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Investigation of antimicrobial activity and *entA* and *entB* genes in *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from naturally fermented Turkish white cheeses

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Abstract In this research, the antagonistic effects of *Enterococcus faecalis* and *E. faecium* bacteria isolated from naturally fermented Turkish white cheeses, produced without starter culture, against *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *Staphylococcus aureus*, and *E. faecalis* were evaluated. The presence of *entA* and *entB* genes was also detected in the isolates, which had antimicrobial activity. Total 71 strains of *E. faecalis* and 7 strains of *E. faecium* were tested; 20 of *E. faecalis* and none of *E. faecium* strains showed antimicrobial activity against the tested bacteria using agar spot method. Among *E. faecalis* strains, which had antimicrobial activity, three strains contained both *entA* and *entB* genes, two strains carried only *entA* gene, and five strains had only *entB* gene. These cheese-sourced enterococcal strains or their enterocins should be considered to be used for food preservation especially in the dairy industry.

Keywords: *Enterococcus*, antimicrobial, bacteriocin, genes, cheese

Introduction

Enterococci, especially *Enterococcus faecium* and *E. faecalis*, affect the characteristic taste and flavor of fermented cheeses because of their enzymatic activities such as protease and lipase (1-4). *E. faecium* K77D strain can be used as starter culture in the fermentation of dairy products. In addition, some strains of enterococci are used as probiotic culture, e.g., *E. faecium* SF 68 and *E. faecium* PR88 strains (2-6). However, some enterococci are recognized as nosocomial pathogen because of their resistance to a wide range of antibiotics, in addition to the presence of virulence factors (3-5,7-9). Therefore, it is recommended that food-borne enterococci should be tested for the presence of virulence genes and resistant characteristics against certain antibiotics (5,10,11).

Enterococcus species, mostly *E. faecium* and *E. faecalis* strains, can synthesize antimicrobial peptides called enterocins from ribosomes (2,4,12,13). Their antimicrobial spectrum includes usually Gram-positive and Gram-negative pathogenic and food-spoilage bacteria (13). The mechanisms of these antibacterial peptides may include pore formation in bacterial cell, damage in cell DNA and 16S rDNA, and inhibition of peptidoglycan synthesis (12).

Enterococci may produce multiple bacteriocins such as enterocins AS-48, L50A, A, B, Q, P, L50B, 1071A, and 1071B (14-16). Enterocins A and B were commonly found in the same isolates (14,15).

In a previous study, the *E. faecium* and *E. faecalis* isolates from naturally fermented Turkish white cheeses, which were obtained from *Canakkale*, *Hatay*, *Balıkesir*, and *Erzurum* regions, were investigated for safety evaluation in terms of resistant characteristics against antibiotics, virulence genes, and plasmid profiles (17). In this study, antimicrobial activity potentials of *E. faecalis* and *E. faecium* isolates against Gram-positive bacteria such as *Listeria monocytogenes*, *L. innocua*, *L. ivonovii*, *Staphylococcus aureus*, and *E. faecalis* were determined. In addition, the presence of *entA* and *entB* genes in *E. faecalis* isolates, which showed antibacterial activity, was detected.

Materials and Methods

Isolation and identification of enterococcal strains In this study, *E. faecalis* (*n*=71) and *E. faecium* (*n*=7) strains were isolated from naturally fermented Turkish white cheeses and identified as species level by morphological, physiological, and biochemical tests (API 20 STREP (bioMérieux, Marcy l'Etoile, France), as well as by 16S rDNA sequencing analysis using with 907r and 27f universal primers in a

previous study (17). The strains were stored at −20°C in 30% glycerol containing Brain Heart Infusion (BHI, Himedia, India) broth.

Determination of potential antimicrobial activity of the strains Antimicrobial activity of *E. faecium* and *E. faecalis* isolates from naturally fermented Turkish cheeses were investigated using agar spot and well diffusion methods against *L. monocytogenes* ATCC 7644, *L. ivanovii* ATCC 19119, *L. innocua* ATCC 33090, *S. aureus* ATCC 6538*, S. aureus* ATCC 25923*,* and *E. faecalis* ATCC 29212.

Agar spot test was performed by spotting 3 μL of activated *E. faecium* or *E. faecalis* isolates to BHI agar surface and incubating aerobically at 37°C for 18–24 h. The cultures were covered with 10 mL of BHI soft agar (0.7% agar) inoculated with 10 μL of the test culture (*L. ivanovii* ATCC 19119*, L. monocytogenes* ATCC 7644, *L. innocua* ATCC 33090*, S. aureus* ATCC 6538*, S. aureus* ATCC 25923*,* or *E. faecalis* ATCC 29212). After incubation, the inhibition zone diameters around the test cultures were evaluated (18).

Cell-free supernatants (CFS), obtained by centrifugation at 12,000× *g* at 4°C for 10 min, were used in the well diffusion methods for the determination of antimicrobial activity of the strains. CFS were neutralized to pH 7.0 with 1 M NaOH, and 30 μL of each CFS was placed into wells containing 70 μL of activated test culture in 10 mL BHI broth containing 0.7% agar. The cultures were incubated aerobically at 37°C for 18–22 h. The clear inhibition zones around the spots and/or wells were examined (19,20).

To determine inhibitory activity due to hydrogen peroxide, 1 μL of catalase enzyme (Sigma-Aldrich, St. Louis, MO, USA) at 1 mg/mL was added into CFS; the residual inhibitory effect was then evaluated by well diffusion technique (21).

To test proteinaceous nature of the inhibitors, the active CFS were subjected to 1 μL of trypsin and proteinase K enzymes (Sigma-Aldrich) at 1 mg/mL at 37°C during 2 h. Then, the activity was detected against test culture. The lack of inhibition zones indicated protease effect to CFS. The heat effect was evaluated by heating the supernatants at 60°C, 100°C, and 121°C for 15 min, following which, the inhibitory effect was checked by the well diffusion technique (21).

Investigation of *entA* and *entB* genes in the strains Antagonistic *E. faecalis* strains (*n*=20) were activated in BHI broth for 24 h at 37°C, and the genomic DNAs of all strains were extracted as described before (17) by phenol–chloroform extraction procedure for the detection of enterocin encoded genes. Enterocin encoded *entA* and *entB* genes were examined using PCR with specific primers listed in Table 1 (21). PCR amplification was performed in 25 μL reaction mixture including 150 ng/mL of DNA, 1X PCR-buffer (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA), 3 mM $MgCl_2$ (Fermentas) for *entA* and 2.5 mM $MgCl_2$ for *entB*, 200 μM dNTPs (Fermentas) 10 pmols of primers (Integrated DNA Technologies, Coralville, IA, USA), and 2.5 U of Taq DNA polymerase (Fermentas). The amplification program was applied at 95°C for 2 min followed by 35 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, followed by a final extension at 72°C for 5 min in Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific). Agarose gel (2%) (Sigma-Aldrich) electrophoresis stained with ethidium bromide (Sigma-Aldrich) was applied to PCR products, and the product size was estimated using a DNA ladder (100 bp) (Fermentas).

Table 1. Specific primers for *entA* and *entB* genes (20)

Enterocin Genes	Oligonucleotide Sequence (5′→3′)	Product Size (bp)
entA	f: AAATATTATGGAAATGGAGTGTAT r: GCACTTCCCTGGAATTGCTC	126
entB	f: GAAAATGATCACAGAATGCCTA r: GTTGCATTTAGAGTATACATTTG	159

Results and Discussion

Turkish white cheese is usually produced from raw or pasteurized milk, with or without the use of starter cultures (22). *Enterococcus* species may be found in the microbial flora of fermented cheeses, indicating fecal contamination or unhygienic conditions during production process (5). Enterococcal strains have a significant role in the flavor development of fermented cheeses owing to their protease and lipase enzymes (5,23).

Enterococci may also generate antimicrobial metabolites, such as bacteriocins called enterocins, hydrogen peroxide, or organic acids (2,4,24). Enterocin A and B are the most common enterocins produced by enterococcal strains (14,15). Franz *et al.* (25) reported that enterocin A can be classified as a class II.1 bacteriocin and showed antimicrobial activity against *Listeria* spp., especially *L. monocytogenes,* and also *Lactobacillus, Enterococcus*, *Pediococccus* spp. Enterocin B was also included in the class II.3 bacteriocins, and it was produced by several enterococcal strains, which produced enterocin A. This enterocin was effective against *Enterococcus* and *Lactobacillus* spp., *L. monocytogenes*, *Staphylococcus aureus*, and *Clostridium perfringens* (14,23).

In this research, none of the *E. faecium* strains showed antimicrobial activity, while 20 *E. faecalis* strains showed antimicrobial activity against tested bacteria. After treatment of CFS of antagonistic *E. faecalis* strains with pH adjustment, catalase, protease, and heat, only 10 strains showed antimicrobial activity against tested bacteria (Table 2). Three strains had antimicrobial activity against *L. monocytogenes* ATCC 7644, while one, five, nine, and seven strains showed antimicrobial activities with different zone diameter against *S. aureus* ATCC 6538, *L. ivanovii* ATCC 19119*, L. innocua* ATCC 33090, and *E. faecalis* ATCC 29212, respectively. Most of the strains (*n*=7) also showed multi-antimicrobial activities against tested food-borne pathogens and food-spoilage bacteria. Most of the antimicrobial activity zones formed against *L. ivanovii* ATCC 19119 and *E. faecalis* ATCC 29212 test bacteria. Enterocin structural *entA* and *entB* genes

Table 2. Safety evaluation and zone diameters of *entA* and/or *entB* positive *E. faecalis* isolates against test bacteria (mm)

Isolate no	*L. monocytogenes* ATCC 7644	*L. innocua* ATCC 33090	*L. ivanovii* ATCC 19119	*E. faecalis* ATCC 29212	*S. aureus* 6538	*entA* gene	*entB* gene	Virulence genes[1)]	Antibiotic resistance[1)2)]
P_{1-3}		8	15	17		+	+	*cpd, cop, ccf, cad, efaAfs, gelE, espfs, espfm*	E_I, VA_I, K_R
P_{1-4}				11		+		*cpd, cop, ccf, cad, efaAfs, gelE, espfs, espfm*	E_R, TE_R, C_R
P_{3-2}		11	11	15	11	+		*agg2, cpd, cop, ccf, cad, efaAfs, gelE, espfs*	E_I, K_I
P_{9-2}			15				+	*agg2, cpd, cop, ccf, cad, efaAfs, gelE, espfs, espfm*	
P_{9-3}		9	15	15			+	*cpd, cop, ccf, cad, efaAfs, gelE, espfs, espfm*	
P_{9-4}			15	17			+	*agg2, cpd, cop, ccf, cad, efaAfs, gelE, espfs, espfm*	
P_{9-5}	8		15			+	+	*cpd, cop, ccf, cad, efaAfs, gelE, espfs, espfm*	
P_{10-5}			13			+	+	*cpd, cop, ccf, cad, efaAfs, gelE, espfs, espfm*	E_I, TE_R
P_{11-3}	11	9	10	15			+	*cpd, cop, ccf, cad, efaAfs, gelE, cylM, espfs, espfm*	E_I
P_{11-4}	15	14	12	15			+	*cpd, cop, ccf, cad, efaAfs, gelE, cylM, espfs, espfm*	E_I, C_I

[1)]These data were taken from a previous study by Ozmen Togay *et al.* (17).

[2)]E_R, Erythromycin resistance; E_I, Intermediate level erythromycin resistance; K_R, Kanamycin resistance; K_I, Intermediate level kanamycin resistance; TE_R, Tetracycline resistance; TE_I, Intermediate level tetracycline resistance; VA_I, Intermediate level vancomycin resistance; C_R, Chloramphenicol resistance; C_I, Intermediate level chloramphenicol resistance.

were determined in 10 of *E. faecalis* strains, which had antimicrobial activity (Fig. 1). Three of *E. faecalis* strains (P_{1-3}, P_{9-5}, P_{10-5}) had both of *entA* and *entB* genes, while two of *E. faecalis* strains carried only *entA* gene; five of the strains had only *entB* gene (Table 2). *E. faecalis* P_{1-3}, *E. faecalis* P_{9-5}, and *E. faecalis* P_{10-5} strains showed antimicrobial activity against *Listeria inocua* ATCC 33090, *L. ivanovii* ATCC 19119, and *E. faecalis* ATCC 29212; *L. monocytogenes* ATCC 7644 and *Listeria ivanovii* ATCC 19119; and *L. ivanovii* ATCC 19119, respectively. *E. faecalis* P_{3-2}, *E. faecalis* P_{11-3}, and *E. faecalis* P_{11-4} strains presented the widest range of antimicrobial activity against tested food-borne pathogen and food-spoilage bacteria. Some studies report on the determination of antimicrobial activity potential and enterocin-encoding genes in *Enterococcus* species isolated from cheese samples (12-14,26-29). Silvetti *et al.* (13) reported antagonistic effect against *L. monocytogenes* of *E. faecalis* strains isolated from Valtellina Casera cheese, Formagèla Valseriana cheese, and Formaggella Val di Scalve cheese samples obtained in Italy. Renye *et al.* (27) also mentioned antilisterial activity and enterocin-encoded genes (*entA*, *entP, entL50AB,* and *entB*) in *E. faecium* isolates from cheeses. Similarly, 3 of *E. faecalis* isolates contained multiple enterocin structural genes (*entA* and *entB*), and these isolates showed antagonistic effect against *Listeria* spp. (*L. monocytogenes*, *L. innocua*, and *L. ivanovii*) (Table 2). Tuncer (28) also found three of *E. faecium* and one of *E. durans* isolates as bacteriocin producer from Turkish Tulum cheese samples, and these strains usually showed antimicrobial activity against *L. innocua*. Favaro *et al*. (12) determined bacteriocinogenic *E. faecium* strains from homemade Bulgarian white brine cheese. The bacteriocin-encoding genes (*entA*, *entB*, *entP*, and *entL50B*) and the inhibitory spectrum of the strains were evaluated, which showed antilisterial activity. Veljovic *et al.* (29) detected enterocin production and antimicrobial activity against *L. monocytogenes* in two of *E. faecalis* isolates from cheeses.

In the present study, the *E. faecalis* strains that carried *entA* and/or *entB* genes could not show antimicrobial activity against all tested Gram-positive pathogen bacteria such as *Listeria monocytogenes*, *E. faecalis*, and *S. aureus*. Ineffective expression of antimicrobial characteristics in phenotypical assay and expression of genes encoding bacteriocins was dependent on many factors related to growth conditions (13). It was thought that a lack expression of *entA* and *entB* genes in these *E. faecalis* isolates occurred or the isolates carried incomplete or nonfunctional bacteriocin genes or produced additional bacteriocins except *entA* and *entB* as mentioned by Abriouel *et al.* (30).

Antagonistic *E. faecalis* strains that had *entA* and *entB* genes in this study could carry some virulence genes and showed high and/or

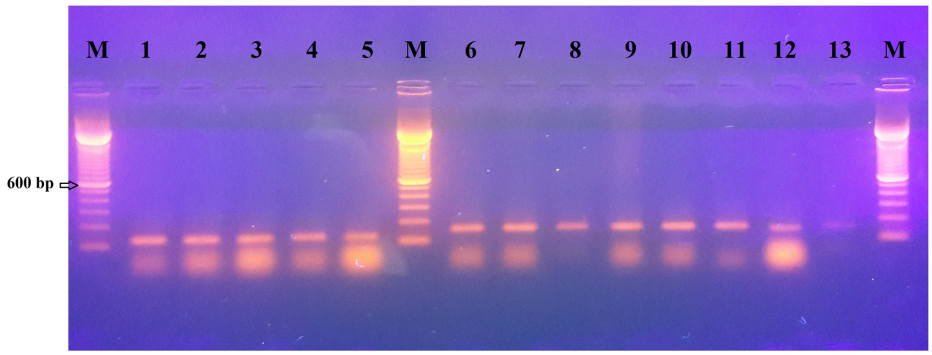

Fig. 1. PCR amplification of enterocin structural genes in *E. faecalis* strains isolated from cheese (*entA* positive strains: 1. P_{1-3}, 2. P_{1-4}, 3. P_{3-2}, 4. P_{9-5}, 5. P_{10-5}; *entB* positive strains: 6. P_{1-3}, 7. P_{9-2}, 8. P_{9-3}, 9. P_{9-4}, 10. P_{9-5}, 11. P_{10-5}, 12. P_{11-3}, 13. P_{11-4}).

intermediate level resistance characteristics against some antibiotics such as erythromycin, kanamycin, vancomycin, tetracycline, and chloramphenicol, based on the results in a previous study (17) (Table 2).

The *cpd, ccf, cob,* and *cad* genes coding for sex pheromones, the *efaAfs* gene for a cell wall adhesin, the *gelE* gene coding for extracellular metalloendopeptidase in enterococci, and at least one of the surface-protein-encoded genes (*espfs* and *espfm*) were present in all *entA*- and *entB*-encoded *E. faecalis* isolates. Only two *E. faecalis* strains (P_{11-3} and P_{11-4}) had *cylM* gene, which was one of the cytolysin coding genes (*cylA*, *cylB*, and *cylM*) (Table 2). Özden Tuncer *et al.* (14) and Eaton and Gasson (31) mentioned that the sex pheromones were not considered as virulence factors. Semedo *et al.* (32) stated that the *efaAfs* gene was commonly found in enterococci. In the previous study (17) none of the *E. faecalis* isolates showed β-haemolytic activity, although some isolates carried haemolysin-related genes (*cylM, cylB,* and *cylA*). As mentioned in the previous study, the cytolysin determinants (*cylA, cylB,* and *cylM*) might be the silent genes (31,32). In addition, *E. faecalis* strains which have enterocin encoding (*entA* and *entB*) genes and having antagonistic effect, were mostly found susceptible to tested antibiotics; moreover, vancomycine resistance encoding *vanA* and *vanB* genes were not detected in any isolate. However, some strains were found resistant to certain antibiotics (17). There were some studies about bacteriocinogenic enterococci that carried virulence determinants and resistant to certain antibiotics (16,33). Sanchez Valenzuela *et al.* (33) reported two bacteriocin producers and enterocin gene-encoded (*entA*, *entP*, *entB*, and *ent107*) *E. faecalis* isolates from cheese samples that carried virulence determinants and were resistant to some antibiotics such as erythromycin, tetracycline, rifampicin, ciprofloxacin, and levofloxacin. In another study, Moraes *et al.* (16) determined that most of enterocin gene encoded (*entA*, *entB*, *entP*, *entAS48*, and *entL50AB*) *Enterococcus* spp. strains isolated from raw milk and cheese samples were found to be positive for numerous tested virulence genes primarily *efaA*, *asa1*, and *gelE*. Moraes *et al.* (16) emphasized that these bacteriocin producer enterococcal isolates had application potential for food preservation; however, these isolates demonstrated the contradictory characteristics because of their virulence potentials.

To conclude, antimicrobial activity, bacteriocin-producing potential, and safety of enterococci are dependent on strain. Although some of *entA* and *entB* encoding *E. faecalis* strains may have potential risk factors for food safety, such as certain virulence determinants and antibiotic resistance characteristics, the enterocins produced by these isolates may be useful for food preservation. New enterocin producer strains from naturally fermented Turkish white cheeses were found in this study. Further studies are needed to investigate the purification of the enterocins from these isolates.

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