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Occurrence of enterocin genes, virulence factors, and antibiotic resistance in 3 bacteriocin-producer *Enterococcus faecium* strains isolated from Turkish tulum cheese

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Abstract: In this study, the occurrence of enterocin genes, virulence factors, and antibiotic resistance in the bacteriocin-producer *Enterococcus faecium* EYT17, EYT31, and EYT39 strains was investigated. Polymerase chain reaction (PCR) studies showed that all of the *E. faecium* strains carried the enterocin A and B structural genes (*entA* and *entB*), while *E. faecium* EYT17 and EYT31 strains also carried the enterocin P structural gene (*entP*). None of the strains exhibited β-haemolysis or gelatinase activity. PCR analysis revealed that EYT17, EYT31, and EYT39 strains were clear of potential virulence determinants, except for *ccf* and *efaAfm*. The *ccf* gene was found in all of them. The *efaAfm* gene was only found in the EYT17 strain. All of the strains were found sensitive to ampicillin, chloramphenicol, gentamicin, norfloxacin, penicillin, streptomycin, tetracycline, sulphamethoxazole/trimethoprim, and vancomycin by disc diffusion method. *E. faecium* strains only exhibited intermediary resistance to erythromycin (15 μg). The results of this study showed that the multiple enterocin-producer *E. faecium* EYT17, EYT31, and EYT39 strains are safe and these strains may be used for food preservation.

Key words: *Enterococcus faecium*, enterocin, virulence factors, antibiotic resistance, food safety

1. Introduction

The enterococci, a group of lactic acid bacteria, are important in environmental, food, and clinical microbiology. These bacteria are also associated with different traditional European cheeses, especially artisanal cheeses produced in Mediterranean countries, made with raw or pasteurised milk (1,2). *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterococcus durans* are the most prevalent species in milk and cheese products (3). Several studies have suggested that dairy strains of enterococci play a fundamental role in the ripening of traditional cheeses, probably by their proteolysis, lipolysis, and citrate breakdown, hence contributing to their typical taste and flavour (3,4). Additionally, many enterococci strains display the ability to produce bacteriocin, specifically called enterocin, which exhibits an inhibitory effect against various foodborne pathogens or food spoilage bacteria, thus offering a tool for improving food safety (5). The frequently encountered enterocins produced by enterococci strains are enterocins A, B, P, AS-48, L50A, L50B, 1071A, 1071B, and Q (6).

Some enterococci strains are opportunistic human pathogens, which are among the prevalent bacteria involved in nosocomial infections such as bacteraemia, endocarditis, and urinary tract infections (4). Antibiotic resistance of enterococci is a contributing factor for their pathogenesis. Vancomycin-resistant enterococci are especially a major problem in treating human infections, since this antibiotic is considered as a last resort in treating multiple drug-resistant enterococci (3,4). In addition, cytolysin/haemolysin is a bacterial toxin that is encoded by an operon localised on a pheromone-responsive plasmid or on the chromosome. Cytolysin shows haemolytic and bactericidal activity. It is thought to play an important role in human infections (7). Moreover, several genes for virulence factors such as gelatinase (*gelE*), cell wall adhesin (*efaAfm* and *efaAfs* from *E. faecium* and *E. faecalis*, respectively), sex pheromone (*cpd*, *cob*, *ccf*, and *cad*), collagen adhesion (*ace*), enterococcal surface protein (*espfm* and *espfs* from *E. faecium* and *E. faecalis*, respectively), aggregation substance (*agg*), and cytolysin (*cylM*, *cylB*, and *cylA*) have been characterised in enterococci (8–11).

In a previous study, Tuncer (12) isolated 3 bacteriocin-producer strains from artisanal Turkish tulum cheese and phenotypically identified them as *Enterococcus faecium*. These strains exhibited inhibitory activity against a broad range of bacteria including lactobacilli, lactococci, enterococci, and also *Listeria innocua*, *Bacillus cereus*,

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and *Pseudomonas fluorescens*. In the present study, we identified these strains at species level by 16S rDNA homology and determined the presence of their enterocin genes. Furthermore, we investigated the incidence of 14 virulence factor genes (*gelE*, *efaAfm*, *efaAfs*, *cpd*, *cob*, *ccf*, *cad*, *ace*, *espfm*, *espfs*, *agg*, *cylA*, *cylB*, and *cylM*), haemolysis, gelatinase activity, and antibiotic resistances of these strains for detection of their safety.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Three bacteriocin-producer *E. faecium* (EYT17, EYT31, and EYT39) strains isolated from Turkish tulum cheese (12) were grown overnight in de Man-Rogosa-Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37 °C. All cultures were stored at –20 °C in MRS broth with 20% sterile glycerol added.

2.2. DNA isolation

Total DNA was extracted from overnight *E. faecium* cultures as previously described by Cancilla et al. (13).

2.3. Genetic identification of strains

Genetic identification of strains was done to the species level by 16S rDNA sequencing using polymerase chain reaction (PCR) with universal primers pA (5'-AGA GTT TGA TCC TGG CTC AG-3') and pE' (5'-CCG TCA ATT CCT TTG AGT TT-3') as described previously by Edwards et al. (14). PCR amplifications were performed in 50-µL mixtures using 3 µL of DNA solution, 1 µL of each primer, 20 µL of nuclease-free water, and 25 µL of PCR master mix (Fermentas, Vilnius, Lithuania). Samples were subjected to an initial cycle of denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, and elongation at 72 °C for 90 s, and a last cycle at 72 –C for 10 min using a programmable DNA thermocycler (Techne TC3000, Cambridge, UK). The amplification products were analysed by electrophoresis on 1.5% agarose gel at 120 V for 1.5 h in Tris-acetate-EDTA buffer and revealed in ethidium bromide (20 µg/mL). The gel was photographed under UV light. The size of amplified fragments was determined by comparison with an O'GeneRuler™ 100-bp DNA ladder (Fermentas). Sequencing of the 16S rDNAs was performed in RefGen (METU Technopolis, Ankara, Turkey). Similarity searches were conducted using the BLAST software of the National Center for Biotechnology Information (Bethesda, MD, USA).

2.4. PCR detection of known enterocin genes

PCR amplification of well-known structural genes of enterocin A (*entA*), enterocin B (*entB*), enterocin P (*entP*), enterocin L50A/B (*entL50A-entL50B*), bacteriocin 31 (*bac31*), enterocin AS48 (*entAS48*), enterocin 1071A/1071B (*ent1071A-ent1071B*), and enterocin Q (*entQ*) was performed using specific enterocin PCR primers as reported in Table 1. PCR for structural genes of enterocins was performed as an initial cycle of denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation 94 °C for 1 min, annealing at an appropriate temperature for 1 min, and elongation at 72 °C for 40 s (15). The amplification products were analysed by electrophoresis on 1.5% agarose gel at 120 V for 1.5 h in Tris-acetate-EDTA buffer and revealed in ethidium bromide (20 µg/mL). The gel was photographed under UV light.

Table 1. Polymerase chain reaction primers, annealing temperatures, and product sizes for detection of enterocin genes.

Gene	Primer sequences (5' to 3')	PCR annealing temperature (°C)	Product size (bp)	Reference
entA	f: AAA TAT TAT GGA AAT GGA GTG TAT r: GCA CTT CCC TGG AAT TGC TC	56	126	(15)
entB	f: GAA AAT GAT CAC AGA ATG CCT A r: GTT GCA TTT AGA GTA TAC ATT TG	50	162	(15)
entP	f: TAT GGT AAT GGT GTT TAT TGT AAT r: ATG TCC CAT ACC TGC CAA AC	50	120	(15)
entL50A/B	f: TGG GAG CAA TCG CAA AAT TAG r: ATT GCC CAT CCT TCT CCA AT	52	98	(11)
bac31	f: TAT TAC GGA AAT GGT TTA TAT TGT r: TCT AGG AGC CCA AGG GCC	50	123	(15)
entAS48	f: GAG GAG TTT CAT GAT TTA AAG A r: CAT ATT GTT AAA TTA CCA AGC AA	50	340	(15)
entQ	f: ATG AAT TTT CTT CTT AAA AAT GGT ATC GCA r: TTA ACA AGA AAT TTT TTC CCA TGG CAA	56	105	(11)
ent1071A/B	f: CCT ATT GGG GGA GAG TCG GT r: ATA CAT TCT TCC ACT TAT TTT T	51	343	(11)

2.5. Haemolytic and gelatinase activity

The *E. faecium* strains were grown overnight in MRS broth at 37 °C. For determination of haemolytic activity, strains were streaked onto Columbia Agar plates (Laboratorios Conda S.A., Madrid, Spain) containing 5% (v/v) of either sheep blood or human blood. The plates were incubated at 37 °C for 48 h under aerobic conditions. Presence of zone of clearing around the colonies was interpreted as β-haemolysis (8).

Production of gelatinase was determined on Todd-Hewitt agar (Merck) containing 30 g of gelatine (Merck) per litre as described by Eaton and Gasson (9).

2.6. PCR detection of virulence genes

The virulence factors in 3 bacteriocin-producer *E. faecium* strains were investigated by PCR amplification, revealing the presence of genes encoding for gelatinase (*gelE*), cell wall adhesin (*efaAfm* and *efaAfs*), sex pheromone (*cpd*, *cob*, *ccf*, and *cad*), collagen adhesin (*ace*), enterococcal surface protein (*espfm* and *espfs*), aggregation substance (*agg*), and cytolysin (*cylM*, *cylB*, and *cylA*). The primers used in this assay are listed in Table 2. PCR for virulence genes was performed as an initial cycle of denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C (56 °C for gene *agg*) for 30 s, and elongation at 72 °C for 1 min (16). The amplification products were analysed by electrophoresis on 1.5% agarose gel at 120 V for 1.5 h in Tris-acetate-EDTA buffer and revealed in ethidium bromide (20 μg/mL). The gel was photographed under UV light.

2.7. Antibiotic resistance

The antibiotic susceptibility patterns of the *E. faecium* strains were detected by the disc diffusion method on Muller-Hinton agar (Merck) as described by Cariolato et al. (8). The antibiotics used were ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (120 μg), norfloxacin (10 μg), penicillin G (10 U), streptomycin (300 μg), tetracycline (30 μg), sulphamethoxazole/trimethoprim (30 μg), and vancomycin (30 μg). All antibiotic discs were obtained from Oxoid (Basingstoke, UK). Susceptibility or resistance was determined according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (17).

Table 2. Polymerase chain reaction primers and product sizes for detection of virulence determinants.

Gene	Primer sequences (5' to 3')	Product size (bp)	Reference
gelE	f: ACC CCG TAT CAT TGG TTT r: ACG CAT TGC TTT TCC ATC	419	(16)
efaAfm	f: AAC AGA TCC GCA TGA ATA r: CAT TTC ATC ATC TGA TAG TA	735	(16)
efaAfs	f: GAC AGA CCC TCA CGA ATA r: AGT TCA TCA TGC TGT AGT A	705	(16)
cpd	f: TGG TGG GTT ATT TTT CAA TTC r: TAC GGC TCT GGC TTA CTA	782	(16)
cob	f: AAC ATT CAG CAA ACA AAG C r: TTG TCA TAA AGA GTG GTC AT	1405	(16)
ccf	f: GGG AAT TGA GTA GTG AAG AAG r: AGC CGC TAA AAT CGG TAA AAT	543	(16)
cad	f: TGC TTT GTC ATT GAC AAT CCG r: ACT TTT TCC CAA CCC CTC AA	1299	(16)
ace	f: AAA GTA GAA TTA GAT CCA CAC r: TCT ATC ACA TTC GGT TGC G	350	(11)
espfm	f: TTG CTA ATG CAA GTC ACG TCC r: GCA TCA ACA CTT GCA TTA CCG AA	955	(16)
espfs	f: TTG CTA ATG CTA GTC CAC GAC C r: GCG TCA ACA CTT GCA TTG CCG AA	933	(16)
agg	f: AAG AAA AAG AAG TAG ACC AAC r: AAA CGG CAA GAC AAG TAA ATA	1553	(9)
cylM	f: CTG ATG GAA AGA AGA TAG TAT r: TGA GTT GGT CTG ATT ACA TTT	742	(16)
cylB	f: ATT CCT ACC TAT GTT CTG TTA r: AAT AAA CTC TTC TTT TCC AAC	843	(16)
cylA	f: TGG ATG ATA GTG ATA GGA AGT r: TCT ACA GTA AAT CTT TCG TCA	517	(16)

3. Results and discussion

3.1. Genetic identification of strains

Bacteriocin-producer EYT17, EYT31, and EYT39 strains were identified previously as *E. faecium* based on phenotypic characteristics (12). In this study, these bacteriocin-producer strains were identified at the species level by 16S rDNA sequence homology. PCR product of an approximately 900-bp DNA fragment was amplified from all 3 strains. Sequencing of PCR amplification products of the EYT17, EYT31, and EYT39 strains showed 99% homology with the *E. faecium* genome deposited in GenBank. These results confirmed the biochemical identification of these strains.

3.2. Detection of enterocin structural genes

The purified DNAs of bacteriocin-producer *E. faecium* EYT17, EYT31, and EYT39 strains were used as a template in PCR amplifications to determine the existence of structural genes encoding 8 enterocins (Table 1). All 3 *E. faecium* strains carried the enterocin A and B structural genes, while *E. faecium* EYT17 and EYT31 strains also carried the enterocin P structural gene. None of the evaluated strains showed PCR amplification fragments for enterocin L50A/B, bacteriocin 31, enterocin AS48, enterocin 1071A/1071B, and enterocin Q structural genes (Table 3). Previous studies showed that PCR detection of more than one bacteriocin encoding gene in the *Enterococcus* strains isolated from fermented foods is not unusual (6,11,15), as confirmed in this study. According to the classification scheme of Franz et al. (18), enterocin A and enterocin P are grouped in class II.1 (pediocin-like bacteriocins), which has a very effective antilisterial activity, and enterocin B is grouped into class II.3 (linear nonpediocin-like bacteriocins). Casaus et al. (19) reported that enterocin B exhibited synergistic activity with enterocin A. The number of survivors was drastically reduced when a mixture of the 2 bacteriocins (enterocin A and B) was added to the cells. In a previous study, Tuncer (12) reported that *E. faecium* EYT17 and EYT31 strains (enterocin A, B, and P producers) showed the same antibacterial activity spectrum and these strains exhibited broader inhibitory activity spectrum than the *E. faecium* EYT39 strain (enterocin A and B producer). Enterocins produced in *E. faecium* EYT17, EYT31, and EYT39 strains probably act in a synergistic mode of action, as reported by Casaus et al. (19). Further investigations are also needed for the determination of the synergistic mode of action of these bacteriocins.

3.3. Haemolytic and gelatinase activity

Haemolytic activity of bacteriocin-producer *E. faecium* strains was tested on sheep blood and human blood agars. None of the *E. faecium* strains showed β-haemolytic activity on either of the 2 blood agars. Different researchers reported that *Enterococcus* strains isolated from a variety of fermented food products exhibited no β-haemolytic activity, similar to our results (10,20). Haemolysin production can increase the severity of enterococcal infections, and the presence of genes involved in haemolysin/cytolysin production is also considered a risk factor (21). The β-haemolytic enterococci isolates are considered undesirable in foods, and their use as starter cultures in food fermentations is not recommended (22). None of our strains exhibiting β-haemolytic phenotype might be considered as advantageous for the safety of the strains.

Gelatinase activity of *E. faecium* strains was tested on Todd-Hewitt agar containing gelatine (30 g/L). Gelatinase activity was not detected in *E. faecium* strains. The same findings were reported by Eaton and Gasson (9) and Franz et al. (23). None of the *E. faecium* strains involved in either study showed gelatinase activity. On the other hand, a high number of *E. faecalis* strains isolated from food were detected to be producing gelatinase in these 2 studies. Gelatinase production by *E. faecium* species is not common (23), as confirmed in this study.

Table 3. Detection of enterocin structural genes in 3 bacteriocin-producer *Enterococcus faecium* strains from Turkish tulum cheese.

Enterocin structural genes (PCR amplification)	*Enterococcus faecium* strains		
	EYT17	EYT31	EYT39
entA	+	+	+
entB	+	+	+
entP	+	+	-
entL50A/B	-	-	-
bac31	-	-	-
entAS48	-	-	-
entQ	-	-	-
ent1071A/B	-	-	-

3.4. Detection of virulence genes

Bacteriocin-producer *E. faecium* EYT17, EYT31, and EYT39 strains were screened for the presence of 14 known virulence determinants. PCR analysis revealed that these strains were clear of potential virulence determinants, except for *ccf* and *efaAfm*. The structural genes of 12 virulence factors, namely gelatinase (*gelE*), cell wall adhesin (*efaAfs*), sex pheromone (*cpd*, *cob*, and *cad*), collagen adhesin (*ace*), enterococcal surface protein (*espfm* and *espfs*), aggregation substance (*agg*), and cytolysin (*cylM*, *cylB*, and *cylA*) were not found in any of the multiple enterocin-producer strains (Table 4). The *ccf* gene was found in all 3 strains. The PCR amplification of the *ccf* gene yielded an amplicon of the expected 543-bp size (Figure, lane 5). The adhesion-like *E. faecium* antigen A gene (*efaAfm*) was only found in the EYT17 strain, amplifying a 735-bp fragment (Figure, lane 2). The sex pheromones are not considered a virulence factor. On the other hand, the enterococci strains with the sex pheromone (*cpd*, *cob*, *ccf*, and *cad*) determinants have the potential to acquire the respective sex pheromone plasmids and, hence, the associated virulence determinants (9). The exact role of *efaAfm* as a virulence factor is still unknown (11). The *efaAfm* gene has not yet been conclusively shown to contribute to pathogenesis in animal studies, in contrast to the adhesion-like *E. faecalis* antigen A (*efaAfs*) (15). Furthermore, the absence of *cylMBA* and *gelE* genes in *E. faecium* EYT17, EYT31, and EYT39 strains agrees with our observation that these strains lack β-haemolytic and gelatinase activity. Recently, observations similar to our results were reported by different researchers. These studies showed that a large number of *E. faecium* strains of food origin had *ccf* and *efaAfm* genes (9,11,18,24,25). Basanta et al. (25) showed that the only virulence genes in the multiple enterocin-producer *E. faecium* L50 strain are *ccf* and *efaAfm*, as confirmed in this study. The low amounts of virulence factors contained in *E. faecium* EYT17, EYT31, and EYT39 strains are advantages.

3.5. Antibiotic resistance

In this study, the disc diffusion method was used to determine the antibiotic resistance patterns of the *E. faecium* EYT17, EYT31, and EYT39 strains. All of the strains were sensitive to ampicillin, chloramphenicol, gentamicin, norfloxacin, penicillin, streptomycin, tetracycline, sulphamethoxazole/trimethoprim, and vancomycin. The *E. faecium* strains only exhibited intermediary resistance to erythromycin (15 μg). The resistance against antibiotics is an important factor for the evaluation of the safety of enterococci (16). These bacteria have natural and acquired resistance to antibiotics. Antibiotic-resistant enterococci are widespread in meat products, dairy products, and ready-to-eat foods (26). Susceptibility to clinically relevant antibiotics of enterococci of food origin is very important for consumer health. Thus, the complete susceptibility to clinically important antibiotics of the multiple enterocin-producing *E. faecium* EYT17, EYT31, and EYT39 strains is advantageous.

In conclusion, the multiple enterocin encoding genes were detected in bacteriocinogenic *E. faecium* EYT17, EYT31, and EYT39 strains. These strains were found

Table 4. Incidence of virulence factors in 3 bacteriocin-producer *Enterococcus faecium* strains from Turkish tulum cheese.

Virulence determinants (PCR amplification)	*Enterococcus faecium* strains		
	EYT17	EYT31	EYT39
gelE	-	-	-
efaAfm	+	-	-
efaAfs	-	-	-
cpd	-	-	-
cob	-	-	-
ccf	+	+	+
cad	-	-	-
ace	-	-	-
espfm	-	-	-
espfs	-	-	-
agg	-	-	-
cylM	-	-	-
cylB	-	-	-
cylA	-	-	-

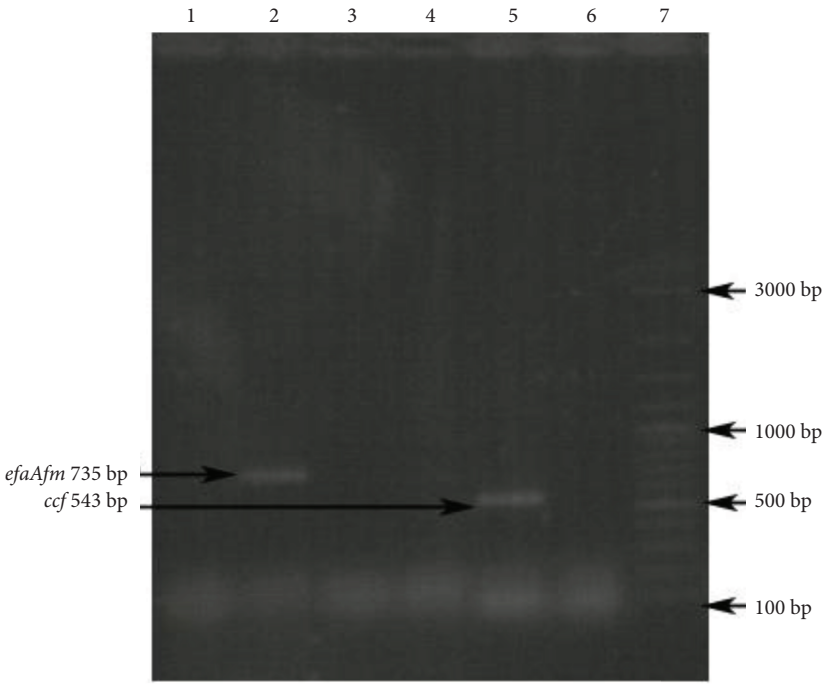

Figure. PCR screening for some virulence genes from *Enterococcus faecium* EYT17. Lane 1, *gelE*; lane 2, 735-bp fragment of the *efaAfm* gene; lane 3, *cpd*; lane 4, *cob*; lane 5, 543-bp fragment of the *ccf* gene; lane 6, *cad*; lane 7, 100-bp DNA ladder (Fermentas).

clear of potential virulence determinants, except for *ccf* and *efaAfm*. In addition, all of these strains were found completely susceptible to clinically important antibiotics. These findings suggest that multiple enterocin-producing *E. faecium* EYT17, EYT31, and EYT39 strains have low risk factors for consumer health and these strains may be used for food preservation. Further studies should be made to evaluate the application of these strains as adjunct cultures in the dairy fermentation process.

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References

1. Giraffa G. Enterococci from foods. FEMS Microbiol Rev 26: 163–171, 2002.
2. Franz CMAP, Stiles ME, Schleifer KH et al. Enterococci in foods - a conundrum for food safety. Int J Food Microbiol 88: 105–122, 2003.
3. Ogier JC, Seror P. Safety assessment of dairy microorganisms: the *Enterococcus* genus. Int J Food Microbiol 126: 291–301, 2008.
4. Morandi S, Brasca M, Andrighetto C et al. Technological and molecular characterisation of enterococci isolated from north-west Italian dairy products. Int Dairy J 16: 867–875, 2006.
5. Khan H, Flint S, Yu PL. Enterocins in food preservation. Int J Food Microbiol 141: 1–10, 2010.
6. Edalatian MR, Najafi MBH, Mortazavi SA et al. Production of bacteriocins by *Enterococcus* spp. isolated from traditional, Iranian, raw milk cheeses, and detection of their encoding genes. Eur Food Res Technol 234: 789–796, 2012.
7. Koch S, Hufnagel M, Theilacker C et al. Enterococcal infections: host response, therapeutic, and prophylactic possibilities. Vaccine 22: 822–830, 2004.
8. Cariolato D, Andrighetto C, Lombardi A. Occurrence of virulence factors and antibiotic resistances in *Enterococcus faecalis* and *Enterococcus faecium* collected from dairy and human samples in North Italy. Food Control 19: 886–892, 2008.
9. Eaton TJ, Gasson MJ. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl Environ Microb 67: 1628–1635, 2001.
10. Yoon MY, Kim YJ, Hwang HJ. Properties and safety aspects of *Enterococcus faecium* strains isolated from *Chungkukjang*, a fermented soy product. LWT-Food Sci Technol 41: 925–933, 2008.
11. Ben Belgacem Z, Abriouel H, Ben Omar N et al. Antimicrobial activity, safety aspects, and some technological properties of bacteriocinogenic *Enterococcus faecium* from artisanal Tunisian fermented meat. Food Control 21: 462–470, 2010.

12. Tuncer Y. Some technological properties of phenotypically identified enterococci strains isolated from Turkish tulum cheese. Afr J Biotechnol 8: 7008–7016, 2009.

13. Cancilla MR, Powell IB, Hillier AJ et al. Rapid genomic fingerprinting of *Lactococcus lactis* strains by arbitrarily primed polymerase chain reaction with ^{32}P and fluorescent labels. Appl Environ Microb 58: 1772–1775, 1992.

14. Edwards U, Rogall T, Blocker H et al. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 17: 7843–7853, 1989.

15. Yousif NMK, Dawyndt P, Abriouel H et al. Molecular characterization, technological properties and safety aspects of enterococci from Husuwa, an African fermented sorghum product. J Appl Microbiol 98: 216–228, 2005.

16. Reviriego C, Eaton T, Martín R et al. Screening of virulence determinants of *Enterococcus faecium* strains isolated from breast milk. J Hum Lact 21: 131–137, 2005.

17. Clinical and Laboratory Standards Institute. CLSI M100-S21. Performance Standards for Antimicrobial Susceptibility Testing: 21st Informational Supplement. Clinical and Laboratory Standards Institute. Wayne, PA, USA; 2011.

18. Franz CMAP, van Belkum MJ, Holzapfel et al. Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiol Rev 31: 293–310, 2007.

19. Casaus P, Nilsen T, Cintas LM et al. Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiology 143: 2287–2294, 1997.

20. Özmen Toğay S, Çelebi Keskin A, Açık L et al. Virulence genes, antibiotic resistance and plasmid profiles of *Enterococcus faecalis* and *Enterococcus faecium* from naturally fermented Turkish foods. J Appl Microbiol 109: 1084–1092, 2010.

21. Valenzuela AS, Ben Omar N, Abriouel H et al. Risk factors in enterococci isolated from foods in Morocco: determination of antimicrobial resistance and incidence of virulence traits. Food Chem Toxicol 46: 2648–2652, 2008.

22. Gomes BC, Esteves CT, Palazzo ICV et al. Prevalence and characterization of *Enterococcus* spp. isolated from Brazilian foods. Food Microbiol 25: 668–675, 2008.

23. Franz CMAP, Muscholl-Silberhorn AB, Yousif NMK et al. Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. Appl Environ Microb 6: 4385–4389, 2001.

24. Martín M, Gutérrez J, Herranz C et al. Genes encoding bacteriocins and their expression and potential virulence factors of enterococci isolated from pigeons (*Columba palumbus*). J Food Protect 69: 520–531, 2006.

25. Basanta A, Sanchez J, Gomez-Sala B et al. Antimicrobial activity of *Enterococcus faecium* L50, a strain producing enterocins L50 (L50A and L50B), P and Q, against beer-spoilage lactic acid bacteria in broth, wort (hopped and unhopped), and alcoholic and non-alcoholic lager beers. Int J Food Microbiol 125: 293–307, 2008.

26. Foulquié Moreno MR, Sarantinopoulos P, Tsakalidou E et al. The role and application of enterococci in food and health. Int J Food Microbiol 106: 1–24, 2006.