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Enterococcus faecalis and *E. faecium* in Dairy Production Line: Antibiotic Resistance Profile and Virulence Characteristics

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Abstract

This study investigated the prevalence, antimicrobial resistance and virulence of *Enterococcus faecalis* and *E. faecium* , in two dairy plants, posing public health risks. Overall, from 219 samples, 42 were contaminated with *E. faecium* (26/42, 62%) or *E. faecalis* (16/42, 38%), which confirmed via PCR and BD Phoenix automated system. Antibiotic resistance was found in 48% of the enterococci isolates, with high rates for azithromycin (31% *E. faecalis*, 27% *E. faecium*), erythromycin (44% *E. faecalis*, 27% *E. faecium*), and tetracycline (19% *E. faecalis*, 12% *E. faecium*). ciprofloxacin and vancomycin resistances were rare (below 10% for both). Notably, 25% of the resistant isolates were multidrug-resistant (MDR) with a Multiple Antibiotic Resistance (MAR) index of 0.3. Virulence analysis showed that 69% of 42 isolates harboured at least one *esp* or *efaA*, or both genes which are essential for enterococcal pathogenesis, while 67% of isolates produced biofilms. Most antibiotic-resistant isolates (60%) were biofilm producers and harboured associated virulence genes. In conclusion, antibiotic-resistant *E. faecalis* and *E. faecium* along the dairy production line might pose potential food safety and public health concerns, due to their presence in biofilm communities, which enhance antibiotic resistance and facilitate horizontal gene transfer.

Keywords: Antimicrobial resistance, Biofilm ability, Dairy industry, *Enterococcus* sp., Virulence factors

1. Introduction

Enterococci, a major genus of lactic acid bacteria (LAB), are ubiquitous commensal microorganisms present in plants, soil, water, food, and the gastrointestinal tracts of both humans and animals (Terzić-Vidojević et al., 2021). Some enterococcal strains have the role as starter cultures due to their proteolytic and lipolytic activity therefore they can break down milk components into other components (acetaldehyde, acetoin, and diacetyl) which ripens the cheese and are used in production of various cheese types (Chajęcka-Wierzchowska et al., 2020a). They are also well-rounded bacteriocin producers, harbouring plasmids with bacteriocin-encoding genes, and can be applied to probiotic preparations (Urshev & Yungareva, 2020). However, the use of *Enterococcus* strains as probiotics has recently become controversial because of their easily acquired mobile genetic elements related to antibiotic resistance and virulence factors from other bacteria (Almeida-Santos et al., 2025; Hegstad et al., 2010; Hollenbeck & Rice, 2012; Krawczyk et al., 2021; Monteiro Marques et al., 2023; Oruc et al., 2021; Palmer et al., 2010; Sparo et al., 2012; Vignaroli et al., 2011). Importantly,

Enterococcus species are resilient and versatile due to their ability to survive a broad range of habitats (a wide range of pH and temperature, etc.), making them well-adapted to several food systems and the healthcare environment (Chajęcka-Wierzchowska et al., 2020a; Výrostková et al., 2021; Amidi-Fazli and Hanifian, 2022; Apostolakos et al., 2022; Soares et al., 2023).

They are responsible for life-threatening nosocomial infections, including endocarditis, urinary tract infections, and bacteraemia (Apostolakos et al., 2022; Chajęcka-Wierzchowska et al., 2020; Soares et al., 2023; Urshev & Yungareva, 2020). Their natural resistance to penicillin, ampicillin, and most cephalosporins, and the ability to quickly adapt clinically used antimicrobial agents like quinupristin-dalfopristin, linezolid, daptomycin, and tigecycline, amplifies their recognition of being a major nosocomial pathogen (Ahmed & Baptiste, 2018; Arias & Murray, 2012; Niebel et al., 2015; Van Tyne & Gilmore, 2014). There are nearly 50 species within the genus *Enterococcus*, with *E. faecium* and *E. faecalis* being the most frequent nosocomial opportunistic pathogens (Ahmed & Baptiste, 2018; Bonacina et al., 2017; Ramos et al., 2020). Additionally, *E. faecium* is featured on the WHO priority list of ESKAPE pathogens thanks to its potential drug resistance mechanisms, such as drug inactivation, drug target site alteration, or modification (Rice, 2010; Santajit & Indrawattana, 2016). Especially, vancomycin-resistant enterococci (VRE), may also show resistance against glycopeptide and other antimicrobials and acts as reservoirs of multidrug-resistance (MDR) among other bacteria via horizontal transfer (Ahmed & Baptiste, 2018; M. H. Kim et al., 2021). The only presence of antimicrobial resistance in a strain does not invariably signify its pathogenic potential; however, when coupled with other virulence factors, it renders the strain more dangerous for public health (Chajęcka-Wierzchowska et al., 2017). Enterococci species possess various key virulence genes that contribute to their pathogenesis, including enterococcal surface proteins (*esp*), collagen-binding protein (*ace*), gelatinase (*gelE*), aggregation substance (*asa1*), hyaluronidase (*hyl*), and endocarditis antigen (*efaA*). These genes are crucial for adhering to and colonizing host cells, invading tissues, and resisting non-specific defensive mechanisms (Foulquié Moreno et al., 2006; Golob et al., 2019; Vankerckhoven et al., 2004). The enterococci are frequently found within bacterial biofilms that contribute to their virulence and antibiotic resistance by offering stability and protection to the bacterial population while facilitating various bacterial interactions (Conwell et al., 2022; Del Pozo & Patel, 2007; Santajit & Indrawattana, 2016; Sharma et al., 2014). Previous studies have reported the presence of antibiotic-resistant *E. faecalis* and *E. faecium* , along with their virulence factors, in dairy products (Cariolato et al., 2008; Gaglio et al., 2016a; Silvetti et al., 2019; Palmeri et al., 2020;

Gołaś-Prądzyńska et al., 2022; Jahansepas et al., 2022; Kim et al., 2022; Nasiri and Hanifian, 2022; Paschoalini et al., 2023). However, detailed data about the contamination pathways of enterococci within the dairy product production line is still required to provide insights into enabling rapid remedial action to prevent the spread of these pathogens. This study was designed to enhance understanding of the contamination sources of *E. faecalis* and *E. faecium* strains in dairy products. To this end, dairy-related samples were comprehensively analysed from farm to fork for the prevalence of these strains along with their antibiotic resistance, biofilm capability, and virulence traits.

2. Materials and Methods

2.1 Sampling

This study was conducted at two large commercial dairy plants and their corresponding farms in Kayseri, Turkey, between August 2020 and May 2021. The sampling details of the visited dairy plants and the corresponding dairy farms in this study are presented in Table 1. Briefly, a total of 219 samples were collected, consisting of 20 raw bulk-tank milk samples, 40 dairy product samples (25 cheese and 15 yogurt), 98 nasal swabs (23 from workers and 75 from cows), 23 hand swabs from workers (13 from farm workers and 10 from plant workers), and 38 swabs from equipment surfaces (12 from farm equipment and 26 from dairy plants).The milk samples (approximately 50 mL) were aseptically collected into the sterile 50-mL screw-capped plastic bottles from each farm and dairy plant. Cheese, yogurt, cream, and milk samples were taken according to ISO (17728:2015). The nasal swab was taken from both nostrils of the animals and humans involved in the study with sterile cotton-tipped swabs. All human subjects provided samples voluntarily, attesting to their absence of recent antimicrobial treatment within the preceding three months. The farm owners also permitted animal sampling and confirmed that their animals had not undergone antimicrobial therapy in the past month. Nasal samples were collected from both nares of cattle, farm workers, and dairy workers using a sterile transportation swab with Stuart medium (BTR, Ankara, Türkiye). The swab was inserted into a 2-5 cm depth in one nasal cavity and rotated five times in the mucosal surfaces for 5 s to take nasal samples (Centeno-Martinez et al., 2022). Hand swab samples were taken in working hours from farm and plant workers' forefingers and thumbs, without previous notification. Workers' nasal samples were collected with a sterile transportation swab; after instructing the workers, they placed the swabs inside the nostril entrance and rotated three times (Woodall et al., 2021).

Farm and plant equipment samples were collected according to ISO (18593:2004) on a sterile transportation swab.

The study was approved by the ethics committee (Reference: 2019/155 and 19/036), and all participating workers provided informed consent and volunteered to take part in the research.

2.2 Isolation of *Enterococcus* spp.

In this study, *Enterococcus* spp. was isolated and identified from the samples according to Yoon et al. (2020). Briefly, swab samples were added to 5 mL of Enterococcosel broth (Becton Dickinson, USA). Additionally, 10 g of each dairy product sample (cheese, milk, and yogurt) was mixed with 90 mL of Enterococcosel broth separately for enrichment and incubated at 35 °C for 24 h. Afterward, 100 µL of each culture was inoculated onto Enterococcosel agar (Becton Dickinson, USA) and incubated for 24-48 h at 37 °C. After the incubation, beige colonies with black halos on the plates were considered *Enterococcus* spp., and at least three presumptive colonies were inoculated onto blood agar (Oxoid, England) for molecular analysis and Phoenix identification. Genomic DNA from the isolates was extracted with an InstaGene matrix kit (Bio-Rad, USA), following the manufacturer's protocol.

2.3 Confirmation of *Enterococcus* spp. Isolates by Real Time- PCR (qPCR)

Phenotypically characterized enterococci isolates were confirmed by qPCR using primers for 23S rRNA, as outlined by Sanderson et al.(2019). The PCR assay was conducted using a 25 µL reaction mixture composed of 10 µL Green SuperMix Low ROX (Quanta Biosciences, Inc., Gaithersburg, MD, USA), 0.5 µL of each primer (10 pmol), 5 µL of DNA, and 9 µL of sterile distilled water. The PCR was conducted on the Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad, California, USA), according to the procedure detailed in Table S1, and evaluated in CFX Maestro Software (BioRad, USA).

2.4 Identification of *E. faecalis* and *E. faecium* by Multiplex PCR (m-PCR)

This PCR protocol was done using species-specific primers designed to target the *ddl* (d-Ala–d-Ala ligase) genes of each *Enterococcus* species, according to the method proposed by Dutka-Malen et al. (1995). In brief, the PCR reaction was set up in a 25-µL volume, comprising 12.5 µL of 2x Dream Taq Green PCR Master Mix (Thermo, USA), 8.5 µL of DNase/RNase-free water (Thermo, USA), 1 µL (25 pmol) of each primer, and 2 µL of the DNA template. The detailed PCR conditions employed for this study are detailed in Table S1.

2.5 BD Phoenix CPO detect assay

In this study, all Enterococci isolates were further evaluated by the Phoenix ™100 system (BD Diagnostic Systems, Sparks, USA) to confirm species-level identification (ID) and perform antimicrobial susceptibility testing (AST). The test was applied using the BD Phoenix CPO Combo panel (PMIC/ID-95, Cat. No: 448614) following the manufacturer's instructions. The PMIC/ID-95 panel includes the following antibiotics: amoxicillin-clavulanate, amikacin, linezolid, teicoplanin, vancomycin, ciprofloxacin, chloramphenicol, erythromycin, tetracycline, and azithromycin. Isolates where then evaluated as resistant, intermediate, or susceptible according to their MIC value determined by CLSI (2023).

2.6 Detection of Vancomycin Resistance and putative virulence genes

E. faecalis and *E. faecium* isolates were also analysed for vancomycin resistance genes (*vanA, B, C1 and C2*), as well as putative virulence factor genes (*ace*, *gelE*, *efaA, asa1, esp$_{fs}$, and esp$_{fm}$*) using mPCR. Reaction mixtures (25 µL total volume) were prepared using 2x DreamTaq Green PCR Master Mix (Thermo, USA), with the PCR conditions provided in Tables S2 and S3.

2.7 Evaluation of biofilm forming ability of Isolates

The biofilm formation ability of the isolates was detected as described by Stepanović et al. (2000). In brief, Enterococcus isolates were incubated overnight at 37°C in tryptic soy broth (TSB) supplemented with 2% glucose (Merck, Germany). Following incubation, the cultures were adjusted to a turbidity equivalent to a 0.5 McFarland standard. Next, 100 µL of each culture was inoculated into a sterile 96-well polystyrene microtiter plate containing 100 µL of TSB. The wells containing only TSB were used as the negative control in the test. The plates were incubated for 24 hours at 37°C. After incubation, the contents of each well were removed, washed three times with 200 µL of phosphate-buffered saline, fixed with 96% ethanol, and stained for 5 minutes with 2% crystal violet. The cell-bound dye was then resuspended with 160 µL of 33% (v/v) glacial acetic acid. Finally, the optical density (OD) of each well was calculated at 570 nm with an ELISA plate reader (Thermo, Finland). The cut-off value (ODc) was calculated by adding three standard deviations of the optical measurements to the mean value of the negative control. Biofilm forming abilities were calculated after the measurement as follows: $OD \leq ODC$ = no biofilm forming ability, $ODC < OD \leq 2 \times ODC$ = weak, $2 \times ODC < OD \leq 4 \times ODC$ = moderate, and $4 \times ODC < OD$ = strong.

2.8 Statistical analysis

Descriptive analyses were performed on the rates obtained in this study to determine the distribution of *E. faecalis* and *E. faecium* isolates across different sample types, antibiotic resistance rates, and biofilm formation ability. A chi-square test, based on Pearson's chi-square and continuity correction, was applied. A p-value of <0.05 was considered statistically significant. All data were analysed using The Jamovi Project v 2.3.

3. Results

In this study, 75 (34%) of 219 samples analysed were found to be positive for *Enterococcus* spp.: 54% (7/13) of farm personnel hands, 58% (7/12) of farm equipment, 43% (32/75) of cow nasal swabs, 45% (9/20) of bulk tank milk, 39% (10/26) of plant equipment, 31% (4/13) of farm personnel noses, 20% (2/10) of plant personnel noses, and 16% (4/25) of cheese samples. The prevalence of enterococci between different sample types were statistically insignificant (p>0.05).

The BD Phoenix system and m-PCR identified 42 (56%) of the 75 *Enterococcus* spp. isolates as either *E. faecalis* (26/42, 62%) or *E. faecium* (16/42, 38%), while the remaining 33 isolates were unidentified species. In detail, *E. faecium* was mainly isolated from farm personnel noses and plant equipment (23.1% each), farm equipment (16.7%), and cow nasal swabs (16%), whereas *E. faecalis* was more common on farm personnel hands (23.1%), in cheese (12%), and in bulk tank milk (10%) samples, with a few isolates identified from farm equipment (8.3%) and cow nasal swabs (9.3%). There was no statistically significant difference (p > 0.05) in the isolation rates of *E. faecium* and *E. faecalis* from different sources. The isolation frequencies of *E. faecium* and *E. faecalis* for each dairy plant and sample type are presented in Table 1.

Of the 42 isolates, 20 (48%), including 12 (46%) *E. faecium* and 8 (50%) *E. faecalis*, were resistant to one or more antibiotics tested. All *E. faecalis* and *E. faecium* isolates (100%) were susceptible to amoxicillin-clavulanic acid, linezolid, teicoplanin and chloramphenicol. Resistance to other antibiotics ranged from 4 % to 27% for *E. faecium* and 6% to 44% for *E. faecalis*. The highest antimicrobial resistance rates for medically important antibiotics, such as azithromycin, erythromycin, and tetracycline, were 31%, 44%, and 19% for *E. faecalis*, and 27%, 27%, and 12% for *E. faecium* , respectively (Figure 1). Nevertheless, ciprofloxacin resistance was rare and detected in only 2 (8%) of the *E. faecium* isolates. Vancomycin resistance was also found in only one isolate from both species, despite 15 isolates (37.5%) carrying the *vanA* gene, with 7 (26.9%) being *E. faecium* and 8 (50%) being *E. faecalis*. However, all the antibiotic resistance rates between *E. faecium* and *E. faecalis* were statistically

non-significant (p>0.05). Moreover, 5 (25%) of resistant isolates displayed MDR, with a MAR index higher than 0.2. Among the MDR isolates, four were from cow nasal swabs and one was from bulk tank milk (Table 3). Additionally, both *E. faecalis* and *E. faecium* strains isolated from cheese and farm personnel hands were susceptible to all antibiotics tested, whereas the *E. faecalis* isolate from the bulk tank milk sample was MDR (erythromycin, tetracycline, and azithromycin).

In this study, 29 (69%) of the enterococci isolates carried at least one of the tested virulence genes, with *efaA* being the most common (28/42, 67%). Additionally, the *esp* genes were detected in 9 (21.4%) isolates. None of the isolates were positive for the *ace*, *gel*E, and *asa*1 genes. The *efaA* was found in 9 (35%) *E. faecium* , in 11 (69%) *E. faecalis* isolates, and combination with the *esp* gene in 4 (15.4%) *E. faecium* and 4 (25%) *E. faecalis* isolates. Out of the 42 isolates tested, 28 (67%) produced biofilm, with 6 (14.3%) classified as strong, 17 (40.5%) as moderate, and 5 (12%) as weak biofilm producers. The frequency of biofilm formation was higher in *E. faecalis* (81.3%) than in *E. faecium* (57.7%), with no statistically significant difference ($p > 0.05$) (Table 3).

4. Discussion

Enterococci are commonly present in the food industry, particularly in dairy products, thanks to their exceptional ability to resist and/or grow in adverse environments (Gołaś-Prądzyńska et al., 2022a; Paschoalini et al., 2023; Silvetti et al., 2019). *Enterococcus s*pecies, especially *E. faecium* and *E. faecalis*, can act as reservoirs for AMR and virulence genes (Torres et al., 2018; Yang et al., 2019), and can also transmit these genes to other species and different bacterial genera. Therefore, they are increasingly recognized as significant human pathogens and pose significant challenges to public health and food safety (Gołaś-Prądzyńska et al., 2022a; Paschoalini et al., 2023; Yang et al., 2019; Zhao et al., 2024). Our study assessed the antimicrobial resistance and virulence characteristics of *E. faecalis* and *E. faecium* isolated from the production chain of dairy products, from farm to fork. *Enterococcus* spp. was isolated not only from dairy products but also from dairy farms and dairy plants, mainly from cow noses, equipment, and personnel, highlighting possibility of cross contamination by different *Enterococcus* species at different step in dairy production chain.

Despite several earlier studies reporting that *E. faecalis* is generally more commonly isolated from dairy products (Gołaś-Prądzyńska et al., 2022a; Hosseini et al., 2016; Jahansepas et al., 2022; Výrostková, Regecová, Dudriková, Marcinčák, Vargová, Kováčová, & Mal'ová, 2021;

Yuksel et al., 2015), *E. faecium* was predominant (62%) in the dairy processing chain in this study, which is similar to the results of other studies (Bulajic et al., 2015; Chajęcka-Wierzchowska et al., 2020a; Deng et al., 2021; Paschoalini et al., 2023). It was noted that 46% of *E. faecium* and 50% of *E. faecalis* were resistant to at least one of the antibiotics tested, exhibiting generally similar resistance patterns. Of the resistant isolates, 25% exhibited an MDR phenotype, with a MAR index of 0.3, indicating that these isolates originated from a source where antibiotics are overused. A similar rate of MDR strains was reported by Gołaś-Prądzyńska et al. (Gołaś-Prądzyńska et al., 2022); however, El-Zamkan et al. (2021) demonstrated a higher rate (67.6%), the difference attributed to the fact that their isolates were obtained from bovine and goat milk with mastitis.

The highest antimicrobial resistance rates were observed for erythromycin (44% in *E. faecalis* and 27% in *E. faecium*), azithromycin (31% in *E. faecalis* and 27% in *E. faecium*), and tetracycline (19% in *E. faecalis* and 12% in *E. faecium*). It was noted higher resistance to erythromycin particularly in isolates from cow nasal swabs, in the current study. Enterococci are among the bacterial pathogens that can cause bovine mastitis. Erythromycin and other macrolides are frequently used to treat clinical and subclinical mastitis, and this resistance to erythromycin might be the result (Gao et al., 2019; Lee et al., 2023; Zwald et al., 2004). Earlier studies have reported prevalence of erythromycin-resistant enterococci ranging from 14.3% to 75% (Bulajic et al., 2015; Gaglio et al., 2016; Chajęcka-Wierzchowska et al., 2020; Výrostková et al., 2021; Salamandane et al., 2023). Our tetracycline resistance rates in *E. faecium* and *E. faecalis* were relatively lower than those reported in other studies (21% to 97%)(Różańska et al., 2019; Bastião Rocha et al., 2022; Gołaś-Prądzyńska et al., 2022; Paschoalini et al., 2023; Salamandane et al., 2023). Azithromycin, a broad-spectrum macrolide antibiotic with long persistence, can be used for the treatment of subclinical bovine mastitis (Lucas et al., 2010). It has been reported that drinking milk with high levels of azithromycin residues could severely affect the human immune and cardiovascular systems (Kurjogi et al., 2019). Azithromycin resistance has been detected in 60% of *E. faecalis* isolates from milk samples with mastitis (Bag et al., 2022). In light of this information, it is possible that the animals in the dairy farms where sampling was conducted had previously received azithromycin treatment for mastitis. Furthermore, the fact that azithromycin-resistant isolates were predominantly observed in equipment suggests cross-contamination of resistant strains.

Moreover, both isolates were generally susceptible to clinically relevant antibiotics, including ampicillin, teicoplanin, linezolid, vancomycin, chloramphenicol, and ciprofloxacin in this

study. These results are significant due to their continued effectiveness as the preferred therapy for enterococcal infections in the studied region. The chloramphenicol usage is forbidden in Türkiye, on animal husbandry since 2002 (Kasimoglu-Dogru et al., 2010). Therefore, low resistance of enterococci against chloramphenicol can also be seen in previous studies conducted in Türkiye (Çitak et al., 2004; Sanlibaba & Senturk, 2018). Previous investigations have reported both *E. faecium* and *E. faecalis*, were susceptible to chloramphenicol (Palmeri et al., 2021; Gołaś-Prądzyńska et al., 2022).

Similarly, Gołaś-Prądzyńska & Rola (2021) and Rocha et al. (2022) also found that the enterococci strains isolated from milk were susceptible to linezolid. It has been reported that resistance to linezolid and vancomycin, which are effective in the treatment of enterococcal infections, is rare or even not detected in enterococci isolates from animals and animal-derived foods (Bortolaia & Guardabassi, 2023; Güngör et al., 2024; Maleki et al., 2021; Różańska et al., 2019; Xuan et al., 2021). In recent years, there has been an increase in the incidence of enterococci with high levels of resistance, with Vancomycin-resistant *E. faecium* being listed as a high-priority pathogen by the WHO (WHO, 2024). In this study, vancomycin resistance was identified in only one isolate of each species; however, the *vanA* gene was detected in 15 isolates (37.5%), of which 7 (26.9%) were *E. faecium* and 8 (50%) were *E. faecalis*. These findings suggest that the presence of the *vanA* gene in vancomycin-susceptible isolates, as determined by traditional phenotypic susceptibility testing, could indicate vancomycin-variable enterococci (VVE) (Li et al., 2022). The ability of VVEs to evade phenotypical antimicrobial susceptibility tests makes them more dangerous to public health in two ways: they can revert to vancomycin resistance upon vancomycin treatment (Kohler et al., 2018; Thaker et al., 2015), and *vanA* and other operons (*vanB, vanG, vanM*, and *vanN*) are transferable between bacteria, which makes *vanA*-positive isolates responsible for disseminating vancomycin resistance genes (Ahmed & Baptiste, 2018). It should be noted that there was a positive correlation between the low vancomycin resistance of the isolates and their susceptibility to linezolid, which is one of the most effective antibiotics for the treatment of VRE and is approved by the US Food and Drug Administration (Bialvaei et al., 2017; Paschoalini et al., 2023; Tokano et al., 2024).

Over the years, the prevalence of VRE has noticeably decreased since its initial reports in 1988, particularly following the ban on avoparcin which is a vancomycin analogue used as a growth promoter in animals, in 1997 in European countries including Türkiye (Sanlibaba & Senturk, 2018). However, reports of VRE have been increasing in recent years (Tokano et al., 2024). Additionally, studies in Türkiye have indicated high prevalence rates of VRE in dairy products,

with rates of 84.8% and 86.1% reported by Çitak et al. (2004) and Yüksel et al. (2015), respectively.

Identification of virulence genes contained in these isolates is important for understanding their complex pathogenic potential (Badul et al., 2021; Chajęcka-Wierzchowska et al., 2017). In this study, the *efaA* gene was the most prevalent in the isolates, occurring in 35% of *E. faecium* isolates and 69% of *E. faecalis* isolates. In contrast, the *esp* gene was found in only one *E. faecium* isolate (3.9%) and was present in combination with *efaA* in the other isolates. The *efaA* and *esp* genes are essential for bacterial adhesion to eukaryotic cells and surfaces, facilitating the colonization of host tissues and contributing to biofilm formation (Ghazvinian et al., 2024; Medeiros et al., 2014; Popovic et al., 2018). These genes are more prevalent in clinical isolates but have been reported in food, animal, clinical, and environmental samples in earlier studies consistent with our results (Anderson et al., 2016; Badul et al., 2021; Eaton & Gasson, 2001; Ghazvinian et al., 2024; Mannu et al., 2003; Medeiros et al., 2014; Popovic et al., 2018). It is noteworthy that the *esp* gene was more frequently found among *E. faecalis* strains compared to *E. faecium* isolates and isolates positive for *esp* exhibited moderate to strong biofilm-forming capability, consistent with previous studies. (Ghazvinian et al., 2024; Popovic et al., 2018; Soares et al., 2014). Our findings highlighted a significant relationship between the presence of virulence genes in isolates and their ability to produce biofilm. Biofilm formation by *Enterococcus* species have a major role in the pathogenicity due to it contribute to their virulence and antibiotic resistance (Gajewska et al., 2023; Hashem et al., 2017; Pirbonyeh et al., 2024; Popovic et al., 2018). In the current study, most antibiotic-resistant isolates were found in biofilm communities (67%) and contained virulence factor genes important for pathogenesis; among them, 14.3%, 40.5%, and 12% were classified as strong, moderate, and weak biofilm formers, respectively. These findings suggest that the isolates recovered in our study may pose a significant public health risk due to the potential acquisition of these microorganisms and their mobile genetic elements through the food chain, direct contact with animals, or environmental contamination. Furthermore, *E. faecalis* isolates exhibited higher biofilm formation than *E. faecium* isolates, in line with findings from previous studies (Gajewska et al., 2023; Pereira et al., 2017).

5. Conclusions

This study indicates that *E. faecalis* and *E. faecium* , along with the dairy production line, exhibited either no or low resistance to critically important antibiotics, such as linezolid,

ciprofloxacin, ampicillin, and vancomycin. Despite the low resistance, the presence of multidrug-resistant isolates, particularly within biofilm communities harbouring virulence factor genes in the dairy processing line from farm to final products, makes these isolates a potential public health concern due to the risk of transmission of these resistant bacteria to humans through the food chain. Given the rising antimicrobial resistance of enterococci and their notable capacity to acquire and transfer virulence genes, implementing stringent control and effective monitoring measures are essential to protect public health and ensure dairy product safety. Additionally, adopting multifaceted and sustainable strategies are crucial to mitigating the spread of antimicrobial-resistant bacteria in dairy farming. Strengthening farm hygiene protocols, ensuring good veterinary practices, enhancing biosecurity measures, such as rigorous sanitation, systematic water quality monitoring, and improved animal welfare, as well as providing training for farm personnel on hygiene practices, are fundamental to limiting resistance development and transmission. Additionally, it is necessary to ensure adherence to existing guidelines on antibiotic use in Good Dairy Farming Practices and to place greater emphasis on antimicrobial resistance (AMR) surveillance programs for food animals.

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Author contributions

D.A.G.: Formal Analysis, Investigation, Resources, Data Curation, Writing- Original Draft Preparation, Visualization. N.E.O.: Conceptualization, Methodology, Resources, Writing–Review & Editing, Project Administration, Funding acquisition. C.G.: Investigation, Visualization. K.K.: Investigation, Visualization. Y.O.: Investigation, Visualization. F.D.: Investigation.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical statement

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This study was approved by the Human Research Ethics Committee (Reference: 2019/155) and the Local Ethics Committee for Animal Experiments (Reference:19/036) of Erciyes University, Kayseri Türkiye.

Data availability statement

Data will be made available on reasonable request.

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Table 1. The distribution of *E. faecalis* and *E. faecium* among different sources in two dairy plant's continuum

Sample type	Plant A			Plant B			Total		
	No. of Samples	No. of *E. faecium* (%)	No. of *E. faecalis* (%)	No. of Samples	No. of *E. faecium* (%)	No. of *E. faecalis* (%)	No. of Samples	No. of *E. faecium* (%)	No. of *E. faecalis* (%)
Cheese	15	1 (6.7)	-	10	-	3 (30)	25	1 (4)	3 (12)
Yogurt	5	-	-	10	-	-	15	-	-
Butter	5	-	-	5	-	-	10	-	-
Milk	5	-	-	5	-	1 (20)	10	-	1 (10)
Cow nasal swab	35	2 (5.7)	3 (9)	40	10 (25)	4 (10)	75	12 (16)	7 (9.3)
Farm equipment	6	2 (33.3)	1 (17)	6	-	-	12	2 (16.7)	1 (8.3)
Plant equipment	13	4 (30.8)	1 (8)	13	2 (15.4)	-	26	6 (23.1)	1 (3.8)
Farm personnel nose	5	1 (20)	-	8	2 (25)	-	13	3 (23.1)	-
Farm personnel hand	5	-	2 (40)	8	1 (12.5)	1 (12.5)	13	1 (7.7)	3 (23.1)
Plant personnel nose	6	-	-	4	1 (25)	-	10	1 (10)	-
Plant personnel hand	6	-	-	4	-	-	10	-	-
Total	**106**	**10 (9.4)**	**7 (6.6)**	**113**	**16 (14.2)**	**9 (8)**	**219**	**26 (11.9)**	**16 (7.3)**

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Table 2. Biofilm formation ability and observed virulence genes in Enterococci isolates from different samples

Enterococcus spp. isolates	Biofilm formation				Virulence genes					
	Weak (%)	Moderate (%)	Strong (%)	Total (%)	*efaA*$_{fs}$ (%)	*efaA*$_{fm}$ (%)	*esp*$_{fs}$ (%)	*esp*$_{fm}$ (%)	*efaA*$_{fs}$+ *esp*$_{fs}$ (%)	*efaA*$_{fm}$+ *esp*$_{fm}$ (%)
***E. faecalis* (n=16)**	3 (18.8)	5 (31.3)	5 (31.3)	13 (81.3)	15 (93.8)	-	4 (25)	-	4 (25)	-
***E. faecium* (n=26)**	2 (7.7)	12 (46.2)	1 (3.8)	15 (57.7)	-	13 (50)	-	5 (19.2)	-	4 (15.4)
Total (n=42)	5 (12)	17 (40.5)	6 (14.3)	28 (67)	15 (35.7)	13 (31)	4 (9.3)	5 (12)	4 (9.5)	4 (9.5)

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Table 3. The biofilm ability, virulence and *vanA* genes, antimicrobial resistance profiles and MAR indexes of the isolates

Isolate No.	*Enterococcus* spp.	Source	Biofilm ability	Virulence genes	*vanA*	Antimicrobial resistance profile	MAR index
1	*E. faecium*	Farm equipment	ND	$efaA_{fm}$	-	AZM	0.1
2	*E. faecium*	Farm equipment	Moderate	-	+	ND	-
3	*E. faecium*	Farm personnel nose	Weak	-	+	ND	-
4	*E. faecium*	Farm personnel nose	Weak	$efaA_{fm}$	-	AZM	0.1
5	*E. faecium*	Farm personnel nose	Strong	esp_{fm}	-	E	0.1
6	*E. faecium*	Farm personnel hand	ND	$efaA_{fm}$	-	ND	-
7	*E. faecium*	Plant equipment	Moderate	-	-	AZM	0.1
8	*E. faecium*	Plant equipment	ND	-	-	E, TE	0.2
9	*E. faecium*	Plant equipment	Moderate	-	+	E, AZM	0.2
10	*E. faecium*	Plant equipment	Moderate	$efaA_{fm}$	+	VAN, AZM	0.2
11	*E. faecium*	Plant equipment	Moderate	$efaA_{fm}$, esp_{fm}	-	ND	-
12	*E. faecium*	Plant equipment	ND	$efaA_{fm}$	-	ND	-
13	*E. faecium*	Cow nasal swab	ND	$efaA_{fm}$	-	ND	-
14	*E. faecium*	Cow nasal swab	ND	$efaA_{fm}$	+	E, TE	0.2
15	*E. faecium*	Cow nasal swab	Moderate	-	-	CIP, E, AZM	0.3
16	*E. faecium*	Cow nasal swab	Moderate	-	-	ND	-
17	*E. faecium*	Cow nasal swab	ND	-	+	ND	-
18	*E. faecium*	Cow nasal swab	Moderate	$efaA_{fm}$, esp_{fm}	-	ND	-
19	*E. faecium*	Cow nasal swab	Moderate	-	-	TE	0.1
20	*E. faecium*	Cow nasal swab	ND	-	-	CIP, E, AZM	0.3
21	*E. faecium*	Cow nasal swab	ND	-	-	ND	-
22	*E. faecium*	Cow nasal swab	ND	-	-	ND	-
23	*E. faecium*	Cow nasal swab	Moderate	$efaA_{fm}$, esp_{fm}	-	ND	-

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24	*E. faecium*	Cow nasal swab	ND	*efaA*$_{fm}$	-	ND	-
25	*E. faecium*	Plant personnel nose	Moderate	*efaA*$_{fm}$	-	E	0.1
26	*E. faecium*	Cheese	Moderate	*efaA*$_{fm}$, *esp*$_{fm}$	+	ND	-
27	*E. faecalis*	Farm equipment	ND	*efaA*$_{fs}$	+	E, AZM	0.2
28	*E. faecalis*	Farm personnel hand	Moderate	*efaA*$_{fs}$	-	ND	-
29	*E. faecalis*	Farm personnel hand	Weak	*efaA*$_{fs}$	+	ND	-
30	*E. faecalis*	Farm personnel hand	Strong	*efaA*$_{fs}$, *esp*$_{fs}$	+	ND	-
31	*E. faecalis*	Plant equipment	Moderate	*efaA*$_{fs}$	+	VAN, AZM	0.2
32	*E. faecalis*	Cow nasal swab	Moderate	*efaA*$_{fs}$	+	E	0.1
33	*E. faecalis*	Cow nasal swab	Moderate	*efaA*$_{fs}$	+	ND	-
34	*E. faecalis*	Cow nasal swab	Weak	*efaA*$_{fs}$	-	ND	-
35	*E. faecalis*	Cow nasal swab	Weak	-	-	AMP, TE, E	0.3
36	*E. faecalis*	Cow nasal swab	Strong	*efaA*$_{fs}$	-	E	0.1
37	*E. faecalis*	Cow nasal swab	Strong	*efaA*$_{fs}$, *esp*$_{fs}$	-	E, TE, AZM	0.3
38	*E. faecalis*	Cow nasal swab	Moderate	*efaA*$_{fs}$	-	E, AZM	0.2
39	*E. faecalis*	Cheese	ND	*efaA*$_{fs}$	-	ND	-
40	*E. faecalis*	Cheese	Strong	*efaA*$_{fs}$, *esp*$_{fs}$	+	ND	-
41	*E. faecalis*	Cheese	ND	*efaA*$_{fs}$	-	ND	-
42	*E. faecalis*	Milk	Strong	*efaA*$_{fs}$, *esp*$_{fs}$	+	E, TE, AZM	0.3

AMC: Amoxicillin-clavulanic acid, **AMP:** Ampicillin, **LNZ:** Linezolid, **TEC:** Teicoplanin, **VAN:** Vancomycin, **CIP:** Ciprofloxacin, **C:** Chloramphenicol, **E:** Erythromycin, **TE:** Tetracycline, **AZM:** Azithromycin, **ND:** Not detected

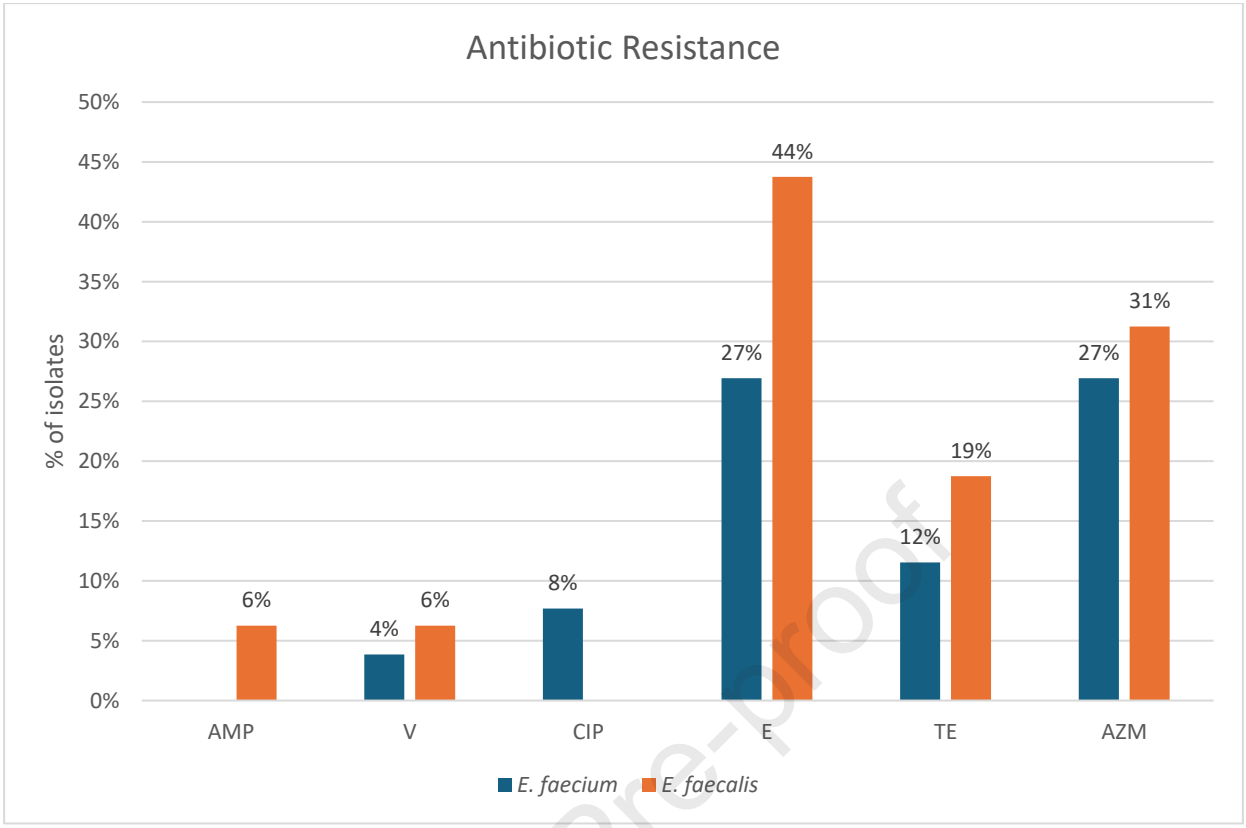


Figure 1. Rate of resistant *E. faecalis* and *E. faecium* against antibiotics: **AMP:** Ampicillin, **V:** Vancomycin, **CIP:** Ciprofloxacin, **E:** Erythromycin, **TE:** Tetracycline, **AZM:** Azithromycin

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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