations in the occurrence of *E. faecalis* in cheese. Differences in prevalence may be attributed to variations in milk origin, production practices, hygiene standards, ripening conditions, and regulatory frameworks. In Türkiye, the widespread consumption of artisanal cheeses manufactured under traditional conditions may partially explain the relatively frequent detection of enterococci. While such products are valued for their distinctive sensory properties, the limited application of standardized hygienic controls during production may increase the risk of contamination by opportunistic pathogens.

The relatively high proportion of *E. faecalis* (33.67%) detected in the present study deserves particular attention from a public health perspective. *Enterococcus faecalis* is widely recognized as an opportunistic pathogen and is among the leading causes of nosocomial infections, including urinary tract infections, bacteremia, endocarditis, and wound infections [60]. Moreover, *E. faecalis* strains isolated from food products, especially traditional cheeses, have frequently been reported to harbor virulence determinants and antimicrobial resistance genes, raising concerns about their potential role as reservoirs for the dissemination of clinically relevant traits through the food chain [40]. Although enterococci are considered part of the natural microbiota of many artisanal cheeses and may contribute to flavor development during ripening. The presence of *E. faecalis* at notable levels, as observed in this study, may pose a potential risk, particularly for immunocompromised individuals and vulnerable populations [47,59]. Several studies have demonstrated that food-derived *E. faecalis* strains can exhibit phenotypic and genotypic similarities to clini-

Table 1. Isolation sources and hemolytic activity of *Enterococcus faecalis* strains.

Number	Cheese samples	*Enterococcus faecalis* strains	Hemolytic activity
1	White	*E. faecalis* E1	β-hemolytic
2	White	*E. faecalis* E4	β-hemolytic
3	Tulum	*E. faecalis* E10	γ-hemolytic
4	Ezine	*E. faecalis* E14	β-hemolytic
5	Tulum	*E. faecalis* E15	α-hemolytic
6	Lor	*E. faecalis* E17	γ-hemolytic
7	White	*E. faecalis* E20	α-hemolytic
8	Tulum	*E. faecalis* E30	β-hemolytic
9	Lor	*E. faecalis* E33	β-hemolytic
10	Örgü	*E. faecalis* E35	γ-hemolytic
11	White	*E. faecalis* E36	β-hemolytic
12	Tulum	*E. faecalis* E38	β-hemolytic
13	Tulum	*E. faecalis* E40	β-hemolytic
14	Lor	*E. faecalis* E45	β-hemolytic
15	White	*E. faecalis* E47	β-hemolytic
16	Ezine	*E. faecalis* E49	α-hemolytic
17	Civil	*E. faecalis* E52	β-hemolytic
18	Örgü	*E. faecalis* E55	γ-hemolytic
19	White	*E. faecalis* E56	β-hemolytic
20	Ezine	*E. faecalis* E60	α-hemolytic
21	Örgü	*E. faecalis* E62	γ-hemolytic
22	Tulum	*E. faecalis* E63	β-hemolytic
23	Tulum	*E. faecalis* E64	β-hemolytic
24	Ezine	*E. faecalis* E70	β-hemolytic
25	Ezine	*E. faecalis* E72	β-hemolytic
26	White	*E. faecalis* E75	γ-hemolytic
27	White	*E. faecalis* E78	β-hemolytic
28	Civil	*E. faecalis* E80	α-hemolytic
29	Tulum	*E. faecalis* E83	γ-hemolytic
30	Civil	*E. faecalis* E86	β-hemolytic
31	Tulum	*E. faecalis* E89	γ-hemolytic
32	Örgü	*E. faecalis* E92	α-hemolytic
33	Civil	*E. faecalis* E97	β-hemolytic

cal isolates, suggesting a possible epidemiological link between food consumption and human colonization or infection [2,61,62]. In Türkiye, where the consumption of traditionally produced cheeses made from raw or minimally processed milk is widespread, inadequate hygienic practices during production and ripening may further increase the likelihood of contamination with opportunistic enterococci [58]. Therefore, the relatively high proportion of *E. faecalis* identified in the present study highlights the necessity of continuous and systematic monitoring of enterococcal populations in traditional cheeses. Although enterococci are often regarded as technologically relevant members of cheese microbiota, the predominance of *E. faecalis* warrants particular attention due to its well-documented capacity to harbor virulence determinants and acquire antimicrobial resistance. From a One Health perspective, the detection of *E. faecalis* in artisanal cheese matrices represents a critical convergence point between animal husbandry practices, food processing environments, and human exposure pathways. In this context, traditional cheeses may function not only as direct vehicles for consumer exposure but also as ecological niches that facilitate the persistence and potential dissemination of resistance genes and virulence traits across the human-animal-environment interface [28,29]. Therefore, integrating species-level identification with comprehensive profiling of virulence factors and antimicrobial resistance patterns is essential to accurately assess the public health significance of *E. faecalis* in dairy products and to support risk-based food safety management strategies.

Evaluation of the antimicrobial resistance profiles of the 33 *E. faecalis* strains demonstrated that resistance was most prevalent against rifampicin (100%; 33/33), kanamycin (100%; 33/33), ciprofloxacin (90.90%; 30/33), enrofloxacin (84.84%; 28/33), trimethoprim (78.79%; 26/33), and ampicillin (60.60%; 20/33). Moderate resistance rates were observed for norfloxacin (57.58%; 19/33),

Table 2. Antibiotic resistance profiles of *Enterococcus faecalis* isolates and comprehensive evaluation of Multidrug-Resistance (MDR) and multiple antibiotic resistance (MAR) index.

Antibiotics	R		I		S	
	n	%	n	%	N	%
Trimethoprim	26	78.79	5	15.15	2	6.06
Rifampicin	33	100	0	0	0	0
Norfloxacin	19	57.58	4	12.12	10	30.30
Ciprofloxacin	30	90.90	0	0	3	9.10
Chloramphenicol	9	27.27	5	15.15	19	57.58
Clindamycin	10	30.30	6	18.18	17	51.52
Erythromycin	6	18.18	26	78.79	1	3.03
Tetracycline	18	54.55	5	15.15	10	30.30
Vancomycin	0	0	0	0	33	100
Gentamicin	3	9.10	15	45.45	15	45.45
Kanamycin	33	100	0	0	0	0
Streptomycin	13	39.40	10	30.30	10	30.30
Ampicillin	20	60.60	5	15.15	8	24.25
Methicillin	15	45.45	6	18.18	12	36.37
Penicillin G	18	54.55	4	12.12	11	33.33
Cefazolin	15	45.45	0	0	18	54.55
Cefoxitin	17	51.52	3	9.10	13	39.38
Enrofloxacin	28	84.84	2	6.06	3	9.10

MDR of strains	n	%	MAR index
Resistance to 3 antibiotics	4	12.12	0.166
Resistance to 4 antibiotics	6	18.18	0.222
Resistance to 5 antibiotics	12	36.36	0.277
Resistance to ≥6 antibiotics	11	33.33	0.333

Based on the measured inhibition zones, isolates were classified as susceptible (S), intermediate (I), or resistant (R).

tetracycline (54.55%; 18/33), penicillin G (54.55%; 18/33), cefoxitin (51.52%; 17/33), cefazolin (45.45%; 15/33), methicillin (45.45%; 15/33), streptomycin (39.40%; 13/33), and clindamycin (30.30%; 10/33), while comparatively lower levels of resistance were detected for chloramphenicol (27.27%; 9/33), erythromycin (18.18%; 6/33), and gentamicin (9.10%; 3/33). Notably, none of the isolates exhibited resistance to vancomycin (Table 2). Antimicrobial resistance patterns of *E. faecalis* isolates revealed marked variability in resistance frequencies across the tested antibiotics. Chi-square (χ^2) analysis demonstrated that resistance rates differed significantly between antibiotic classes ($p < 0.05$), indicating a non-uniform distribution of resistance across the tested antimicrobial agents.

The antimicrobial resistance patterns observed among the 33 *E. faecalis* strains evaluated in this study clearly highlight the multifaceted resistance potential of this species. The uniform resistance to rifampicin and kanamycin (100%) strongly reflects an intense, sustained selective pressure on these enterococcal populations, which aligns with the well-documented ability of *E. faecalis* to acquire and maintain resistance traits under antimicrobial exposure [63]. These findings suggest that antimicrobial use in animal production systems, coupled with possible environmental contamination during processing, may play a significant role in shaping the observed resistance patterns. In traditional cheese production environments, suboptimal hygiene standards combined with potential exposure to antimicrobials may further contribute to the persistence and dissemination of resistant *E. faecalis* strains along the food chain. High-level resistance to rifampicin may develop rapidly during therapy, particularly when rifampicin is used in combination regimens or in environments with sustained antibiotic exposure [64]. Similarly, the complete resistance to kanamycin observed in this study likely reflects the widespread dissemination of aminoglycoside-modifying enzymes, a phenomenon reported in both clinical and non-clinical *E. faecalis* isolates [47]. These findings support the view that aminoglycoside resistance in enterococci is no longer restricted to hospital-associated strains but is broadly established across different ecological reservoirs.

The markedly high resistance rates to fluoroquinolones, particularly ciprofloxacin and enrofloxacin, observed in *E. faecalis* isolates in the present study are of particular concern given the species' prominent role in the food chain and its relevance to human health. In *E. faecalis*, fluoroquinolone resistance has been predom-

inantly associated with point mutations in the quinolone resistance–determining regions of the DNA Gyrase Subunit A (*gyrA*) and DNA Topoisomerase IV Subunit A (*parC*) genes, along with increased efflux pump activity, mechanisms that collectively confer reduced susceptibility to this antimicrobial class [65]. The selection and maintenance of these resistance traits are widely considered to result from sustained exposure to fluoroquinolones in livestock production, enabling resistant *E. faecalis* strains to persist and disseminate through foods of animal origin [47,48,54]. Although a direct causal relationship between the intake of contaminated foods and *E. faecalis* infections has not been definitively established, the consistent recovery of resistant *E. faecalis* from widely consumed foods supports the hypothesis that the food chain represents a plausible, albeit indirect, route for the dissemination of fluoroquinolone resistance [66].

Moderate resistance rates to β-lactam antibiotics, including ampicillin, penicillin G, cefoxitin, and cefazolin, further illustrate the adaptive flexibility of *E. faecalis*. Although enterococci exhibit intrinsic low susceptibility to cephalosporins, resistance to ampicillin is particularly noteworthy, as this antibiotic remains a cornerstone in the treatment of enterococcal infections [64]. Ampicillin resistance has been associated with structural alterations in penicillin-binding proteins, especially Penicillin-Binding Protein 4 (PBP4), which reduces antibiotic binding affinity and compromises therapeutic efficacy [63]. The increasing frequency of ampicillin-resistant *E. faecalis* reported worldwide raises concerns regarding the future effectiveness of first-line therapies.

Resistance to tetracycline and erythromycin, although comparatively lower, remains epidemiologically significant [67]. In contrast, the relatively low resistance rates observed for gentamicin and clindamycin suggest that these agents may retain partial effectiveness against certain *E. faecalis* strains. However, this finding should be interpreted cautiously, as even low resistance levels may rapidly increase under selective pressure. Moreover, the emergence of high-level aminoglycoside resistance, which abolishes synergistic effects with cell wall–active antibiotics, continues to represent a major clinical challenge [68].

One of the most notable findings of this study is the complete susceptibility of all isolates to vancomycin. The absence of Vancomycin-Resistant Enterococci (VRE) is encouraging, particularly considering the global dissemination of *vanA* and *vanB* vancomycin-resistance gene clusters in hospital settings [60]. This result suggests that vancomycin remains an effective therapeutic option for severe enterococcal infections in the context of the isolates examined. Nevertheless, previous studies have emphasized that vancomycin resistance can emerge rapidly in response to antimicrobial pressure, highlighting the importance of continuous surveillance [64].

Overall, the resistance patterns identified in this study indicate the presence of MDR *E. faecalis* strains and underscore the critical need for rational antibiotic use and sustained antimicrobial resistance monitoring (Table 2). Specifically, 33.33% of the isolates were resistant to six or more antibiotics, while 36.36% showed resistance to five antibiotics, indicating a considerable MDR burden among cheese-derived enterococci. From a public health perspective, *E. faecalis* should be regarded not only as an opportunistic pathogen but also as a significant reservoir of resistance determinants capable of dissemination across clinical, food-related, and environmental settings [67]. The MAR index values ranged from 0.166 to 0.333, reflecting substantial antimicrobial exposure. The overall mean MAR index was calculated as 0.27 ± 0.06, exceeding the threshold value of 0.2, which is commonly associated with isolates originating from high-risk contamination sources. This finding underscores the considerable antimicrobial resistance burden among *E. faecalis* isolates recovered from traditional cheese samples. Studies examining enterococci recovered from traditionally manufactured cheeses in regions such as Türkiye, Iran, Egypt, and parts of Southern Europe have documented comparable MAR index values and high frequencies of multidrug resistance, particularly in products prepared using artisanal methods [32,40,58]. By contrast, cheeses produced at an industrial scale under standardized processing conditions and more rigorous hygienic oversight tend to harbor enterococcal populations with lower MAR indices and reduced resistance prevalence [57,59]. Collectively, these differences highlight the influence of production systems, raw material sources, and hygiene management on the development and persistence of antimicrobial resistance in cheese-associated enterococci.

4. Limitations

This study has some limitations that should be acknowledged. The cheese samples were collected from a single geographical region (Ankara, Türkiye), which may limit the generalizability of the findings to other regions or different traditional cheese production systems. In addition, although both phenotypic and molecular methods were applied for species identification, more advanced genomic approaches, such as whole-genome sequencing, could not be performed, which could provide deeper insight into the genetic diversity and antimicrobial resistance mechanisms of the isolates. Finally, antimicrobial susceptibility testing was carried out using the disk diffusion method only, and Minimum Inhibitory Concentration (MIC) values were not determined, which may offer more detailed information on resistance levels.

5. Conclusions

This study provides information on the prevalence and antimicrobial resistance profiles of *E. faecalis* isolated from traditional cheeses marketed in the Ankara region of

Türkiye. In contrast to studies addressing enterococci in broader contexts, this work focuses specifically on traditional dairy products, contributing to a better characterization of their associated microbiota and resistance patterns at a regional level. The results indicate that traditional cheeses may harbor *Enterococcus* spp., with *E. faecium* and *E. faecalis* identified as the predominant species. The occurrence of *E. faecalis*, a species of clinical relevance as an opportunistic pathogen, together with the detection of antimicrobial-resistant isolates, suggests the presence of resistant enterococci in these food products. These findings highlight the importance of hygienic practices in dairy production and support the need for continued monitoring of enterococci and their antimicrobial resistance profiles in traditional cheese products to better understand their potential role within the food chain.

Availability of Data and Materials

All data are provided in this publication.

Author Contributions

Pınar ŞANLIBABA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. Seda SEYİRT: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. Başar Uymaz Tezel: Conceptualization, Data curation, Validation, Visualization, Writing – original draft, Writing – review & editing. All authors contributed to critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

This manuscript was revised with the assistance of ChatGPT (GPT 4o), which was used to improve clarity and rephrase selected sentences during the writing process. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

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