Technological and safety properties of bacteriocin-producing *Enterococcus* strains isolated from traditional Turkish cheeses

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RESEARCH ARTICLE

Abstract

The aim of this study was to identify bacteriocin-producing lactic acid bacteria (LAB) isolated from traditional Turkish cheeses, evaluate their antibacterial properties and technological characteristics, and determine the safety properties of isolates. The isolated strains were identified as *Enterococcus faecium* DP8.3, DP9.3, and *Enterococcus mundtii* DP35.1, which are genetically different from each other. The bacteriocins produced by these isolates exhibited stability under high temperatures and maintained their antibacterial activity across a broad pH spectrum. The bacterial strains were fast acid producers, lacked proteolytic or lipolytic activity, were susceptible to antibiotics, and did not show antibiotic resistance or virulence genes. In addition, all the *Enterococcus* strains decarboxylated tyrosine and showed *tdc* gene expression. According to the technological properties and safety assessment of the strains, it is believed that they can be used as protective adjunct cultures in the food industry.

Keywords: bacteriocin; cheese; enterococci; safety assessment; technological property

Introduction

Physical, chemical, and biological methods are commonly used to control bacterial growth in foods (Yi *et al.*, 2020). Over the past decade, there has been growing interest in using natural protective agents as alternatives to chemical additives in the food industry. This shift is driven by changing consumer perceptions regarding health and the rising demand for healthier food options (Kaškonienė *et al.*, 2017). Lactic acid bacteria (LAB) are key contributors in this area, as they produce antimicrobial compounds such as organic acids, diacetyl, hydrogen peroxide, ethanol, and bacteriocins. These compounds inhibit the growth of harmful and spoilage bacteria, thereby enhancing the safety, shelf life, and quality of fermented food products. Bacteriocins are ribosomally synthesized antimicrobial metabolites that inhibit the growth of microorganisms, including strains closely related to bacteriocin-producing bacteria (O'Connor *et al.*, 2020). As key bioprotective agents, bacteriocins are considered safe for human consumption due to the minimal impact on human microbiota. They are also stable in the food matrix, exhibiting resistance to heat, pH variation, and food-related enzymes (Johnson *et al.*, 2018). This stability enables the production of food products that align with the consumer preferences for natural ingredients (O'Connor *et al.*, 2020).

Various LAB have been identified in fermented products (Avcı and Özden Tuncer, 2017; Gök Charyyev *et al.*, 2019; Sağlam and Uçan Türkmen, 2022). LAB play a crucial role, especially in the production and ripening of dairy products, contributing to the improvement of their flavor, aroma, and texture (Güley *et al.*, 2023; Sağlam and

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Uçan Türkmen, 2022). Various traditional cheeses are produced worldwide. In Türkiye, studies have examined traditional cheeses produced in different regions and their microflora (Güley *et al.*, 2023; Sağlam and Uçan Türkmen, 2022). Previous studies reported that species belonging to the genera *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Enterococcus*, and *Bifidobacterium* are commonly observed in traditional Turkish cheeses (Erginkaya *et al.*, 2018; Güley *et al.*, 2023; Sağlam and Uçan Türkmen, 2022). Enterococci are LAB that are usually detected in the digestive tracts of humans and animals. They can also be present in various foods and environmental settings. Depending on the strain, enterococci can function as starter or adjunct cultures, probiotics, spoilage bacteria, and opportunistic pathogens (Graham *et al.*, 2020). During fermentation, enterococci play a significant role in shaping the taste, aroma, and texture of dairy products (Graham *et al.*, 2020). Their lipolytic and proteolytic activities, along with citrate metabolism, contribute to the ripening process and enhance the organoleptic properties of these products (Jaouani *et al.*, 2015). Enterococci are essential as protective cultures due to their ability to produce bacteriocins, specifically referred to as enterocins (Graham *et al.*, 2020). Studies have shown that bacteriocin-producing enterococci have been isolated from various food sources, including milk and dairy products (Avcı and Özden Tuncer, 2017; Cavicchioli *et al.*, 2017), meat and meat products (Altınkaynak and Tuncer, 2020), cereal-based beverage boza (Gök Charyyev *et al.*, 2019), as well as fish and seafood (Iseppi *et al.*, 2019). However, enterococci are considered opportunistic pathogens that play a role in the development of nosocomial infections. The recognition of enterococci as opportunistic pathogens is influenced by factors such as escalating antibiotic resistance and diverse virulence determinants. Enterococci can display both intrinsic and acquired resistance to antibiotics (Graham *et al.*, 2020). This antibiotic resistance limits medical applications, complicating the treatment of enterococcal infections. Virulence factors, which are genetically encoded, can lead to pathogenic effects on the host tissue and enable enterococci to resist the host's defense system, further exacerbating the challenge of managing these infections. Enterococcal virulence factors include those that bind to the host tissue and extracellular matrix protein, confer resistance and avoidance to the host immune system, and cause cell and tissue damage and sex pheromones (Chajęcka-Wierzchowska *et al.*, 2017). Biogenic amine production is another significant aspect of enterococci that can potentially impact consumer health. These amines are formed by the decarboxylation of amino acids via microbial activities and can be harmful to humans. The production of biogenic amines varies by strain, making it an important consideration when selecting starter or bioprotective cultures (Barbieri *et al.*, 2019).

The aim of this study was to isolate bacteriocin-producing LAB from various traditional Turkish cheeses and investigate their technological and safety properties.

Materials and Methods

Cheese samples

For this investigation, 40 samples of traditionally made Turkish cheeses, including goat, sheep, Turkish Beyaz (white), ripened Turkish Beyaz, Tulum, and Çökelek cheese, were used as isolation materials. All samples were obtained from the Isparta province of Türkiye.

Screening of presumptive bacteriocin-producing LAB isolates

Bacteriocin-producing LAB were screened from the cheese samples using the method applied by Öztürk *et al.* (2023), with minor modifications. Ten grams of each cheese sample was homogenized in 90 mL of sterile saline solution (0.85% NaCl, w/v) and serially diluted to 10^{-5}. Of each dilution, 100 µL, was spread onto Enterococcosel agar (BBL, Becton Dickinson and Company, Sparks, USA) and de Man Rogosa and Sharpe (MRS) agar (Biokar, France), and then Petri dishes, incubated at 37°C for 24–48 hours. After that, tryptone soy soft agar medium (0.5% yeast extract; TSBYE, LAB M, Lancashire, United Kingdom) inoculated with 100 µL of the indicator bacteria *Listeria monocytogenes* ATCC 7644 or *Enterococcus faecium* ATCC 51559 was spread homogeneously on Petri dishes, which were incubated for 24 hours at 37°C, after which colony growth of indicator bacteria was observed. At the end of the incubation period, the colonies that showed a clear zone with sharp edges were evaluated as presumptive bacteriocin producers. After being confirmed as LAB by Gram staining and catalase testing, the isolates were cultured in MRS broth and stored at -20°C adding 20% (v/v) sterile glycerol.

Evaluation of the antimicrobial activity spectrum of presumptive bacteriocin-producing LAB isolates

The isolates were examined for their antibacterial activity against the 18 indicator bacteria listed in Table 1, using the sterile toothpick technique defined by Van Belkum *et al.* (1989). The overnight cultures of the isolates grown in MRS broth were streaked on MRS agar and incubated at 37°C for 24 hours. The growing colonies were transferred to MRS agar using a sterile toothpick and incubated under the same conditions. Of the indicator strain

Table 1. Growth medium and incubation temperature of indicator strains.

Indicator strains	Growth medium[a] and incubation temperature
Lactococcus lactis subsp. *lactis* LMG 2910	GM17, 30ºC
Enterococcus faecalis LMG 2708	MRS, 37ºC
Enterococcus faecalis ATCC 29212	MRS, 37ºC
Enterococcus faecalis ATCC 51299	MRS, 37ºC
Enterococcus faecium ATCC 51559	MRS, 37ºC
Listeria innocua LMG 2813	GM17, 30ºC
Listeria monocytogenes ATCC 7644	TSBYE, 37ºC
Listeria monocytogenes ATCC 19115	TSBYE, 37ºC
Escherichia coli ATCC 25828	TSBYE, 37ºC
Escherichia coli ATCC 25922	TSBYE, 37ºC
Salmonella Typhimurium ATCC 14028	TSBYE, 37ºC
Salmonella Enteritidis ATCC 13076	TSBYE, 37ºC
Staphylococcus aureus ATCC 25923	TSBYE, 37ºC
Staphylococcus aureus ATCC 43300	TSBYE, 37ºC
Staphylococcus aureus ATCC 6538	TSBYE, 37ºC
Pediococcus pentosaceus LMG 2001	TSBYE, 37ºC
Bacillus cereus ATCC 10876	TSBYE, 37ºC
Pseudomonas aeruginosa ATCC 15442	TSBYE, 37ºC

[a]GM17: M17 broth (containing 0.5% glucose); MRS: de Man Rogosa and Sharpe broth; TSBYE: Tryptone soy broth (containing 0.5% yeast extract).

culture grown in the liquid medium indicated in Table 1, 100 µL was added to a soft agar medium and poured onto Petri dishes. After incubation for 18 hours, the antibacterial activity of the isolates was evaluated by measuring the inhibition zone diameter against the indicator bacteria.

Examination of the protein nature of the antimicrobial substances

The protein nature of the antimicrobial substances synthesized by the isolates was examined following the method of Ryan *et al.* (1996), using pepsin (pH 3.0), proteinase K (pH 7.0), α-chemotrypsin (pH 7.0), trypsin (pH 7.0), and catalase (pH 7.0) enzyme solutions, which were prepared at a final concentration of 50 mg/mL. All enzymes were obtained from Sigma-Aldrich (Steinheim, Germany). Twenty microliters of the filtered cell-free supernatant and 20 µL of each enzyme were spotted 1 cm apart on MRS agar plates. *E. faecalis* ATCC 51299 was used as an indicator bacteria. The Petri dishes were incubated for 24 hours at 37°C, and then the zone shapes were examined. The formation of a half-moon-shaped inhibition zone on the side where the enzyme was applied indicates that the antimicrobial substance is likely protein-based.

Identification of bacteriocin-producing isolates

Isolation of genomic DNA

For the extraction of genomic DNA, we followed the method suggested by Cancilla *et al.* (1992). Initially, 500 µL of the active cultures was centrifuged, and the resulting cell pellet was resuspended in 500 µL of lysis buffer, followed by incubation at 37°C for 30 min. Next, we added 30 µL of 10% sodium dodecyl sulfate (Serva, Heidelberg, Germany) and incubated the mixture at 80°C for 10 min. The lysate was then mixed with 700 µL of phenol-chloroform (1:10) and centrifuged. The upper phase was carefully transferred to new Eppendorf tubes, and 700 µL of chilled 2-propanol (Merck, Darmstadt, Germany) was added. After another centrifugation, the genomic DNA pellets obtained were dissolved in 50 µL of Tris-EDTA (pH 8.0).

16S rDNA sequence analysis

Polymerase chain reaction (PCR) was performed to amplify the 16S rDNA region of the isolates using universal bacterial primers as described by Edwards *et al.* (1989) (Table 2). The DNA sequence analysis of the PCR products was performed by BM Software Consulting and Laboratory Systems Ltd. (Ankara, Türkiye). The similarity of the 16S rDNA sequences among the samples was analyzed using the National Center for Biotechnology Information BLAST program.

Identification of isolates using PCR with specific primers for the *Enterococcus* genus and species

Polymerase chain reaction was performed using specific primers for the *Enterococcus* genus (Sahoo *et al.*, 2015), as well as for *E. faecium* and *E. mundtii* (Jackson *et al.*, 2004) to confirm the identification of the isolates determined by 16S rDNA sequence analysis (Table 2). The PCR was conducted in a gradient thermal cycler (TurboCylcler 2, Blue-Ray Biotech. Corp., Taipei City, Taiwan). The amplified PCR fragments were then electrophoresed on 2% (w/v) agarose gels. For these PCR experiments, *E. faecium* ATCC 51559 and *E. mundtii* YB6.30 (Altınkaynak and Tuncer, 2020) were used as positive controls.

Genotyping of Enterococcus *isolates using random amplified polymorphic DNA PCR*

The random amplified polymorphic DNA (RAPD) PCR method recommended by Cocolin *et al.* (2009) was employed for genotyping the bacteriocin-producing *Enterococcus* isolates. The PCR was conducted following the protocol outlined byRossetti and Giraffa (2005). The amplified PCR fragments were electrophoresed on 2% (w/v) agarose gels. The GelJ program was used to ascertain the genetic similarities of the isolates (Heras *et al.*, 2015).

Table 2. Primer sequences, PCR protocols, and product size used for the identification of bacteriocin-producing strains.

Genes	Primers sequence (5′ to 3′)	PCR protocol	Product size (bp)	References
16S rRNA	AGAGTTTGATCCTGGCTCAG CCGTCAATTCCTTTGAGTTT	94°C for 2 min x1; 94°C for 30 s, 55°C for 60 s, 72°C for 90 s x30; 72°C for 10 min x1	921	Edwards *et al.* (1989)
Enterococcus tuf	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	95°C for 15 min x1; 94°C for 30 s, 64°C for 30 s, 72°C for 30 s x30; 72°C for 10 min x1	112	Sahoo *et al.* (2015)
E. faecium sodA	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	95°C for 4 min x1; 95°C for 30 s, 55°C for 60 s, 72°C for 30 s x30; 72°C for 10 min x1	215	Jackson *et al.* (2004)
E. mundtii sodA	CAGACATGGATGCTATTCCATCT GCCATGATTTTCCAGAAGAAT	95°C for 4 min x1; 95°C for 30 s, 60°C for 60 s, 72°C for 30 s x30; 72°C for 10 min x1	98	Jackson *et al.* (2004)

Effects of enzymes, heat, and pH treatments on antimicrobial activity

Cell-free neutralized supernatant (CFNS) samples were used to evaluate the effects of enzymes and heat treatments on bacteriocin activity. To prepare the CFNS, isolates were cultured at 37°C for 18 hours, after which active cultures were centrifuged at 3059 x g (Sigma 2-16P, rotor no. 12,141, Germany) for 15 min. The pH of the supernatant was adjusted to 6.5–7.0 using 6N NaOH, and the neutralized supernatant was sterilized by filtration (Minisart NML, Sartorius Stedim Biotech, Germany). To determine the effect of enzyme treatments on antimicrobial activity, solutions of proteinase K, trypsin, α-chymotrypsin, pepsin, α-amylase, lipase, catalase, and lysozyme, each at a final concentration of 1 mg/mL, were added to the CFNS samples. The tubes were then incubated for 2 hours at 37°C. Subsequently, the enzyme activities were stopped by heat treatment at 100°C for 5 min (Franz *et al.*, 1997).

Through heat treatment of the CFNS samples at 100°C for 5, 10, 15, and 20 min, and at 121°C for 15 min, the impact of temperature on antibacterial activity was investigated. To determine the effect of pH changes on antimicrobial activity, active cultures were centrifuged at 3059 x g for 15 min. The pH of the culture supernatants was adjusted between pH 2.0 and 11.0 and then filtered. Culture supernatants without pH adjustment were used as controls. The effects of the pH treatments on antimicrobial activity were calculated using the critical dilution method, and the adjusted cell-free supernatant samples were examined against *E. faecalis* ATCC 51299 using an agar spot test (Franz *et al.*, 1997).

Screening of enterocin genes

The PCR protocol specified in previous studies was used to determine the presence of well-known enterocin structural genes *entA*, *entB*, *entP*, *entQ*, *entL50A/B*, *ent1071A/B*, *entCRL35*, *bac31*, *entAS48*, and *munKS*, in the *Enterococcus* strains (Belgacem *et al.*, 2010; Settanni *et al.*, 2014; Yousif *et al.*, 2005; Zendo *et al.*, 2005). The primer sequences and PCR protocols used for the detection of enterocin structural genes in the bacteriocin-producing strains are presented in Table 3. The resulting PCR products were analyzed using 2% (w/v) agarose gel electrophoresis. For the PCR experiments, enterocin A-, B-, and P-producing *E. faecium* EYT17 (Özden Tuncer *et al.*, 2013) and mundticin KS-producing *E. mundtii* YB6.30 (Altınkaynak and Tuncer, 2020) were included as positive controls.

Technological properties of the bacteriocin-producing *Enterococcus* strains

Acid production

The acid production abilities of the *Enterococcus* strains were assessed in MRS broth at 37°C. The pH of the cultures was measured at 0, 6, and 24 hours of incubation using a pH meter (WTW 3110, Weilheim, Germany). Acid production was quantified by calculating the difference (ΔpH) between the initial and post-incubation pH values. Based on these ΔpH values, the acid production abilities of the cultures were classified as follows: fast (ΔpH > 1.5), moderate (ΔpH 1.0–1.5), and slow (ΔpH < 1.0) (Bradley *et al.*, 1992; Özkalp *et al.*, 2007).

Proteolytic and lipolytic activities

The proteolytic activities of the strains were tested using calcium caseinate agar. Bacteriocin-producing active cultures were dropped onto 10 µL calcium caseinate agar and incubated at 37°C for 72 hours. At the end of the incubation, zone formation around the colony was accepted as a positive proteolytic activity (Martín *et al.*, 2006). The lipolytic activities of the strains were evaluated using Luria-Bertani agar (LB, Sigma Aldrich, 28713) containing 0.2% (w/v) $CaCI_2$ and 0.1% (w/v) Tween 80 (Moraes *et al.*, 2012), and Spirit Blue agar (BD Difco 295020, France) containing a lipase-reagent (BD Difco 215335) (Landeta *et al.*, 2013). The formation of opaque zones around the colonies growing in both mediums was examined.

Table 3. Primer sequences, PCR protocols, and product size used for the detection of bacteriocin genes.

Genes	Primers sequence (5′ to 3′)	PCR protocol	Product size (bp)	References
entA	AATATTATGGAAATGGAGTGTAT GCACTTCCCTGGAATTGCTC	94°C for 5 min x1; 94°C for 60 s, 56°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1	126	Yousif *et al.* (2005)
entB	GAAAATGATCACAGAATGCCTA GTTGCATTTAGAGTATACATTTG	94°C for 5 min x1; 94°C for 60 s, 50°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1	162	Yousif *et al.* (2005)
entP	TATGGTAATGGTGTTTATTGTAAT ATGTCCCATACCTGCCAAAC	94°C for 5 min x1; 94°C for 60 s, 50°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1	120	Yousif *et al.* (2005)
entL50A/B	TGGGAGCAATCGCAAAATTAG ATTGCCCATCCTTCTCCAAT	94°C for 5 min x1; 94°C for 60 s, 52°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1	98	Belgacem *et al.* (2010)
bac31	TATTACGGAAATGGTTTATATTGT TCTAGGAGCCCAAGGGCC	94°C for 5 min x1; 94°C for 60 s, 50°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1	123	Yousif *et al.* (2005)
entAS48	GAGGAGTTTCATGATTTAAAGA CATATTGTTAAATTACCAAGCAA	94°C for 5 min x1; 94°C for 60 s, 50°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1	340	Yousif *et al.* (2005)
entQ	TGAATTTTCTTCTTAAAAATGGTATCGCA TTAACAAGAAATTTTTTCCCATGGCAA	94°C for 5 min x1; 94°C for 60 s, 56°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1	105	Belgacem *et al.* (2010)
ent1071A/B	CCTATTGGGGGAGAGTCGGT ATACATTCTTCCACTTATTTTT	94°C for 5 min x1; 94°C for 60 s, 51°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1	343	Belgacem *et al.* (2010)
munKS	TGAGAGAAGGTTTAAGTTTTGAAGAA TCCACTGAAATCCATGAATGA	94°C for 3 min x1; 94°C for 60 s, 55°C for 30 s, 72°C for 60 s x30; 72°C for 7 min x1	380	Zendo *et al.* (2005)
entCRL35	GCAAACCGATAAGAATGTGGGAT TATACATTGTCCCCACAACC	94°C for 3 min x1; 94°C for 60 s, 55°C for 30 s, 72°C for 3.4 min x30; 72°C for 4 min x1	490	Settanni *et al.* (2014)

Safety properties of the bacteriocin-producing *Enterococcus* strains

Antibiotic resistance

The antibiotic resistance of the *Enterococcus* strains was determined against ampicillin (2 and 10 µg), imipenem (10 µg), tigecycline (15 µg), penicillin G (10 U), vancomycin (30 µg), teicoplanin (30 µg), erythromycin (15 µg), tetracycline (30 µg), doxycycline (30 µg), minocycline (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), norfloxacin (10 µg), nitrofurantoin (100 and 300 µg), rifampin (5 µg), chloramphenicol (30 µg), quinupristin-dalfopristin (15 µg), linezolid (30 µg), gentamycin (120 µg), and streptomycin (300 µg; Oxoid Ltd., Basingstoke, United Kingdom), using the disk diffusion method on Mueller Hinton agar (LAB M). Strains were classified as resistant, intermediary, or susceptible according to the European Committee on Antimicrobial Susceptibility Testing clinical breakpoint table (EUCAST, 2021) and the Clinical and Laboratory Standards Institute (CLSI, 2016) guidelines.

Detection of antibiotic resistance genes

The presence of erythromycin (*ermA*, *ermB*, and *ermC*), tetracycline (*tetK*, *tetL*, *tetM*, *tetO*, and *tetS*), high-level aminoglycoside [*aac(6′)-Ie-aph(2″)-Ia*, *aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2″)-Id*], and vancomycin resistance genes (*vanA*, *vanB*, *vanC1*, *vanC2*, *vanC3*, *vanD*, *vanE*, and *vanG*) was determined with PCR using specific primers (Depardieu *et al.*, 2004; Dutka-Malen *et al.*, 1995; Lemcke and Bülte, 2000; Niu *et al.*, 2016; Ouoba *et al.*, 2008; Vakulenko *et al.*, 2003). The primer sequences and PCR protocols used for the detection of antibiotic-resistant genes in bacteriocin-producing strains are detailed in Table 4. In the PCR experiments, *E. faecium* ATCC 51559 (*vanA*$^+$), *E. faecalis* ATCC 51299 [*aph(3′)-IIIa* $^+$, *aac(6′)-Ie-aph(2″)-Ia*$^+$, *ant(6′)-Ia*$^+$, and *vanB*$^+$], *E. gallinarum* DYE45 (*ermA*$^+$ and *ermB*$^+$), *E. casseliflavus* DYE26 (*tetS*$^+$, *vanC2*$^+$, and *vanC3*$^+$), *E. gallinarum* DYE22 (*vanC1*$^+$ and *vanD*$^+$) (Akpınar Kankaya and Tuncer, 2020), *E. faecium* FYE41 (*ermC*$^+$, *tetM*$^+$, and *tetL*$^+$) (Demirgül and Tuncer, 2017), *E. faecium* MSE53.1 [*ant(4′)-Ia*$^+$, *aph(2″)-Ib*$^+$, and *aph(2″) Ic*$^+$] (Yalçın *et al.*, 2023) were used as positive controls.

Hemolytic and gelatinase activities

The hemolytic and gelatinase activities of the strains were monitored on Columbia agar supplemented with 5% (v/v) sheep blood (Liofichem, Roseto degli Abruzzi, Italy) and Todd Hewitt agar (Liofilchem) containing 3% (w/v) gelatin, following the method described by Eaton and Gasson (2001).

Detection of virulence factor genes

Aggregation protein (*agg*) (Eaton and Gasson, 2001), hyaluronidase (*hyl*) (Vankerckhoven *et al.*, 2004), cell wall adhesins ($efaA_{fm}$ and $efaA_{fs}$), cytolysin (*cylM*, *cylB*, and *cylA*), extracellular surface protein (esp_{fm} and esp_{fs}), gelatinase (*gelE*), sex pheromones (*cpd*, *cob*, *ccf*, and *cad*)

Table 4. Primer sequences, PCR protocols, and product size for the detection of antibiotic resistance genes.

Genes	Primers sequence (5′ to 3′)	PCR Protocol	Product size (bp)	References
ermA	AAGCGGTAAAACCCCTCTGAG TCAAAGCCTGTCGGAATTGG	94°C for 2 min x1; 94°C for 60 s, 55°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1	442	Ouoba *et al.* (2008)
ermB	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	94°C for 2 min x1; 94°C for 60 s, 52°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1	425	Ouoba *et al.* (2008)
ermC	ATCTTTGAAATCGGCTCAGG CAAACCCGTATTCCACGATT	94°C for 2 min x1; 94°C for 60 s, 48°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1	295	Ouoba *et al.* (2008)
tetK	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA	94°C for 2 min x1; 94°C for 60 s, 55°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1	718	Ouoba *et al.* (2008)
tetL	GTTGCGCGCTATATTCCAAA TTAAGCAAACTCATTCCAGC	94°C for 2 min x1; 94°C for 60 s, 54°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1	788	Ouoba *et al.* (2008)
tetM	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	94°C for 2 min x1; 94°C for 60 s, 45°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1	656	Ouoba *et al.* (2008)
tetO	GATGGCATACAGGCACAGAC CAATATCACCAGAGCAGGCT	94°C for 2 min x1; 94°C for 60 s, 55°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1	614	Ouoba *et al.* (2008)
tetS	TGGAACGCCAGAGAGGTATT ACATAGACAAGCCGTTGACC	94°C for 2 min x1; 94°C for 60 s, 55°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1	660	Ouoba *et al.* (2008)
aph(3′)-IIIa	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG	94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1	523	Vakulenko *et al.* (2003)
ant(4′)-Ia	CAAACTGCTAAATCGGTAGAAGCC GGAAAGTTGACCAGACATTACGAACT	94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1	294	Vakulenko *et al.* (2003)
ant(6′)-Ia	ACTGGCTTAATCAATTTGGG GCCTTTCCGCCACCTCACCG	94°C for 3 min x1; 94°C for 30 s, 56°C for 30 s, 72°C for 60 s x35; 72°C for 5 min x1	577	Niu *et al.* (2016)
aac(6′)-Ie-aph(2″)-Ia	CAGGAATTTATCGAAAATGGTAGAAAAG CACAATCGACTAAAGAGTACCAATC	94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1	369	Vakulenko *et al.* (2003)
aph(2″)-Ib	CTTGGACGCTGAGATATATGAGCAC GTTTGTAGCAATTCAGAAACACCCTT	94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1	867	Vakulenko *et al.* (2003)
aph(2″)-Ic	CCACAATGATAATGACTCAGTTCCC CCACAGCTTCCGATAGCAAGAG	94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1	444	Vakulenko *et al.* (2003)
aph(2″)-Id	GTGGTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC	94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1	641	Vakulenko *et al.* (2003)
vanA	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	94°C for 2 min x1; 94°C for 60 s, 54°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1	732	Dutka-Malen *et al.* (1995)
vanB	ACGGAATGGGAAGCCGA TGCACCCGATTTCGTTC	94°C for 2 min x1; 94°C for 60 s, 54°C for 60 s, 72°C for 60 s x30; 72°C for 7 min x1	647	Depardieu *et al.* (2004)
vanC1	GAAAGACAACAGGAAGACCGC TCGCATCACAAGCACCAATC	94°C for 5 min x1; 94°C for 30 s, 58°C for 30 s, 72°C for 30 s x30; 72°C for 5 min x1	796	Lemcke and Bülte (2000)
vanC2	CGGGGAAGATGGCAGTAT CGCAGGGACGGTGATTTT	94°C for 5 min x1; 94°C for 30 s, 58°C for 30 s, 72°C for 30 s x30; 72°C for 5 min x1	484	Lemcke and Bülte (2000)
vanC3	GCCTTTACTTATTGTTCC GCTTGTTCTTTGACCTTA	94°C for 5 min x1; 94°C for 30 s,58°C for 30 s, 72°C for 30 s x30; 72°C for 5 min x1	224	Lemcke and Bülte (2000)
vanD	TGTGGGATGCGATATTCAA TGCAGCCAAGTATCCGGTAA	94°C for 3 min x1; 94°C for 60 s,54°C for 60 s, 72°C for 60 s x30; 72°C for 7 min x1	500	Depardieu *et al.* (2004)
vanE	TGTGGTATCGGAGCTGCAG ATAGTTTAGCTGGTAAC	94°C for 3 min x1; 94°C for 60 s, 54°C for 60 s, 72°C for 60 s x30; 72°C for 7 min x1	430	Depardieu *et al.* (2004)
vanG	CGGCATCCGCTGTTTTTGA GAACGATAGACCAATGCCTT	94°C for 3 min x1; 94°C for 60 s, 54°C for 60 s, 72°C for 60 s x30; 72°C for 7 min x1	941	Depardieu *et al.* (2004)

(Reviriego *et al.*, 2005), and gene-encoding collagen-binding protein (*ace*) (Belgacem *et al.*, 2010) in the bacteriocin-producing *Enterococcus* strains were determined with PCR. The primer sequences and PCR protocols used for the detection of virulence factor genes in the bacteriocin-producing strains are described in Table 5. *E. faecalis* ATCC 29212 (*agg*$^+$, *gelE*$^+$, *efaA*$_{fs}$$^+$, *efaA*$_{fm}$$^+$, *esp*$_{fm}$$^+$, *esp*$_{fs}$$^+$, *cpd*$^+$, *cob*$^+$, *ccf*$^+$, *cad*$^+$, *ace*$^+$, *acm*$^+$, *cylM*$^+$, *cylB*$^+$, *cylA*$^+$, and *hyl*$^+$) was used as a positive control strain.

Table 5. Primer sequences, PCR protocols, and product size for the detection of virulence factors and amino acid decarboxylase genes.

Genes	Primers sequence (5′ to 3′)	PCR Protocol	Product size (bp)	References
gelE	ACCCCGTATCATTGGTTT ACGCATTGCTTTTCCATC	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	419	Reviriego *et al.* (2005)
efaAfm	AACAGATCCGCATGAATA CATTTCATCATCTGATAGTA	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	735	Reviriego *et al.* (2005)
efaAfs	GACAGACCCTCACGAATA AGTTCATCATGCTGTAGTA	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	705	Reviriego *et al.* (2005)
espfm	TTGCTAATGCAAGTCACGTCC GCATCAACACTTGCATTACCGAA	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	955	Reviriego *et al.* (2005)
espfs	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGAA	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	933	Reviriego et al. (2005)
cpd	TGGTGGGTTATTTTTCAATTC TACGGCTCTGGCTTACTA	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	782	Reviriego *et al.* (2005)
cob	AACATTCAGCAAACAAAGC TTGTCATAAAGAGTGGTCAT	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	1405	Reviriego *et al.* (2005)
ccf	GGGAATTGAGTAGTGAAGAAG AGCCGCTAAAATCGGTAAAAT	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	543	Reviriego *et al.* (2005)
cad	TGCTTTGTCATTGACAATCCG ACTTTTTCCCAACCCCTCAA	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	1299	Reviriego *et al.* (2005)
ace	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCG	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	350	Belgacem *et al.* (2010)
agg	AAGAAAAAGAAGTAGACCAAC AAACGGCAAGACAAGTAAATA	95°C for 5 min x1; 95°C for 30 s, 56°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	1533	Eaton and Gasson (2001)
cylM	CTGATGGAAAGAAGATAGTAT TGAGTTGGTCTGATTACATTT	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	742	Reviriego *et al.* (2005)
cylB	ATTCCTACCTATGTTCTGTTA AATAAACTCTTCTTTTCCAAC	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	843	Reviriego *et al.* (2005)
cylA	TGGATGATAGTGATAGGAAGT TCTACAGTAAATCTTTCGTCA	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	517	Reviriego *et al.* (2005)
hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1	276	Vankerckhoven *et al.* (2004)
hdc	GGNATNGTNWSNTAYGAYMGNGCNGA ATNGCDATNGCNSWCCANACNCCRTA	95°C for 10 min x1; 95°C for 30 s, 53°C for 30 s, 72°C for 2 min x30; 72°C for 20 min x1	372	De Las Rivas *et al.* (2006)
ldc	CAYRTNCCNGGNCAYAA GGDATNCCNGGNGGRTA	95°C for 10 min x1; 95°C for 30 s, 53°C for 30 s, 72°C for 2 min x30; 72°C for 20 min x1	1185	De Las Rivas *et al.* (2006)
odc	TWYMAYGCNGAYAARACNTAY TTYGT ACRCANAGNACNCCNGGNGGRTANGG	95°C for 10 min x1; 95°C for 30 s, 53°C for 30 s, 72°C for 2 min x1; 72°C for 20 min x1	1440	De Las Rivas *et al.* (2006)
tdc	TGGYTNGTNCCNCARACNAA RCAYTA ACRTARTCNACCATRTTRAAR TCNGG	95°C for 10 min x1; 95°C for 30 s, 53°C for 30 s, 72°C for 2 min x30; 72°C for 20 min x1	825	De Las Rivas *et al.* (2006)

Biogenic amine production and detection of amino acid decarboxylase genes

The biogenic amine production of the *Enterococcus* strains was investigated using a basal medium containing precursor amino acid (1%, w/v) histidine, lysine, ornithine, or tyrosine separately (Bover-Cid and Holzapfel, 1999). The presence of histidine (*hdc*), lysine (*ldc*), ornithine (*odc*), and tyrosine (*tdc*) decarboxylase genes in the strains was investigated with PCR using the primers reported by De Las Rivas *et al.* (2006). The primer sequences and PCR protocols used for the detection of amino acid decarboxylase genes in the bacteriocin-producing strains are presented in Table 5. *E. gallinarum* DYE22 (*tdc*$^+$) was used as a positive control in the PCR experiments (Akpınar Kankaya and Tuncer, 2022).

Statistical analysis

Antimicrobial activity tests were conducted in triplicate, and the data were analyzed using SPSS version 27. A normality test was performed, and both the Shapiro-Wilk

and Kolmogorov-Smirnov tests indicated that the data did not follow a normal distribution. Therefore, the Kruskal-Wallis test, the nonparametric equivalent of the analysis of variance (ANOVA), was used for statistical analysis.

Results and Discussion

Screening results of the presumptive bacteriocin-producing LAB isolates and their antibacterial activity spectrum

The antibacterial activity screening test revealed that 12 of the presumptive LAB colonies that grew on the Petri dishes exhibited antibacterial activity against the indicator bacteria. Out of these, three colonies showed a clear zone with sharp edges and were thus evaluated as presumptive bacteriocin producers. The isolates with the codes DP8.3, DP9.3, and DP35.1 were identified as LAB by Gram staining and catalase tests, and were subsequently selected for further analysis. The antimicrobial activities of isolates DP8.3, DP9.3, and DP35.1 were determined using the sterile toothpick method. These isolates produced inhibition zones ranging from 2 ± 0.00 to 37 ± 0.00 mm in diameter against the investigated indicator bacteria (Table 6). The isolates displayed activity against vancomycin-resistant *E. faecium* and *E. faecalis*, as well as against food pathogens such as *L. monocytogenes* and *Staphylococcus aureus*. The isolate DP35.1 showed the highest inhibition zones against *L. monocytogenes* ATCC 19115 (Ø 37 ± 0.00 mm) and *L. monocytogenes* ATCC 7644 (Ø 35.33 ± 0.58 mm). The antibacterial activities of the cheese isolates were generally found to be more effective against *Listeria* species compared to other indicator bacteria ($P < 0.05$). Similarly, enterococci isolated from cheeses (Avcı and Özden Tuncer, 2017) and camel milk (Vimont *et al.*, 2017) showed antibacterial activity against the *L. monocytogenes*, *S. aureus*, and *E. faecalis* strains. The isolates demonstrated antimicrobial activity, though at low levels, against Gram-negative bacteria such as *Escherichia*, *Pseudomonas*, and *Salmonella* genera. Gram-negative bacteria are more resistant to bacteriocins owing to their outer membranes (Schelegueda *et al.*, 2015). However, all isolates exhibited an inhibition activity against vancomycin-resistant *E. faecium* ATCC 51559 and *E. faecalis* ATCC 51299. Altınkaynak and Tuncer (2020) reported that mundticin KS-producing *E. mundtii* YB6.30 showed a zone against vancomycin-resistant *E. faecium* ATCC 51559 and *E. faecalis* ATCC 51299, and against *L. monocytogenes*, *L. innocua*, and *S. aureus*. Vancomycin-resistant enterococci (VRE) pose a significant clinical concern, as vancomycin remains one of the last options for the treatment of multidrug-resistant

Table 6. Inhibitory spectrum of bacteriocin-producing strains.

Indicator strains	Inhibition zones of strains (Ø mm)[a]		
	DP8.3	DP9.3	DP35.1
Lactococcus lactis subsp. *lactis* LMG 2910	10.33±0.58^{D}	7±0.00^{E}	18.33±0.58^{E}
Enterococcus faecalis LMG 2708	8±0.00^{E}	7±0.00^{E}	18±0.00^{E}
Enterococcus faecalis ATCC 29212	10±0.00^{D}	5±0.00^{F}	19±0.00^{E}
Enterococcus faecalis ATCC 51299	14.17±0.29^{B}	15.33±0.58^{C}	17±0.00^{E}
Enterococcus faecium ATCC 51559	13±0.00^{B}	10±0.00^{D}	21±0.00^{D}
Listeria innocua LMG 2813	10±0.00^{D}	7.33±0.58^{E}	19.33±0.58^{E}
Listeria monocytogenes ATCC 7644	15±0.00^{A}	24±0.00^{A}	35.33±0.58^{B}
Listeria monocytogenes ATCC 19115	15±0.00^{A}	19.33±0.58^{B}	37±0.00^{A}
Escherichia coli ATCC 25828	5.33±0.58^{F}	4±0.00^{G}	3±0.00^{F}
Escherichia coli ATCC 25922	4±0.00^{G}	5±0.00^{F}	3±0.00^{F}
Salmonella Typhimurium ATCC 14028	5±0.00^{F}	4±0.00^{G}	4±0.00^{F}
Salmonella Enteritidis ATCC 13076	4.17±0.29^{G}	5±0.00^{F}	4±0.00^{F}
Staphylococcus aureus ATCC 25923	11.33±0.58^{C}	12±0.00^{C}	23±0.00^{C}
Staphylococcus aureus ATCC 43300	4±0.00^{G}	4±0.00^{G}	2±0.00^{F}
Staphylococcus aureus ATCC 6538	2.17±0.29^{H}	0±0.00^{H}	4.33±0.58^{F}
Pediococcus pentosaceus LMG 2001	10.17±0.29^{D}	13.67±0.58^{C}	0±0.00^{G}
Bacillus cereus ATCC 10876	0±0.00^{I}	0±0.00^{H}	0±0.00^{G}
Pseudomonas aeruginosa ATCC 15442	10.33±0.58^{D}	13.33±0.58^{C}	20±0.00^{D}

[a]No difference between groups with the same letter ($P > 0.05$).

enterococcal infections. Given the necessity to find alternatives, research is actively exploring new drugs and the potential use of enterocins to combat antibiotic-resistant enterococci (Almeida-Santos *et al.*, 2021; Graham *et al.*, 2020). A promising observation is that all three isolates produced inhibition zones against VRE. These findings suggest that these isolates could serve as potential alternatives to antibiotics for inhibiting VRE, warranting further investigation.

Protein nature of the antibacterial substances

The proteolytic enzyme treatment demonstrated that the antimicrobial substances synthesized by all isolates became inactive when exposed to proteases such as proteinase K, pepsin, trypsin, and α-chemotrypsin. Thus, we concluded that hydrogen peroxide was not the source of the antibacterial activity, as the catalase enzyme did not affect the antimicrobial substances produced by any of the isolates. At the same time, the antimicrobial substances synthesized by the isolates were affected by proteolytic enzymes, proving that antimicrobial substances have protein-based nature. This finding supports the classification of the three isolates as bacteriocin-like substance producers. Previous studies have similarly reported that the protein nature of bacteriocins makes them susceptible to partial or complete degradation by proteolytic enzymes (Gök Charyyev *et al.*, 2019; Öztürk *et al.*, 2023). Similar to our findings, other studies have demonstrated that bacteriocin-producing LAB have been isolated from a variety of food sources, such as milk and dairy products, particularly cheeses (Avcı and Özden Tuncer, 2017; Cavicchioli *et al.*, 2017; Özden Tuncer *et al.*, 2013; Öztürk *et al.*, 2023; Vimont *et al.*, 2017), meat and meat products (Altınkaynak and Tuncer, 2020; Fontana *et al.*, 2015), fish and seafood (Iseppi *et al.*, 2019), the cereal-based beverage boza (Gök Charyyev *et al.*, 2019), and pickles (Qiao *et al.*, 2020; Yi *et al.*, 2020).

Identified bacteriocin-producing isolates

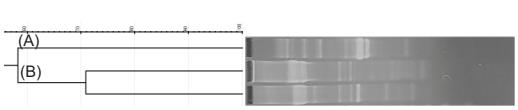
Based on the 16S rDNA sequence analysis, isolates DP8.3 and DP9.3 were identified as *E. faecium*, while DP35.1 was identified as *E. mundtii*. These findings were further validated through PCR analysis using genus- and species-specific primers. The PCR results revealed that all isolates produced 112-base pair (bp) amplicons specific to the *Enterococcus* genus. The isolates DP8.3 and DP9.3 yielded 215-bp fragments specific to *E. faecium*, while the DP35.1 isolate produced a 98-bp fragment specific to *E. mundtii*. Enterococci are commonly found in various food environments, as they can withstand challenging conditions such as high temperatures, salt concentrations, and acid concentrations (Graham *et al.*, 2020). Studies have shown that foodborne bacteriocin-producing *Enterococcus* isolates are mostly from the *E. faecium* and *E. faecalis* species. In line with our findings, Avcı and Özden Tuncer (2017) discovered 11 bacteriocin-producing isolates, six of which were identified as *E. faecium* and five as *E. faecalis*, from traditional Turkish cheeses. Gök Charyyev *et al.* (2019) described the bacteriocinogenic *E. faecium* YT52 strain isolated from boza. Additionally, Qiou *et al.* (2020) identified the TJUQ1 isolate from pickled Chinese celery as *E. faecium*. In addition, *E. mundtii* strains that produce bacteriocins have been found in fresh fish and seafood (Iseppi *et al.*, 2019), sucuk (Altınkaynak and Tuncer, 2020), and sheep and goat colostrum (Öztürk *et al.*, 2023).

Genotyping of *Enterococcus* isolates by RAPD-PCR

The RAPD-PCR genotyping technique is a fast and reliable procedure that has been widely used for distinguishing between the taxonomic and genotypic characteristics of isolates. In addition to the sources of the strains, RAPD-PCR analysis results should be evaluated to determine clonal relationships (Ben Braïek *et al.*, 2019). In this study, an unweighted pair group method with an arithmetic means (UPGMA) dendrogram was developed within the Dice similarity index of the isolates in the GelJ program. The isolates were separated into two distinct clusters, labeled A and B, and showed three patterns (Figure 1). Cluster A included *E. mundtii* DP35.1; and cluster B included *E. faecium* DP8.3 and *E. faecium* DP9.3 isolates. Similarly, the genetic distinguishing of enterococcal isolates was determined by the RAPD-PCR technique by Ben Braïek *et al.* (2019), Rocha *et al.* (2022), and Öztürk *et al.* (2023).

Effects of enzymes, heat, and pH treatments on bacteriocin activity

The characterization of bacteriocin is important for determining its applications and efficacy in the food industry. The proteinase K, trypsin, α-chymotrypsin, and α-amylase enzymes have completely eliminated the antimicrobial activities of the bacteriocins synthesized by all strains (Table 7). In addition, pepsin treatment reduced the bacteriocin activities of the strains by 50%. The bacteriocin produced by *E. faecium* DP9.3 was not affected by lipase or lysozyme treatments. Nevertheless, the use of lipase and lysozyme led to a decrease in the antimicrobial efficacy of the bacteriocins synthesized by *E. faecium* DP8.3 and *E. mundtii* DP35.1. The fact that lipase and/or amylase treatments affect bacteriocin activity indicates that a lipid and/or a carbohydrate moiety in the structure is required for the antimicrobial activity of the bacteriocin, as reported by Müller *et al.* (2009) and



Strain number	Genus	Species
DP35.1	Enterococcus	mundtii
DP9.3	Enterococcus	faecium
DP8.3	Enterococcus	faecium

Figure 1. Dendrogram of RAPD-PCR profiles of bacteriocin-producing *Enterococcus* strains.

Table 7. The effect of pH, enzyme, and heat treatments on the activity of the bacteriocins produced by *Enterococcus* strains.

Treatments	Bacteriocin activity (AU/mL)		
	E. faecium DP8.3	*E. faecium* DP9.3	*E. mundtii* DP35.1
Control	800	400	400
pH 2.0	800	400	400
pH 3.0	800	400	400
pH 4.0	800	400	400
pH 5.0	800	400	400
pH 6.0	800	400	400
pH 7.0	800	400	400
pH 8.0	800	400	400
pH 9.0	800	400	200
pH 10.0	800	200	100
pH 11.0	800	200	0
Proteinase K	0	0	0
Trypsin	0	0	0
α-Chymotrypsin	0	0	0
Pepsin	400	200	200
α-Amylase	0	0	0
Lipase	200	400	0
Catalase	800	400	400
Lysozyme	400	400	200
100°C 5 min	800	400	400
100°C 10 min	800	200	200
100°C 15 min	800	200	100
100°C 20 min	800	100	100
121°C 15 min	0	0	0

Aslam *et al.* (2011). Catalase treatment did not have any effect on the antibacterial activities of the strains. Based on these findings, the presence of hydrogen peroxide did not contribute to the antibacterial activities observed. Instead, the antibacterial substances produced by the *Enterococcus* strains were found to be protein-based in nature. Multiple studies have indicated that the effectiveness of enterocins diminishes when they are exposed to proteolytic enzymes, likely because of their protein nature (Altınkaynak and Tuncer, 2020; Gök Charyyev *et al.*, 2019; Schelegueda *et al.*, 2015; Zendo *et al.*, 2005).

Heat treatment at 100°C for 5, 10, 15, and 20 min did not affect the antibacterial activity of *E. faecium* DP8.3, while the antibacterial activity of *E. faecium* DP9.3 and *E. mundtii* DP35.1 was partially lost at 100°C for 10, 15, and 20 min (Table 7). Researchers have reported that enterocins did not lose (Altnkaynak and Tuncer, 2020; Gök Charyyev *et al.*, 2019) or partially lost (Sonsa-Ard *et al.*, 2015) their activities with heat treatment at 100°C for 5, 10, 15, and 20 min. The antibacterial activities of the strains were completely lost after a 15-minute heat treatment at 121°C. In line with our findings, Schelegueda *et al.* (2015) and Khalkhali and Mojgani (2017) reported that the antimicrobial substances produced by *E. mundtii* Tw56 and *E. faecium* TA0033 have completely lost their activities after they were heated to 121°C for 15 min. On the basis of these findings, the thermal resistance of the antimicrobial substance formed by *E. faecium* DP8.3 may provide a potential advantage for its application in the production of heat-treated foods.

The antibacterial activity of the bacteriocin produced by *E. faecium* DP8.3 was found to be stable between pH 2.0 and 11.0. In contrast, the bacteriocins synthesized by *E. faecium* DP9.3 and *E. mundtii* DP35.1 exhibited stability within the pH ranges of 2.0–9.0 and 2.0–8.0, respectively. However, their antibacterial activities were decreased or eliminated at high alkaline pH levels (Table 7). Schelegueda *et al.* (2015) reported that the bacteriocin produced by the *E. mundtii* Tw56 strain, isolated from fish, remained stable within the pH range of 2.0–10.0. Similarly, Altınkaynak and Tuncer (2020) reported that the bacteriocin produced by the *E. mundtii* YB6.30 strain was stable within the pH range of 2.0–9.0. Khalkhali and Mojgani (2017) found that the bacteriocins produced by the *E. faecium* TA0033 and *E. faecalis* TA102 strains, isolated from human milk, completely lost their antibacterial activity at pH levels of 10.0 and higher.

Detection of enterocin genes

The PCR analysis revealed that the *E. faecium* DP8.3 (*entA*, *entP*, and *munKS*) and DP9.3 strains (*entA*, *entB*, and *munKS*) contain multiple enterocin genes, while the *E. mundtii* DP35.1 strain has only the *munKS* gene. Comparable to our findings, a previous study reported the presence of multiple enterocin genes in enterococci (Avcı and Özden Tuncer, 2017; Khalkhali and Mojgani, 2017; Nami *et al.*, 2019; Öztürk *et al.*, 2023; Toplu and Özden Tuncer, 2023). Many studies have shown that *E. faecium* strains usually have the *entA*, *entB*, or *entP*

genes, while *E. mundtii* strains usually have the *munKS* gene (Öztürk *et al.*, 2023; Schelegueda *et al.*, 2015; Toplu and Özden Tuncer, 2023). Nevertheless, in line with an exceptionally uncommon finding in existing research, the *munKS* gene was identified in *E. faecium* DP8.3 and DP9.3. Aguilar-Galvez *et al.* (2011) reported the expression of the *munKS* gene for the first time in the *E. faecium* CWBI-B1430 strain isolated from artisan-produced Peruvian cheeses. Subsequently, Öztürk *et al.* (2023) identified the expression of the *munKS* gene in the *E. faecium* HC121.4 and HC161.1 strains from goat colostrum.

Technological properties of bacteriocin-producing *Enterococcus* strains

Calculating the ΔpH values of the strains after 6 hours revealed that *E. faecium* DP9.3 was a slow acid producer (ΔpH 0.98 ± 0.025), while the other strains were moderate acid producers (ΔpH 1.46 ± 0.042 for *E. faecium* DP8.3 and ΔpH 1.03 ± 0.007 for *E. mundtii* DP35.1). After 24 hours, the ΔpH values changed to: *E. faecium* DP8.3, 2.05 ± 0.012; *E. faecium* DP9.3, 1.89 ± 0.006; and *E. mundtii* DP35.1, 1.88 ± 0.031. Consequently, all isolates were classified as fast acid producers based on their ΔpH values after 24 hours. Similarly, Jaouani *et al.* (2015) evaluated *Enterococcus* strains; obtained from foods as weak acid producers after 6 hours. Öztürk *et al.* (2023) reported that enterocin-producing enterococci isolated from sheep and goat colostrum were weak acid producers after 6 hours of incubation. After 24 hours, all strains were determined to be moderate acid producers except for the fast acid producer *E. mundtii* HC166.8.

In addition, none of the *Enterococcus* strains exhibited extracellular proteolytic or lipolytic activity. Jaouani *et al.* (2015) reported that five *Enterococcus* strains (5/22, 23%) showed proteolytic activity, but none presented lipolytic activity. In addition, Öztürk *et al.* (2023) reported that all enterocin-producing strains demonstrated extracellular proteolytic and lipolytic activities. Enterococci with proteolytic and lipolytic activities are effective for flavor formation during the ripening of various kinds of cheeses (Graham *et al.*, 2020). Nevertheless, low lipolytic activity is considered a significant benefit, as only a slight breakdown of milk fat is sufficient to initiate flavor development without causing a rancid taste in cheese (Jaouani *et al.*, 2015). Enterococci generally have limited acidifying and proteolytic activities and are not as important as primary starter cultures in dairy fermentation, especially cheese production (Graham *et al.*, 2020). Based on the observation that bacteriocin-producing *Enterococcus* strains have acid-production abilities but do not have lipolytic or proteolytic activity, these strains should be used as protective adjunct cultures in the food industry.

Safety profiles of the *Enterococcus* strains

Enterococci are naturally present as flora in various traditional fermented foods, and they significantly contribute to the taste and aroma of the food products. In addition, they protect against spoilage and harmful bacteria by producing bacteriocins. Bacteriocin-producing enterococci can be used as a preservative culture in foods, but their safety profiles must be characterized accurately (Jaouani *et al.*, 2015). Therefore, the characteristics of isolates, such as antibiotic resistance, resistance gene expressions, virulence factors, and biogenic amine production, should be examined. In our study, all the strains were susceptible to a broad range of antibiotics (Table 8). Similar to our findings, other researchers have shown that bacteriocin-producing *Enterococcus* strains are sensitive to many important antibiotics used in medicine (Bagci *et al.*, 2019; Gok Charyyev *et al.*, 2019; Iseppi *et al.*, 2019). *E. faecium* DP8.3 displayed resistance to teicoplanin and nitrofurantoin antibiotics, whereas *E. mundtii* DP35.1 exhibited resistance to quinupristin-dalfopristin. Compared with the findings of Jaouani *et al.* (2015) and Haghshenas *et al.* (2016), our findings show that the enterocin-producing DP8.3, DP9.3, and DP35.1 strains were resistant to only a limited number of antibiotics. Although it is important to assess the presence of multiple antibiotic resistance in enterococci for safety evaluation, no such resistance was observed in our isolates. Vancomycin, used as a last resort for the treatment of infections caused by multiple antibiotic-resistant enterococci, has raised concerns because of increasing resistance in recent years (Akpınar Kankaya and Tuncer, 2020). Antibiotics such as linezolid, daptomycin, quinupristin/dalfopristin, and tigecycline are used to treat vancomycin-resistant enterococci (Urban-Chmiel *et al.*, 2022). The fact that the isolates show no resistance to both vancomycin and linezolid is an advantage for the reliability of the strains in this study.

No transferable resistance genes were detected in any of the bacteriocin-producing strains. Similarly, various researchers have reported that certain strains of bacteriocin-producing enterococci do not possess resistance genes (Bagci *et al.*, 2019; Valledor *et al.*, 2022). Increasing the resistance of enterococci to clinically important antibiotics is a major problem worldwide in the treatment of enterococcal infections. Furthermore, the potential transfer of resistance genes from LAB to pathogenic bacteria presents a significant threat to human health (Mathur and Singh, 2005). Therefore, we consider it advantageous that the enterococci in our study are sensitive to clinically relevant drugs and do not harbor antibiotic-resistant genes.

The *E. faecium* DP8.3 and DP9.3 strains showed α-hemolytic activity, while *E. mundtii* DP35.1 had a weak α-hemolytic activity on Columbia agar containing 5% sheep blood. Similarly, Avcı and Özden Tuncer (2017)

Table 8. Antibiotic susceptibility of bacteriocin-producing *Enterococcus* strains.

Antibiotics	Consantration µg/disc	*Enterococcus* strains		
		***E. faecium* DP8.3**	***E. faecium* DP9.3**	***E. mundtii* DP35.1**
Ampicillin	2	S[b]	S	S
Ampicillin	10	S	S	S
Imipenem	10	S	S	S
Tigecycline	15	S	S	S
Penicillin G[a]	10	S	S	S
Vancomycin	30	S	I	I
Teicoplanin	30	R	S	S
Erythromycin	15	I	I	I
Tetracycline	30	S	S	S
Doxycycline	30	S	S	S
Minocycline	30	S	S	S
Ciprofloxacin	5	S	S	S
Levofloxacin	5	S	S	S
Norfloxacin	10	S	S	S
Nitrofurantoin	100	R	S	S
Nitrofurantoin	300	R	S	S
Rifampin	5	S	S	S
Chloramphenicol	30	S	S	S
Quinupristin-dalfopristin	15	S	S	R
Linezolid	30	S	S	S
Gentamycin	120	S	S	S
Streptomycin	300	S	S	S

[a]Penicillin G U/disc
[b]S: Susceptible; R: Resistant; I: Intermediary.

reported that some *E. faecium* strains, which are traditional cheese isolates, have an α-hemolytic activity, which may be weak. Gelatinase activity is also a feature that should be examined in the safety evaluation of isolates. Owing to its ability to break down collagen and some bioactive peptides, gelatinase is thought to help start and spread inflammatory processes in enterococcal infections (Jaouani *et al.*, 2015). Gelatinase activity was not observed in any of the bacteriocin-producing *Enterococcus* strains in the present study. Similar to our results, gelatinase activity was not observed in any of the bacteriocin-producing *E. faecium* (Avcı and Özden Tuncer, 2017; Toplu and Özden Tuncer, 2023) or *E. mundtii* strains (Iseppi *et al.*, 2019; Jaouani *et al.*, 2015). The lack of β-hemolytic and gelatinase activities in bacteriocin-producing *Enterococcus* strains is beneficial for their utilization as starter or adjunct cultures in food manufacturing.

None of the *Enterococcus* strains in this study contained virulence genes, consistent with the findings of Ben Braïek *et al.* (2018) and Nami *et al.* (2019). This result aligns with our phenotypic observations, which showed no gelatinase or hemolytic activities. However, previous studies have shown that *Enterococcus* strains isolated from foods can carry various virulence genes (Avcı and Özden Tuncer, 2017; Chajęcka-Wierzchowska *et al.*, 2016). Detection of virulence factors in food-isolated strains does not necessarily mean that they cause diseases in humans. However, because these factors contribute to infection severity, the isolates have pathogenic potential (Chajęcka-Wierzchowska *et al.*, 2017). The absence of virulence factors in the *Enterococcus* strains benefits their safety.

Tyramine is a biogenic amine that can cause a range of acute effects when ingested in large amounts with food. Lactic acid bacteria are the most efficient producers of tyrosine decarboxylase, the enzyme responsible for tyramine formation (Marcobal *et al.*, 2012). The most effective LAB species for producing tyramine belong to the genus *Enterococcus* (Ladero *et al.*, 2012; Marcobal *et al.*, 2012). The ability to produce tyramine is considered a species characteristic of *E. faecalis*, but it is also extremely common among strains of *E. faecium* and *E. durans* (Ladero *et al.*, 2012). In our study, all *Enterococcus* strains decarboxylated tyrosine. The detection of the

tyrosine decarboxylase gene (*tdc*) in all the strains supports the results of the phenotypic decarboxylase activity tests. Similarly, several researchers have indicated that tyramine is frequently produced in enterococci (Avcı and Özden Tuncer, 2017; Jaouani *et al*., 2015; Valledor *et al.*, 2020) and that tyramine-producing strains also have the *tdc* gene (Avcı and Özden Tuncer, 2017; Jaouani *et al*., 2015).

Conclusions

This study focused on bacteriocin-producing LAB isolated from traditional Turkish cheese samples, emphasizing their technological and safety characteristics. Three isolates with a broad spectrum of activities were selected as study materials, and two of these isolates were *E. faecium* and one was *E. mundtii* according to the 16S rRNA gene homology and species-specific PCR analyses. All the strains showed high inhibition zones against VRE and the *L. monocytogenes* strains. The bacteriocins produced by the strains are heat stable and maintain their antibacterial activities in a wide pH range. *E. faecium* DP8.3 and DP9.3 contained multiple enterocin genes. After a 24-hour incubation period, all *Enterococcus* strains were identified as fast acid producers, but none exhibited any extracellular proteolytic or lipolytic activity. The strains were susceptible to clinically important antibiotics and did not harbor antibiotic resistance or virulence factor genes. All the bacteriocin-producing *Enterococcus* strains decarboxylated tyrosine and showed *tdc* gene expression. Their technological features and safety profiles indicate that these strains are suitable as protective adjunct cultures in the food industry. Further studies should investigate the behavior of these bacteria in food applications.

Author Contributions

The author contributed to the conceptualization, methodology, investigation, original draft writing, and the review and editing of the article.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The author declares no conflicts of interest.

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