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Safety Evaluation of Enterocin Producer *Enterococcus* sp. Strains Isolated from Traditional Turkish Cheeses

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Abstract

The purpose of this study was to determine the antimicrobial activity and occurrence of bacteriocin structural genes in *Enterococcus* spp. isolated from different cheeses and also investigate some of their virulence factors. *Enterococcus* strains were isolated from 33 different cheeses. *Enterococcus faecium* (6 strains) and *Enterococcus faecalis* (5 strains) enterocin-producing strains were identified by 16S rDNA analyses. Structural genes *ent*A, *ent*B, *ent*P and *ent*X were detected in some isolates. Multiple enterocin structural genes were found in 7 strains. None of the tested enterococci demonstrated anyβ-haemolytic activity and only one strain had gelatinase activity. Six strains showed multiple antibiotic resistance patterns and in addition, *vanA* and several virulence genes were detected in many strains. Only *E. faecalis* MBE1-9 showed tyrosine decarboxylase activity and *tdc* gene was detected only in this strain.

Key words: *Enterococcus* sp. from cheeses, antibiotic resistance of food borne enterococci bacteriocin production ability, virulence factors

Introduction

Enterococci are an important group of lactic acid bacteria (LAB) that inhabit the digestive tract of humans and animals as well as the surface of waters, soil and plants. They are also found in foods, especially of animal origin, such as traditional cheeses produced in different European and Mediterranean countries (Manolopoulou *et al.*, 2003; Yogurtcu and Tuncer, 2013). *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* are the most frequently isolated strains from traditional cheeses, and they play an important role for ripening of these cheeses based on their proteolysis, lipolysis, citrate breakdown abilities and hence they promote product's unique typical taste and flavor formation (Giraffa, 2002; 2003; Moreno *et al.*, 2006). They can also inhibit the growth of the food-borne pathogens and spoilage microorganisms by producing bacteriocin known as enterocins. The frequently encountered enterocins produced by enterococci strains are enterocins A, B, P, AS-48, L50A, L50B, 1071A, 1071B, and Q (Moreno *et al.*, 2006; Nes *et al.*, 2007; Edalatian *et al.*, 2012; Özden-Tuncer *et al.*, 2013).

Although enterococci have desirable technological and metabolic traits, in recent years they have been found to be associated with clinical infections such as endocarditis, bacteremia and urinary tract. Therefore, enterococci have emerged as an important nosocomial pathogen in recent decades (Franz *et al.*, 2003; Moreno *et al.*, 2006; Peters *et al.*, 2007; Lindenstrauß *et al.*, 2011). Several putative virulence factors have been described in enterococci, such as gelatinase (*gel*E), cell wall adhesin (*efa*A_{fm} and *efa*A_{fs} from *E. faecium* and *E. faecalis*, respectively), sex pheromones (*cpd*, *cob*, *ccf*, and *cad*), collagen adhesin (*ace*), enterococcal surface protein (esp_{fm} and esp_{fs} from *E. faecium* and *E. faecalis*, respectively), aggregation substance (*agg*), and cytolysin (*cyl*M, *cyl*B, and *cyl*A) (Vankerckhoven *et al.*, 2004; Cariolato *et al.*, 2008; Chuang *et al.*, 2009; Barbosa *et al.*, 2010; Ben Belgacem *et al.*, 2010; Lindenstrauß *et al.*, 2011; Yogurtcu and Tuncer, 2013). In addition, enterococci are resistant to several antibiotics due to intrinsic and/or acquire genes located on the chromosomal DNA, plasmids or transposons. The interesting point about antibiotic resistance of enterococci is the increasing number of vancomycin and multiple antibiotic-resistant strains. The reason for the increase in multiple antibiotic resistance in enterococci is transfer of genetic elements carrying antibiotic resistance genes between pathogenic and non-pathogenic bacteria in the human or animal intestinal tract or even in food (Riboldi *et al.*, 2009; Sparo *et al.*, 2011).

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Although the technological and metabolic activity of enterococci is essential for some cheeses, the presence of virulence factors and antibiotic resistance in enterococci should be investigated due to their ability of transferring virulence genes and antibiotic resistance determinants through the food chain. In recent years, virulence factors and antibiotic resistance of clinical isolates were mostly examined, but every day interest in food-borne enterococci and their pathogenity is increasing. From these perspectives, the aim of this study was to isolate enterocin producing enterococci strains from various traditional artisanal cheeses in Turkey, and to determine their enterocin genes, antibiotic resistance patterns and virulence factors. Enterocin production, antibiotic resistance and virulence determinants of enterococci isolated from some cheese types in this study were investigated for the first time.

Experimental

Materials and Methods

Cheese samples. Cheese samples were obtained from various provinces (Denizli, Isparta, Balıkesir, Bolu, Kayseri, Van and Manisa) of Turkey and it has been noted that they are especially homemade. A total of 33 cheeses samples, including traditional Turkish white cheese, Tulum cheese, Manyas Kelle and Van Otlu (Herby) cheese made from cow's, sheep's and goat's milk were used in the present study.

Isolation and phenotypically identification of *Enterococcus* sp. strains. Ten grams of each cheese sample were homogenized in 90 ml sterile saline solution (0.85% NaCl, w/v) with waring blender (8011 ES HGB2WTS3, Torrington, Connecticut, 06790, USA). Serial decimal solutions were prepared in saline solution and spread onto Kanamycin Easculine Azide Agar (Lab M, Ltd., Bury, Lancashire, U.K.) and incubated at 37°C for 48 h. Over an incubation period black colonies of presumptive *Enterococcus* sp. strains were randomly picked up and grown in de Man Rogosa Sharpe (MRS) broth medium (Lab M) at 37°C for 24 h. A total of 100 isolates were identified to the genus level by Gram staining, catalase and cultural tests such as growth in MRS broth (10°C, 37°C, 45°C and pH 9.6), tolerance of 6.5% (w/v) NaCl and resistance to heat (60°C for 30 and 60 min).

Detection of bacteriocin production ability. Antimicrobial activities of the isolates were evaluated towards selected indicator bacteria by using the toothpick method as described by van Belkum *et al.* (1989). Briefly, overnight presumptive *Enterococcus* sp. strains were grown on MRS solid medium at 37°C for 18 h. After incubation time, sterile toothpicks were used to ensure create spots on MRS agar. After the incubation period, an indicator lawn of 5 ml of appropriate soft agar (0.5%, w/v), containing 100 µl indicator bacteria was poured onto the surface and incubated at the required temperature for indicators for 18 h. After incubation, all inhibition zones around the colonies were examined and the zones were measured. The growth medium and incubation temperature of the indicator bacteria are listed in Table I. Stock cultures in appropriate medium with 20% glycerol were stored at –20°C.

The protein nature of antimicrobial substances produced by isolates was determined by proteinase K enzyme assay. The 20 µl proteinase K (20 mg/ml) was dropped near 1 cm of the colonies and then the plates were incubated for 1 h at 37°C. Finally, indicator bacteria were poured onto plate with 5 ml soft agar and incubated at an optimum growth temperature for the indicators. After the incubation period, diminishing of inhibition zones shaped like a half-moon was considered as an indication that the antimicrobial agent produced by *Enterococcus* sp. isolates was affected by the enzyme proteinase K and antimicrobial substances were recognized as bacteriocin (enterocin).

Genomic DNA extraction. Genomic DNA's of bacteriocin producing isolates were extracted from 0.5 ml of overnight cultures using the method of Cancilla *et al.* (1992). DNA precipitates were resuspended in 50 µl Tris-EDTA buffer (pH 8.0).

Identification of enterocin producer isolates. 16S rDNA gene sequence of the isolates were amplified with following specific primers: pA (forward) 5'-AGA GTT TGA TCC TGG CTC AG-3' and pE' (reverse) 5'-CCG TCA ATT CCT TTG AGT TT-3' (Edwards *et al.*, 1989) using in a programmable DNA thermocycler (Techne TC3000, Cambridge, U.K.). Fifty microliter PCR reaction mixture consisted of 3 µl of the bacterial DNA solution, 1 µl of each primer, 20 µl nuclease-free water and 25 µl PCR master mix (Fermentas, Vilnius, Lithuania) and following PCR conditions were ensured: initial denaturation cycle at 94°C for 2 min, next 30 cycles of denaturation 94°C for 30 s, annealing at 55°C for 60 s and elongation at 72°C for 90 s, and a final extension cycle at 72°C for 10 min. Amplification products were separated by electrophoresis on 1% agarose (w/v) gels at 85 V for 1.5 h in Tris-acetate-EDTA buffer. Agarose gels were stained with ethidium bromide (20 µg/ml) and photographed under ultraviolet light using a Nikon D5100 digital camera (Nikon Corp., Japan). The size of amplicons was determined by comparison with O'GeneRuler 100-bp DNA ladder (Fermentas). Sequencing of the 16S rDNA gene was performed in RefGen (ODTU Technocity, Ankara, Turkey). The 16S rDNA homology searches were carried out using BLAST software against the NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) database.

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Table I
Growth medium and incubation temperatures of indicator bacteria, and inhibitory spectrum of *Enterococcus* sp.strains

Indicator strains	Growth medium* and incubation temperature	Inhibition zone (Ø mm)										
		MBE 1–9	MBE 12–3	MBE 15–2	MBE 16–5	MBE 20–5	MBE 22–2	MBE 27–2	MBE 27–3	MBE 29–1	MBE 29–3	MBE 31–4
Lactobacillus plantarum LMG2003	MRS, 37°C	–	–	–	–	–	–	–	–	–	–	–
Lactococcus lactis subsp. *lactis* 1	GM17, 30°C	–	–	–	–	–	–	–	–	–	18	–
Lactococcus lactis subsp. *lactis* 731	GM17, 30°C	7	9	12	16	–	–	13	10	–	10	–
Lactococcus lactis subsp. *lactis* T1	GM17, 30°C	16	13	14	–	–	–	14	13	–	15	–
Lactococcus lactis subsp. *lactis* 105	GM17, 30°C	–	–	–	–	–	–	–	–	–	–	–
Listeria innocua LMG2813	LB, 30°C	7	9	8	6	4	5	9	9	4	6	4
Listeria monocytogenes ATCC15813	LB, 30°C	4	13	17	7	3	5	13	13	3	7	4
Listeria monocytogenes ATCC19115	LB, 30°C	13	5	10	9	6	7	10	6	4	14	6
Listeria monocytogenes ATCC7644	LB, 30°C	12	23	24	10	7	9	26	23	6	11	6
Staphylococcus aureus ATCC25923	TSB, 37°C	7	7	10	9	6	7	9	7	6	8	8
Staphylococcus aureus FRI1003022	TSB, 37°C	7	6	6	5	4	6	7	6	5	6	4
Staphylococcus carnosus LMG2709	TSB, 37°C	8	5	5	6	5	6	7	6	5	15	6
Staphylococcus aureus ATCC29213	TSB, 37°C	11	13	10	15	12	11	10	12	10	12	14
Enterococcus faecalis ATCC29212	GM17, 37°C	8	14	10	6	–	5	8	14	5	8	6
Enterococcus faecalis LMG2708	GM17, 37°C	–	10	–	–	–	–	–	8	–	–	–
Enterococcus faecalis LMG2602	GM17, 37°C	–	15	–	–	–	–	–	–	–	13	–
Bacillus cereus ATCC10876	TSB, 37°C	6	7	5	7	6	6	6	7	5	6	6
Bacillus cereus LMG2732	TSB, 37°C	17	5	16	–	–	–	16	–	–	18	–
Salmonella Typhimurium SL1344	LB, 37°C	10	15	16	14	14	9	12	13	14	17	8
Salmonella Enteritidis ATCC13076	LB, 37°C	7	7	8	7	8	5	16	6	5	7	5
Salmonella Typhimurium ATCC14028	LB, 37°C	–	8	4	4	4	6	–	8	–	–	5
Escherichia coli LMG3083 CFAI (ETEC)	LB, 37°C	5	6	6	4	–	–	6	7	–	7	–
Pediococcus pentosaceus LMG2001	TSB, 37°C	19	15	16	–	–	–	17	14	5	18	–

* MRS: de Man Rogosa Sharpe, GM17: M17 with glucose (5%), LB: Luria Bertani, TSB: Triptic Soy Broth

Table II
PCR primers, annealing temperatures and product sizes for detection of bacteriocin*, virulence** and amino acid decarboxylase*** genes.

Gene	Primer sequence (5’ to 3’)	Annealing temperature (°C)	Product size (bp)	Reference
**ent*A	AAATATTATGGAAATGGAGTGTAT GCACTTCCCTGGAATTGCTC	56	126	Yousif *et al.* 2005
**ent*B	GAAAATGATCACAGAATGCCT A GTTGCATTTAGAGTATACATTTG	50	162	Yousif *et al.* 2005
**ent*P	TATGGTAATGGTGTTTATTGTAAT ATGTCCCATACCTGCCAAAC	50	120	Yousif *et al.* 2005
**entL50A/B*	TGGGAGCAATCGCAAAATTAG ATTGCCCATCCTTCTCCAAT	52	98	Ben Belgacem *et al.* 2010
**bac31*	TATTACGGAAATGGTTTATATTGT TCTAGGAGCCCAAGGGCC	50	123	Yousif *et al.* 2005
**ent*AS48	GAGGAGTTTCATGATTTAAAGA CATATTGTTAAATTACCAAGCAA	50	340	Yousif *et al.* 2005
**entQ*	ATGAATTTTCTTCTTAAAAATGGTATCGCA TTAACAAGAAATTTTTTCCCATGGCAA	56	105	Ben Belgacem *et al.* 2010
**ent1071A/B*	CCTATTGGGGGAGAGTCGGT ATACATTCTTCCACTTATTTTT	51	343	Ben Belgacem *et al.* 2010

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Table II continued

Gene	Primer sequence (5' to 3')	Annealing temperature (°C)	Product size (bp)	Reference
**cyl*L$_{L/S}$	GTGTTGAGGAAATGGAAGCG TCTCAGCCTGAACATCTCCAC	60	324	Brandao *et al.* 2010
**ent*X	GTTTCTGTAAAAGAGATGAAAC CCTCTTAATCATTAACCATAC	50	500	Edalatian *et al.* 2012
***efa*A$_{fm}$	AACAGATCCGCATGAATA CATTTCATCATCTGATAGTA	54	735	Reviriego *et al.* 2005
***efa*A$_{fs}$	GACAGACCCTCACGAATA AGTTCATCATGCTGTAGTA	54	705	Reviriego *et al.* 2005
***ccf*	GGGAATTGAGTAGTGAAGAAG AGCCGCTAAAATCGGTAAAAT	54	543	Reviriego *et al.* 2005
***cpd*	TGGTGGGTTATTTTTCAATTC TACGGCTCTGGCTTACTA	54	782	Reviriego *et al.* 2005
***cob*	AACATTCAGCAAACAAAGC TTGTCATAAAGAGTGGTCA	54	1405	Reviriego *et al.* 2005
***esp*$_{fm}$	TTGCTAATGCAAGTCACGTCC GCATCAACACTTGCATTACCGAA	54	955	Reviriego *et al.* 2005
***esp*$_{fs}$	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGAA	54	933	Reviriego *et al.* 2005
***ace*	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCG	54	350	Ben Belgacem *et al.* 2010
***gel*E	ACCCCGTATCATTGGTT ACGCATTGCTTTTCCATC	54	419	Reviriego *et al.* 2005
***cad*	TGCTTTGTCATTGACAATCCG ACTTTTTCCCAACCCCTCAA	54	1299	Reviriego *et al.* 2005
***agg*	AAGAAAAAGAAGTAGACCAAC AAACGGCAAGACAAGTAAATA	56	1553	Eaton and Gasson 2001
***cyl*A	TGGATGATAGTGATAGGAAGT TCTACAGTAAATCTTTCGTCA	54	517	Reviriego *et al.* 2005
***cyl*B	ATTCCTACCTATGTTCTGTTA AATAAACTCTTCTTTTCCAAC	54	843	Reviriego *et al.* 2005
***cyl*M	CTGATGGAAAGAAGATAGTAT TGAGTTGGTCTGATTACATTT	54	742	Reviriego *et al.* 2005
****hdc*	GGNATNGTNWSNTAYGAYMGNGCNGA ATNGCDATNGCNSWCCANACNCCRTA	53	372	de las Rivas *et al.* 2006
****tdc*	TGGYTNGTNCCNCARACNAARCAYTA ACRTARTCNACCATRTTRAARTCNGG	53	825	de las Rivas *et al.* 2006
****odc*	TWYMAYGCNGAYAARACNTAYTTYGT ACRCANAGNACNCCNGGNGGRTANGG	53	1440	de las Rivas *et al.* 2006
****ldc*	CAYRTNCCNGGNCAYAA GGDATNCCNGGNGGRTA	53	1185	de las Rivas *et al.* 2006

PCR primers, annealing temperatures and product sizes for detection of bacteriocin*, virulence** and amino acid decarboxylase*** genes
Y: C or T; R: A or G; W: A or T; S: C or G; M: A or C; D: A, G or T.

Detection of enterocin genes. The presence of enterocin-encoding genes was detected according to Edalatian *et al.* (2012), by using the most common enterocin primers. Interaction primers, annealing temperatures and the size of amplicons are given in Table II. PCR for enterocin genes were performed using the following parameters: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at an appropriate temperature for 1 min, and elongation at 72°C for 40 s and final extension at 72°C for 10 min. PCR products were analyzed by agarose gel (1.5%, w/v) electrophoresis with reference to O'GeneRuler 100-bp DNA ladder (Fermentas), the bands stained with ethidium bromide and photographed with a Nikon D5100 digital camera under ultraviolet light. *E. faecium* EYT31 strain (*ent*A^{+}, *ent*B^{+} and *ent*P^{+}) obtained from Özden-Tuncer *et al.* (2013), was used as positive control.

Cross-protection activity test. The purpose of this assay was to detect the susceptibility of each strain to

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antimicrobial substances produced by all others. Consequently, enterococci strains were used as producers and indicators. Producer bacteria were spotted on appropriate solid media and then enterocin producer strains were poured onto plates with soft MRS agar as indicators. After incubation at 37°C for 18 h inhibition zones were examined.

Haemolytic and gelatinase activity. *Enterococcus* sp. strains were grown in MRS broth medium at 37°C for 18 h and then streaked onto Columbia Agar (Laboratorios Conda S.A., Madrid, Spain) containing 5% defibrinated sheep blood for determining haemolytic activity. After an incubation period (37°C for 48 h) presence of zones of clearing around the colonies were interpreted as β-haemolysis. The absence of zones around the colonies or green zones around the colonies was interpreted as γ-haemolysis and α-haemolysis, respectively (Cariolato *et al.*, 2008). Gelatinase production abilities of the enterocin producer strains were investigated on Todd-Hewitt agar presence of gelatinase (30 g/l). Briefly, strains were grown in MRS broth medium (at 37°C for 24 h) then streak onto Todd-Hewitt agar (Liofilchem, Roseto degli Abruzzi, Italy). After incubation at 37°C for 24 h, petri dishes were placed 4°C for 5 h before examination for the zone of turbidity around the colonies, indicating hydrolysis (Eaton and Gasson, 2001).

Antibiotic susceptibility. Antibiotic susceptibility of enterocin producer *E. faecium* and *E. faecalis* strains were determined by disc diffusion method on Mueller Hinton agar (Lab M) plates according to the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2012). The antibiotics used in this study were as follows: doxycycline (30 µg), norfloxacin (10 µg), vancomycin (30 µg), chloramphenicol (30 µg), rifampicin (5 µg), minocycline (30 µg), penicillin (10 µg), streptomycin (300 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), ampicillin (10 µg), quinupristin-dalfopristin (15 µg), nitrofurantoin (300 µg), tetracycline (30 µg), gentamicin (120 µg), erythromycin (15 µg), linezolid (30 µg) and teicoplanin (30 µg).

Presence of *van*A and *van*B genes. *E. faecium* and *E. faecalis* strains were searched for the presence of *van*A and *van*B genes with the following primers: VanA1 [5'-GGG AAA ACG ACA ATT GC-3'] and VanA2 [5'-GTACAA TGC GGC CGT TA-3'] for *van*A gene (Dutka-Malen *et al.*, 1995) and VanB [5'-GTG CTG CGA GAT ACC ACA GA-3'] and VanBrev [5'-CGA ACA CCA TGC AAC ATT TC-3'] for *van*B gene (Reviriego *et al.*, 2005). Product size of 732 bp and 1,145 bp were screened for *van*A and *van*B genes, respectively. PCR conditions were performed using the following cycling parameters: initial denaturation cycle at 94°C for 2 min, next 30 cycles of denaturation 94°C for 1 min, annealing at 54°C for 1 min and elongation at 72°C for 1 min, and a final extension cycle at 72°C for 10 min. Electrophoresis of the amplification products was performed on 1.5% (w/v) agarose gel.

Detection of virulence factors. Presence of the *gel*E (gelatinase), *efa*A_{fm} and *efa*A_{fs} (cell wall adhesin for *E. faecium* and *E. faecalis*, respectively), *cpd*, *cob*, *ccf* and *cad* (sex pheromones), *ace* (collagen adhesin), esp_{fm} and esp_{fs} (cell wall-associated protein in *E. faecium* and *E. faecalis*, respectively), *agg* (aggregation substance), *cyl*M, *cyl*B and *cyl*A (cytolysin) genes were investigated by PCR. PCR primers, annealing temperatures and product sizes for detection of virulence factors are listed in Table II. PCR conditions were performed using the following cycling parameters: initial denaturation cycle at 95°C for 5 min, next 35 cycles of denaturation 95°C for 30 s, annealing at an appropriate temperature for 30 s and elongation at 72°C for 1 min, and a final extension cycle at 72°C for 10 min. Electrophoresis of the amplicons was performed on 1.5% (w/v) agarose gel.

Detection of decarboxylase activity and amino acid decarboxylase genes. The amino acid decarboxylase activity of *Enterococcus* sp. strains was estimated according to the method proposed by Bover-Cid *et al.* (1999). Briefly 5 g of amino acid precursors (histidine, lysine, ornithine and tyrosine (Merck) was added to 1 l decarboxylase screening medium. Enterococcal cultures were spotted onto these plates with and without amino acids and incubated at 37°C for 2–5 days. After incubation time, the decarboxylation activity of the *Enterococcus* sp. strains was determined phenotypicaly by yellow to purple color changes on the petri dishes. Tyraminogenic *E. faecium* NYE54 strain obtained from Inoglu and Tuncer (2013) was used as the positive control.

PCR amplification of histidine, lysine, ornithin and tyrosine decarboxylase genes (*hdc*, *ldc*, *odc* and *tdc*, respectively) were carried out based on the method of de las Rivas *et al.* (2012). PCR primers, annealing temperatures and product sizes for detection of amino acid decarboxylase genes are listed in Table II. PCR conditions were performed using the following cycling parameters: initial denaturation cycle at 95°C for 10 min, next 30 cycles of denaturation 95°C for 30 s, annealing at an appropriate temperature for 30 seconds and elongation at 72°C for 2 min, and a final extension cycle at 72°C for 20 min. Electrophoresis of the amplicons was performed on 1.5% (w/v) agarose gel. *E. faecium* NYE54 strain (*tdc*$^{+}$) (Inoglu and Tuncer, 2013) was used as the positive control.

Results

Isolation and phenotypic identification of *Enterococcus* sp. strains. A total of 100 presumptive *Enterococcus* strains isolated from 33 different cheese samples

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on KAA medium and grown in MRS broth. All isolates were Gram-positive, catalase-negative and grown at 10°C, 37°C and 45°C, in the presence of NaCl (6.5%) and at pH 9.6 in MRS broth medium. In addition, all of them were found to be resistant to heat at 60°C for 30 and 60 min.

Detection of bacteriocin production ability. Antimicrobial activities of the isolates were evaluated towards selected indicator bacteria by using toothpick method. The 11 presumptive *Enterococcus* sp. isolates showed inhibition zones against different indicator microorganisms (Table I). The Proteinase K treatment showed that antimicrobial substances of 11 isolates have proteinaceous nature. The isolates were given inhibition zones with different diameter against all *Listeria* and *Staphylococcus* sp. strains tested in this study. On the other hand, inhibition of some Gram negative bacteria (*Salmonella* Typhimurium and *E. coli*) by *Enterococcus* sp. strains was evaluated as a striking result. Based on the obtained results these isolates were considered to be enterocin producers.

Identification of enterocin producer isolates. Presumptive *Enterococcus* sp. strains were identified genotypically by 16S rDNA homology. Sequence similarity of an approximately 900-bp PCR amplification products were determined by BLAST software. The 11 enterocin producer isolates were identified as *E. faecalis* (5) and *E. faecium* (6) with the similarity percentage of 93–99% when 16S rDNA PCR amplicons were compared to GenBank.

Detection of enterocin genes. Enterocin genes were detected by using most common enterocin primers. *E. faecalis* MBE1-9 and *E. faecalis* MBE20-5 strains were not give application with the enterocin primers. The *ent*A gene was detected in 7 strains while 8 strains carried *ent*X gene. The e*nt*B and *ent*P genes were detected in 4 and 2 strains, respectively. The genes coding for enterocin L50A/B, bacteriocin 31, enterocin AS-48, enterocin Q, enterocin 1071 and enterocin *cyl*$L_{L/S}$ were not detected in any *Enterococcus* sp. strains (Table III).

Cross-protection activity test. Based on the data obtained from cross-protection test, all of the *Enterococcus* sp. strains were not inhibited by their enterocins when used as an indicator. *Enterococcus* sp. strains used in the present study showed resistance to own bacteriocins as expected. Observations of cross reaction are given in Table IV.

Haemolytic and gelatinase activity. Three of the strains (*E. faecium* MBE27-2, MBE27-3 and *E. faecalis* MBE29-3) showed α-haemolytic activity while another 4 strains (*E. faecalis* MBE1-9, MBE29-1, MBE31-4 and *E. faecium* MBE12-3) had weak α-haemolytic activity. Non-haemolytic activity was detected in *E. faecium* MBE15-2, MBE16-5, MBE22-2 and *E. faecalis* MBE20-5. Only *E. faecalis* MBE29-3 strain showed gelatinase activity on Todd-Hewitt agar.

Antibiotic susceptibility profiles of *Enterococcus* sp. strains. Antibiotic susceptibility profiles of enterocin producer strains against 18 antibiotics are shown as R (Resistant), I (Intermediate) and S (Sensitive) in Table V. All of the strains were found to be sensitive against gentamicin, teicoplanin, doxycycline, ampicillin, nitrofurantoin, linezolid and levofloxacin while 6 of the 11 *Enterococcus* strains showed multiple antibiotic resistance patterns. All strains found to be susceptible to vancomycin except *E. faecalis* MBE29-1 which has intermediary resistance. Resistance to streptomycin, norfloxacin, tetracycline and chloramphenicol was observed in different strains. The highest resistance rate of 72.72% was found against rifampicin.

Table III
Presence of enterocin genes in the *Enterococcus* sp. strains.

Strains	Source	Structural genes of enterocins									
		xxxxxxx	*entB*	*entP*	*ent1071*	*ent50A/B*	*bac31*	*entAS48*	*entQ*	*entX*	*cylL*$_{L/S}$
E. faecalis MBE1-9	Tulum cheese (sheep milk-Denizli)	–	–	–	–	–	–	–	–	–	–
E. faecium MBE12-3	White cheese (cow milk-Denizli)	+	+	–	–	–	–	–	–	–	–
E. faecium MBE15-2	White cheese (goat milk-Denizli)	+	+	–	–	–	–	–	–	+	–
E. faecium MBE16-5	White cheese (cow milk-Denizli)	+	–	–	–	–	–	–	–	+	–
E. faecalis MBE20-5	Tulum cheese (sheep milk-Denizli)	–	–	–	–	–	–	–	–	–	–
E. faecium MBE22-2	Tulum cheese (sheep milk-Kayseri)	+	–	+	–	–	–	–	–	+	–
E. faecium MBE27-2	Tulum cheese (sheep milk-Bolu)	+	+	+	–	–	–	–	–	+	–
E. faecium MBE27-3	Tulum cheese (sheep milk-Bolu)	+	+	–	–	–	–	–	–	+	–
E. faecalis MBE29-1	Kelle cheese (sheep-cow milk-Manyas/Balıkesir)	–	–	–	–	–	–	–	–	+	–
E. faecalis MBE29-3	Kelle cheese (sheep-cow milk-Manyas/Balıkesir)	–	–	–	–	–	–	–	–	+	–
E. faecalis MBE31-4	White cheese (cow milk-Denizli)	+	–	–	–	–	–	–	–	+	–

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Table IV
Cross protection activity of the enterocin producer *Enterococcus* sp. strains.

Producing strains	Indicator strains										
	MBE 1-9	MBE 12-3	MBE 15-2	MBE 16-5	MBE 20-5	MBE 22-2	MBE 27-2	MBE 27-3	MBE 29-1	MBE 29-3	MBE 31-4
E. faecalis MBE1-9	–	+	+	+	–	+	+	+	+	–	+
E. faecium MBE12-3	–	–	–	+	+	+	–	–	+	+	+
E. faecium MBE15-2	–	–	–	+	–	+	–	–	+	–	+
E. faecium MBE16-5	–	–	–	–	–	–	–	–	+	–	+
E. faecalis MBE20-5	–	–	–	–	–	–	–	–	–	–	–
E. faecium MBE22-2	–	–	–	–	+	–	–	–	+	–	–
E. faecium MBE27-2	–	–	–	+	+	+	–	–	+	–	+
E. faecium MBE27-3	–	–	–	+	+	+	–	–	+	–	+
E. faecalis MBE29-1	–	–	–	–	–	–	–	–	–	–	–
E. faecalis MBE29-3	–	+	+	+	+	+	–	–	+	–	+
E. faecalis MBE31-4	–	–	–	–	–	–	–	–	–	–	–

Table V
Antibiotic susceptibility of enterocin producer *Enterococcus* sp. strains.

Strains	Antibiotics*																	
	S	CN	MH	TEC	DO	CIP	NOR	QD	RD	VA	TE	P	E	AMP	F	LZD	C	LEV
E. faecalis MBE1-9	R[2]	S	S	S	S	S	I	R	S	S	I	S	I	S	S	S	R	S
E. faecium MBE12-3	S	S	S	S	S	I	S	S	R	S	S	S	I	S	S	S	S	S
E. faecium MBE15-2	S	S	S	S	S	R	R	S	R	S	S	R	I	S	S	S	S	S
E. faecium MBE16-5	S	S	S	S	S	I	S	I	R	S	S	R	R	S	S	S	S	S
E. faecalis MBE20-5	S	S	I	S	S	S	S	S	R	S	R	S	I	S	S	S	S	S
E. faecium MBE22-2	S	S	S	S	S	I	S	I	R	S	S	S	R	S	S	S	S	S
E. faecium MBE27-2	S	S	S	S	S	S	S	S	R	S	S	S	I	S	S	S	S	S
E. faecium MBE27-3	S	S	S	S	S	R	I	S	R	S	S	S	I	S	S	S	S	S
E. faecalis MBE29-1	S	S	S	S	S	S	S	R	I	I	S	S	S	S	S	S	S	S
E. faecalis MBE29-3	S	S	S	S	S	S	S	R	I	S	S	S	I	S	S	S	S	S
E. faecalis MBE31-4	S	S	S	S	S	I	S	R	R	S	S	S	S	S	S	S	S	S

* DO: doxycycline (30 μg), NOR: norfloxacin (10 μg), VA: vancomycin (30 μg), C: chloramphenicol (30 μg), RD: rifampicin (5 μg), MH: minocycline (30 μg), P: penicillin (10 μg), S: streptomycin (300 μg), LEV: levofloxacin (5 μg), CIP: ciprofloxacin (5 μg), AMP: ampicillin (10 μg), QD: quinupristin-dalfopristin (15 μg), F: Nitrofurantoin (300 μg), TE: tetracycline (30 μg), CN: gentamicin (120 μg), E: erythromycin (15 μg), LZD: linezolid (30 μg), TEC: teicoplanin (30 μg). Susceptibilities of *Enterococcus* sp. strains were determined according to CLSI 2012. [2]R: Resistant, I: Intermediary, S: sensitive.

Presence of *van*A and *van*B. *E. faecalis* MBE1-9, *E. faecalis* MBE20-5, *E. faecalis* MBE29-1 and *E. faecalis* MBE29-3 strains carry the *van*A gene among the 11 enterocin producer. Remaining enterocin producers do not contain both *van*A and *van*B genes (Table VI).

Detection of virulence factors. The $efaA_{fs}$, *cad* and cyl_M genes were not detected in any strains while *ccf* and *cyl*B genes were amplified by all the strains. The esp_{fm} gene was found only in *E. faecalis* MBE20-5. esp_{fs} was found in *E. faecalis* MBE20-5 and MBE31-4 and *agg* was found in *E. faecalis* MBE1-9 and MBE29-3, respectively. *gel*E gene was detected in all *Enterococcus* stains except *E. faecium* MBE22-2. *cpd*, *cob*, *ace* and *cyl*A were also found in several isolates. Virulence traits of all *Enterococcus* sp. strains are given in Table VI.

Detection of decarboxylase activity and amino acid decarboxylase genes. The isolated DNA of the *Enterococcus* sp. strains was subjected to PCR amplification to detect the presence of the histidine, lysine, ornithin and tyrosine decarboxylase genes (*hdc*, *ldc*, *odc* and *tdc*, respectively). All of the enterocin producer strains except *E. faecalis* MBE1-9, have phenotypically tyrosine decarboxylase activity and *tdc* gene was also detected in the same strains. The *hdc*, *ldc* and *odc* genes were not detected in any strains (Table VI).

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Table VI
Presence of virulens factors, vancomycin and biogenic amine genes in *Enterococcus* sp. strains.

Strains	Virulens factors*													Vanco-mycin**			Biogenic amine***			
	efaAfm	*efaAfs*	*ccf*	*cpd*	*cob*	*cad*	*espfm*	*espfs*	*ace*	*gel*E	*agg*	*cyl*A	*cyl*B	*cyl*M	*van*A	*van*B	*hdc*	*tdc*	*odc*	*ldc*
E. faecalis MBE1-9	–	–	+	+	+	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–
E. faecium MBE12-3	+	–	+	–	–	–	–	–	–	+	–	–	+	–	–	–	–	+	–	–
E. faecium MBE15-2	+	–	+	–	–	–	–	–	–	+	–	–	+	–	–	–	–	+	–	–
E. faecium MBE16-5	–	–	+	+	–	–	–	–	–	+	–	–	+	–	–	–	–	+	–	–
E. faecalis MBE20-5	+	–	+	+	+	–	+	+	+	+	–	+	+	–	+	–	–	+	–	–
E. faecium MBE22-2	+	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–	–
E. faecium MBE27-2	+	–	+	+	–	–	–	–	–	+	–	+	+	–	–	–	–	+	–	–
E. faecium MBE27-3	+	–	+	+	–	–	–	–	–	+	–	–	+	–	–	–	–	+	–	–
E. faecalis MBE29-1	+	–	+	+	+	–	–	–	+	+	–	+	+	–	+	–	–	+	–	–
E. faecalis MBE29-3	–	–	+	+	+	–	–	–	+	+	+	+	+	–	+	–	–	+	–	–
E. faecalis MBE31-4	–	–	+	+	–	–	–	+	+	+	–	–	+	–	–	–	–	+	–	–

*$efaA_{fm}$ and $efaA_{fs}$: cell wall adhesin (from *E. faecium* and *E. faecalis*, respectively); *ccf*, *cpd*, *cob* and *cad*: sex pheromone; esp_{fm} and esp_{fs}: enterococcal surface protein (from *E. faecium* and *E. faecalis*, respectively); *ace*: collagen adhesion; *gel*E: gelatinase; *agg*: aggregation substance; *cyl*M, *cyl*B, and *cyl*A: cytolysin.
***van*A: vancomycin A structural gene; *van*B: vancomycin B structural gene.
****hdc*: histidine decarboxylase gene; *tdc*: tyrosine carboxylase gene; *odc*: ornitin decarboxylase gene; *ldc*: lisin decarboxylase gene.

Discussion

Enterococci easily adapt to several food systems due to their high salt and acid tolerance. It is believed that these bacteria contribute to the flavor and the aroma formation of some different European and Mediterranean cheeses. *E. faecium*, *E. faecalis* and *E. durans* species were frequently isolated from traditional dairy products (Giraffa, 2002; 2003; Martin-Platero *et al.*, 2009; Yogurtcu and Tuncer, 2013; Furlaneto-Maia *et al.*, 2014). In the present study a total of 100 *Enterococcus* strains were isolated from various artisanal cheese samples and 11 strains were determined as enterocin producers and also characterized on species level as *E. faecium* (6 strains) and *E. faecalis* (5 strains). The diversity of *Enterococcus* species from cheese depends on the ecological niche that they were isolated from (Rivas *et al.*, 2012). Similar to other studies we were found that *E. faecium* and *E. faecalis* were mostly isolated enterococcal strains from traditional Turkish cheeses (Tuncer, 2009; Yogurtcu and Tuncer, 2013).

Some *Enterococcus* sp. strains show inhibition activity with their enterocins against food spoilage and food pathogen microorganisms (Khan *et al.*, 2010; Javed *et al.*, 2011; Özden-Tuncer *et al.*, 2013). It was observed that all *E. faecium* and *E. faecalis* strains showed inhibition zones to all *Listeria* sp.and *Staphylococcus* sp. strains used in this study besides closely related species. On the other hand, inhibition zones against 2 *Salmonella* and 1 *E. coli* strains were also determined. Garcia-Cano *et al.* (2014), indicated that 1 *E. faecium* and 1 *E. faecalis* strains isolated from Cotija cheese displayed inhibitor activity against *S. enterica* Typhimurium, *E. coli* besides *L. monocytogenes* and *S. aureus* similar to our findings.

E. faecalis MBE29-1 and *E. faecalis* MBE29-3 strains were found to harbor only *ent*X structural gene while *E. faecium* MBE16-5 and *E. faecalis* MBE31-4 strains carried *ent*A and *ent*X; *E. faecium* MBE15-2 and *E. faecium* MBE 27-3 strains carry *ent*A, *ent*B and *ent*X; *E. faecium* MBE12-3 strain carried *ent*A and *ent*B; *E. faecium* MBE22-2 strain carried *ent*A, *ent*P and *ent*X structural genes. *E. faecium* MBE27-2 was identified as the only producer strain that harbored 4 structural genes (*ent*A, *ent*B, *ent*P and *ent*X) at the same time. It is not unusual that multiple structural enterocin genes may be present in one strain and it is indicated that many *Enterococcus* sp. strains produce more than one bacteriocin similar to our results (Edalatian *et al.*, 2012; Özden-Tuncer *et al.*, 2013; Rehaiem *et al.*, 2014). On the other hand, *E. faecalis* MBE1-9 and MBE20-5 strains have not given the PCR product with the most common enterocin primers tested in this study suggesting that bacteriocins produced by these strains could be new enterocins. Further analyses should be needed for characterization of these enterocins.

However, many *Enterococcus* sp. strains adapt to food fermentation processes as a starter or as a protective culture, they may harbor antibiotic resistance and some virulence determinants that increase the

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risk of pathogenicity of these strains (Martin-Platero *et al.*, 2009; Edalatian *et al.*, 2012). In the present study, 11 enterocin producer strains were found to be resistant to one or more antibiotics. All strains showed sensitivity against vancomycin but four of them were carrying *vanA* genes. Furlaneto-Maia *et al.* (2014), reported that 5 *Enterococcus* sp. isolates harbored *van*A gene, while they were susceptible to vancomycin phenotypically. In addition the same results for ampicillin, tetracycline and linezolid susceptibilities were attained in the study of Pesavento *et al.* 2014. We determined that all enterocin producer strains showed susceptibility against gentamicin, teicoplanin, doxycycline, ampicillin, nitrofurantoin, linezolid and levofloxacin. On the other hand, most of the resistance in the strains obtained against rifampicin and quinupristin-dalfopristin similar to other results obtained in *Enterococcus* sp.strains isolated from Turkish Tulum cheese (Özden-Tuncer *et al.*, 2013).

Only *E. faecalis* MBE29-3 showed gelatinase activity on Todd-Hewitt agar but all the enterocin producers strains except *E. faecium* MBE 22-2 had gene *gel*E. These results suggest that in strains which have no gelatinase activity phenotypically associated with *gel*E the gene was silent or expressed at a low level (Lopes *et al.*, 2006). Similar observations were indicated for clinical and food originated *Enterococcus* isolates (Cariolato *et al.*, 2008; Ben Belgacem *et al.*, 2010; Özden-Tuncer *et al.*, 2013; Inoğlu and Tuncer, 2013; Tuncer *et al.*, 2014; Medeiros *et al.*, 2014). In addition, many of the virulence genes were detected in enterocin producer strains. PCR analysis revealed that all strains contained at least 3 virulence genes. However, sex pheromone genes (*cpd*, *cob*, *ccf* and *cad*) were not accepted as virulence factors their production in *Enterococcus* sp. strains are assume that dissamination of virulence factors, and these genes were detected frequently in *Enterococcus* sp. isolates from cheese (Eaton and Gasson, 2001; Valenzuela *et al.*, 2008; Ben Belgacem *et al.*, 2010; Inoğlu and Tuncer, 2013). Martin *et al.* (2006), confirmed that *efa*A$_{fs}$ and *efa*A$_{fm}$ were only found in *E. faecalis* and *E. faecium*, respectively. However, Barbosa *et al.* (2010) and Inoğlu and Tuncer (2013) were indicated that *efaA*$_{fs}$ gene found in *E. faecium* and *efa*A$_{fm}$ was found in *E. faecalis* strain, as confirmed in this study. The *agg* gene product mediates the establishment of contact between bacteria and host cells and facilitating colonization, therefore *agg* gene harboring strains were unwanted in food. The *agg* gene was detected only in *E. faecalis* strains by other researchers (Eaton and Gasson, 2001; Franz *et al.*, 2001; Martin *et al.*, 2006; Inoğlu and Tuncer, 2013), as confirmed in this study. However, Barbosa *et al.* (2010), showed that *agg* gene may be found in *E. faecium* strains too. The *ace* gene was also having an important role in colonization like *agg* gene and it is reported that *Enterococcus* sp. isolates containing *ace* gene were found to be positively correlated with biofilm formation (Singh *et al.*, 2010, Medeiros *et al.*, 2014). In our study, the strains containing the *agg* and *ace* genes individually and the strains possesing also both genes should be investigated for biofilm formation in a further study.

Cytolysin is a bacterial toxin involved in the formation of haemolytic activity. Several studies confirmed that *Enterococcus* sp. strains harboring cytolysin genes may have β-haemolytic activity (Eaton and Gasson, 2001; Theppangna *et al.*, 2007). However, different researchers found that, non β-haemolytic strains may carry the cytolysin genes similar to the present study (Cosentino *et al.*, 2010; Medeiros *et al.*, 2014). Medeiros *et al.* (2014), showed that *Enterococcus* sp. isolate originated from food had non β-hemolytic activity, but carried *cyl*A gene, as confirmed in this study. It is also reported that in non haemolytic strains cytolysin determinant may behave as silent. On the other hand, the lack of a correlation between phenotypic and genotypic cytolysin production may suggest some genetic rearrangements in *cyl* operon like missing genes (Semedo *et al.*, 2003, Gaspar *et al.*, 2009). In our study *cyl*B gene was detected in all strains while *cyl*A gene was detected only in 5 strains. However, *cyl*M was not detected in any strains and in all strains the lack of β-haemolytic activity was determined.

Another undesirable case for *Enterococcus* sp. strains is biogenic amine production ability (Valenzuela *et al.*, 2008). In the present study, tyrosine decarboxylating ability was determined in 10 *Enterococcus* sp. strains phenotypically and gene *tdc* was also detected in the same strains. Only *E. faecalis* MBE1-9 has no decarboxylating tyrosine does not harbor *tcd* gene as well. Similar to our results, several researchers indicated that tyramine was frequently produced by *Enterococcus* sp. strains (Tuncer, 2009; Komprda *et al.*, 2010; Kalhotka *et al.*, 2012; Inoğlu and Tuncer, 2013). PCR study showed compatibility with the result of the phenotypically tyrosine decarboxylating ability, as confirmed by Inoğlu and Tuncer (2013) and Yüceer and Özden-Tuncer (2015).

Conclusion

Many of the enterococci originating from food may produce enterocins are active against pathogenic or spoilage microorganisms, particularly *Listeria* spp. and *Staphylococcus aureus*. In the present study enterocins were identified using known enterocin primers and multiple enterocin genes were detected in 7 strains. Two enterocins could not be identified and need to be investigated whether they are new bacteriocins.

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However, all enterocin producers were found to have several virulence factors such *i.e. gel*E, *agg*, *cpd*, *cob*, *ace*, *cyl*A and *tdc* genes. In addition 6 strains had multiple antibiotic resistance patterns. Lack of hemolytic and gelatinase activity (except *E. faecalis* MBE29-3) in bacteriocin producing strains are advantageous, although their virulence characteristics make them risky in terms of consumer health.

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