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ORIGINAL ARTICLE

Virulence genes, antibiotic resistance and plasmid profiles of *Enterococcus faecalis* and *Enterococcus faecium* from naturally fermented Turkish foods

S. Özmen Toğay[1,2], A. Çelebi Keskin[3], L. Açık[4] and A. Temiz[1]

1 Department of Food Engineering, Faculty of Engineering, Hacettepe University, Beytepe, Ankara, Turkey
2 Department of Food Engineering, Faculty of Engineering and Architecture, Çanakkale Onsekiz Mart University, Terzioğlu Campus, Çanakkale, Turkey
3 Department of Biology, Faculty of Arts and Science, Kırıkkale University, Kırıkkale, Turkey
4 Department of Biology, Faculty of Arts and Science, Gazi University, Teknikokullar, Ankara, Turkey

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Correspondence
Sine Özmen Toğay, Department of Food Engineering, Faculty of Engineering, Hacettepe University, 06800 Beytepe, Ankara, Turkey.
E-mail: sinevet@hacettepe.edu.tr

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Abstract

Aim: To determine the virulence genes, antibiotic resistance and plasmid profiles of 16 *Enterococcus faecium* and 68 *Enterococcus faecalis* strains isolated from various naturally fermented foods.
Methods and Results: The presence of virulence genes (agg_2, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*, *efaAfs*, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*) and also the genes *vanA* and *vanB* were investigated by polymerase chain reaction (PCR). Antibiotic resistance of the isolates was determined by disc diffusion method. Most of the tested isolates were positive for virulence genes and resistant to some antibiotics. One of the *Ent. faecalis* strains isolated from a cheese sample carried the *vanA* gene and was intermediately resistant to vancomycin. The strains usually contained large plasmids, which might harbour acquired antibiotic resistance.
Conclusion: The study showed that *Ent. faecium* and *Ent. faecalis* strains isolated from naturally fermented Turkish foods may be potential risk factors for consumer health in terms of virulence genes and acquired antibiotic resistance.
Significance and Impact of the Study: The results indicate the importance of enterococcal contamination in terms of the safety of some fermented Turkish foods.

Introduction

Enterococci have a role in improving the typical taste and flavour of many foods, such as cheeses and sausages, by their proteolytic and lipolytic activities (Garcia *et al.* 2002; De Vuyst *et al.* 2003; Klein 2003; Foulquie Moreno *et al.* 2006). The ability of enterococci to produce bacteriocins and to adapt to different environmental conditions are important characteristics for the food industry (De Vuyst *et al.* 2003; Foulquie Moreno *et al.* 2006). In recent years, reports about the use of enterococci as starter cultures, cocultures or probiotics have increased considerably (Franz *et al.* 1999, 2003; De Vuyst *et al.* 2003; Hugas *et al.* 2003; Klein 2003; Foulquie Moreno *et al.* 2006). Besides their beneficial characteristics, some enterococci are recognized as nosocomial pathogens, which have virulence genes and increased resistance to antibiotics (Franz *et al.* 1999; Giraffa *et al.* 2000; Klein 2003; Peters *et al.* 2003; Foulquie Moreno *et al.* 2006; Poeta *et al.* 2006). Cytolysins, gelatinase, serine protease, hyaluronidase, aggregation substance (AS), extracellular surface protein and other adhesins (Ace, EfaA, etc.) are virulence factors in enterococci, especially in *Enterococcus faecium* and *Enterococcus faecalis* strains (Mannu *et al.* 2003; Semedo *et al.* 2003; Sánchez Valenzuela *et al.* 2009).

Enterococci have also been defined as increasingly resistant to multiple antibiotics in recent years (Mannu *et al.* 2003). Antibiotic-resistant enterococci are widespread in foods and this property is transferred between bacteria by plasmids (Franz *et al.* 1999). Discussions have focused on whether pathogenic enterococci can be transmitted by foods. Therefore, it is suggested that enterococci isolated from foods should be tested in terms of potential

virulence genes and antibiotic resistance (Franz *et al.* 1999; Reviriego *et al.* 2005).

The aim of this study was to determine the virulence genes, antibiotic resistance and plasmid profiles of *Ent. faecalis* and *Ent. faecium* strains isolated from naturally fermented cheese, sausage and olive samples produced in Turkey.

Materials and methods

Strain isolation and identification

In this study, 20 samples of cheese, 10 samples of sausage and 20 samples of olives were obtained from domestic markets and local producers in different regions of Turkey. The 25-g food samples were homogenized with 225 ml of a buffered peptone water (PW; Himedia, Mumbai, India) in a sterile stomacher bag, using a Seward 400 laboratory stomacher (West Sussex, UK) at medium speed for 1 min. The olive samples were homogenized after removing the stones aseptically. Decimal dilutions of the food homogenates were made in sterile PW and inoculated on Citrate Azide Tween Carbonate (Himedia) agar (Canzek Majhenic *et al.* 2005) and Kanamycin Aesculin Azide (Fluka, Buchs, Switzerland) agar and then incubated at 37°C for 48 h. Five typical colonies were randomly selected from the highest dilution of each sample and purified twice on Trypticase Soy agar (Merck, Darmstadt, Germany). The pure cultures were identified to the genus level, using Gram staining, catalase test, growth and blackening of Bile Esculin agar (Himedia), growth at 6·5% NaCl, 10°C, 45°C, and pH 9·6. The pure cultures were stored at −20°C in Brain–Heart Infusion (Himedia) broth with 30% glycerol. All isolates were identified to the species level using the API 20 STREP (bioMérieux, Marcy l'Etoile, France) biochemical test kit (Peters *et al.* 2003; Citak *et al.* 2004; Canzek Majhenic *et al.* 2005; Jurkovic *et al.* 2006). The results were confirmed by 16S rDNA sequencing using with 27f (AGAGTTTGATCM TGGCTCAG) and 907r (CCGTCAATTCMTTTRAGTTT) universal primers.

Control strains

The study used *Ent. faecalis* NCIMB 700584 (The National Collection of Industrial, Marine and Food Bacteria, UK) as a positive control strain for virulence genes, *Ent. faecalis* ATCC 29212 as a reference strain and a commercial *Ent. faecium* probiotic control strain from Sweden.

Isolation and analysis of plasmid DNA

Plasmid DNAs of the strains were isolated by the procedure described by Anderson and McKay (1983), separated by 0·8% agarose gel electrophoresis and stained with ethidium bromide. Lambda DNA/*Eco*RI+*Hin*dIII marker (SM0191; Fermentas, St Leon-Rot, Germany) was used as the DNA marker in agarose gel electrophoresis. *Enterococcus faecalis* NCIMB 700584 was not included in this analysis.

PCR for detection of virulence genes

Genomic DNAs of enterococcal strains were isolated according to the method of Miteva *et al.* (1991). PCR primers for the virulence genes (Table 1) were selected according to Reviriego *et al.* (2005). PCR amplifications were performed in 50-μl reaction mixtures using 0·01 mol l^{-1} dNTP mix (Promega, Sunnyvale, CA, USA), 500 U Go Taq Flexi DNA polymerase (Promega), 50 ng of DNA and 20 pmol of each primer obtained from IDT (Integrated DNA Technologies, Coralville, IA, USA).

Samples were subjected to 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 for 30 s and elongation at 72°C for 1 min (Reviriego *et al.* 2005).

Screening for antibiotic resistance

The strains were evaluated for resistance against some antibiotics, including ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg), tetracycline (30 μg), penicillin G (10 μg), gentamycin (10 μg) and vancomycin (30 μg) using a disc diffusion method on Muller–Hinton agar (Merck), as described by the Clinical and Laboratory Standards Institute (CLSI, 2006). All antibiotic discs were purchased from Oxoid (UK). Results were interpreted according to the cut-off levels proposed by Charteris *et al.* (1998) for gentamycin and kanamycin and CLSI (2006) for the other antibiotics.

Screening for *vanA* and *vanB* genes

VanA1 [5′-GGG AAA ACG ACA ATT GC-3′] and VanA2 [5′-GTA CAA TGC GGC CGT TA-3′] primers with the product size of 732-bp were used to screen *vanA* gene in enterococcal strains. VanB [5′-GTG CTG CGA GAT ACC ACA GA-3′] and VanBrev [5′-CGA ACA CCA TGC AAC ATT TC′-3′] primers with the product size of 1145-bp were used to screen *vanB* gene in the strains (Reviriego *et al.* 2005). Primers were obtained from IDT (Integrated DNA Technologies). PCR for *vanA* and *vanB* genes were performed as an initial cycle of denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, elongation at 72°C for 1 min and a last cycle at 72°C for 10 min (Dutka-Malen *et al.* 1995).

Table 1 Polymerase chain reaction primers and products used for detection of virulence genes (Reviriego *et al.* 2005)

Genes	Primers	Sequence (5′–3′)	Product size, bp
agg2	TE32	GTT GTT TTA GCA ATG GGG TAT	1210
	TE33	CAC TAC TTG TAA ATT CAT AGA	
efaAfm	TE37	AAC AGA TCC GCA TGA ATA	735
	TE38	CAT TTC ATC ATC TGA TAG TA	
cpd	TE51	TGG TGG GTT ATT TTT CAA TTC	782
	TE52	TAC GGC TCT GGC TTA CTA	
cob	TE49	AAC ATT CAG CAA ACA AAG C	1405
	TE50	TTG TCA TAA AGA GTG GTC AT	
ccf	TE53	GGG AAT TGA GTA GTG AAG AAG	543
	TE54	AGC CGC TAA AAT CGG TAA AAT	
cad	TE42a	TGC TTT GTC ATT GAC AAT CCG	1299
	TE43a	ACT TTT TCC CAA CCC CTC AA	
efaAfs	TE5	GAC AGA CCC TCA CGA ATA	705
	TE6	AGT TCA TCA TGC TGT AGT A	
gelE	TE9	ACC CCG TAT CAT TGG TTT	419
	TE10	ACG CAT TGC TTT TCC ATC	
cylM	TE13	CTG ATG GAA AGA AGA TAG TAT	742
	TE14	TGA GTT GGT CTG ATT ACA TTT	
cylB	TE15	ATT CCT ACC TAT GTT CTG TTA	843
	TE16	AAT AAA CTC TTC TTT TCC AAC	
cylA	TE17	TGG ATG ATA GTG ATA GGA AGT	517
	TE18	TCT ACA GTA AAT CTT TCG TCA	
espfs	TE34	TTG CTA ATG CTA GTC CAC GAC C	933
	TE36	GCG TCA ACA CTT GCA TTG CCG AA	
espfm	TE104	TTG CTA ATG CAA GTC ACG TCC	955
	TE105	GCA TCA ACA CTT GCA TTA CCG AA	

Determination of haemolytic activity

Haemolytic activity of the strains was determined on blood agar with sheep blood (Salubris, Woburn, MA, USA) as described by Citak *et al.* (2004) and Jurkovic *et al.* (2006).

Results

Distribution of strains

The enterococcal load of the naturally fermented cheese, sausage and olive samples were within the ranges 2–7, 2–4 and 2–5 log CFU g^{-1}, respectively. A total of 87 isolates were selected for this study: 69 isolates from cheese; 8 isolates from sausage; 7 isolates from olive and 3 control strains. The selected isolates of *Ent. faecium* (16) and *Ent. faecalis* (68) were identified at species level (≥90%) by using biochemical test kit API 20 Strep.

Detection of virulence genes

The presence of virulence genes among isolates is shown in Table 2. All of the *Ent. faecalis* and *Ent. faecium* isolates ($n = 87$) in this study, including three control strains, carried between 6 and 13 tested virulence genes. Three *Ent. faecalis* and one *Ent. faecium* isolates were positive for all tested virulence genes.

The *espfs* and *espfm* genes, coding for enterococcal surface protein, were determined in 97 and 60% of *Ent. faecalis* isolates and in 81 and 13% of *Ent. faecium* isolates, respectively. The *efaA*fm and *efaAfs* genes, coding for a cell wall adhesin in enterococci, were found in 67 and 100% of *Ent. faecium* isolates and in 15 and 100% of *Ent. faecalis* isolates, respectively.

The sex pheromone determinants (*cpd*, *cob*, *ccf*, *cad*) were present in all tested *Ent. faecium* and *Ent. faecalis* isolates, except for one *Ent. faecium* strain isolated from sausage. The gene *agg*, coding for the AS, was determined in 88% of *Ent. faecium* and in 57% of *Ent. faecalis* isolates.

Some *Ent. faecium* and *Ent. faecalis* strains had cytolysin determinants (*cylM*, *cylB*, *cylA*) at different levels (Table 2). Certain *Ent. faecalis* strains had *cylM* (43%), *cylB* (26%) or *cylA* (34%) and certain *Ent. faecium* strains had *cylM* (50%), *cylB* (19%) or *cylA* (3%). Some of the *Ent. faecalis* and *Ent. faecium* strains carried three or two of these genes together. Furthermore, all tested *Ent. faecium* and *Ent. faecalis* isolates did not show beta-haemolytic activity on blood agar with sheep blood.

The gene *gelE*, coding for extracellular metalloendopeptidase, was present in all the tested *Ent. faecium* and *Ent. faecalis* isolates.

Table 2 The presence of virulence genes, antibiotic resistance and plasmid contents among *Enterococcus faecium* and *Enterococcus faecalis* isolates

Isolate	Source	Species	Virulence genes	Antibiotic resistance	Plasmid contents
S_{1-1}	Sausage	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	CN_R, E_I, K_R	1
S_{1-3}	Sausage	*Ent. faecium*	*efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, TE_R, K_R	2
S_{1-5}	Sausage	*Ent. faecium*	*efaAfm*, *cpd*, *ccf*, *cad*, *efaAfs*, *gelE*	E_I, K_R	3
S_{2-1}	Sausage	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*	CN_R, E_I, K_R	4
S_{2-5}	Sausage	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	CN_R, E_I, K_R	4
S_{3-3}	Sausage	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	TE_I, K_I	4
S_{6-4}	Sausage	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	CN_R, E_I, K_R	3
S_{6-5}	Sausage	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	K_I	2
P_{1-1}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_R, TE_R, C_R	4
P_{1-2}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_I	6
P_{1-3}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_I, VA_I, K_R	1
P_{1-4}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_R, TE_R, C_R	3
P_{1-5}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_I, VA_I, K_I	5
P_{3-1}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_I, VA_I, K_I	2
P_{3-2}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, K_I	1
P_{3-3}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, VA_I	1
P_{3-4}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, TE_R, VA_I, K_I	3
P_{3-5}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_I, TE_R, VA_I, K_I	3
P_{4-1}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I	1
P_{4-2}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, VA_I, K_I	2
P_{4-3}	Cheese	*Ent. faecium*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	CN_R, E_R, K_R	1
P_{4-4}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I	1
P_{4-5}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*		3
P_{5-1}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, TE_R	3
P_{5-2}	Cheese	*Ent. faecalis*	*agg*$_2$ *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, TE_R	2
P_{5-3}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylA*, *espfs*, *espfm*	E_I, TE_R	1
P_{5-4}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_I, TE_R	1
P_{5-5}	Cheese	*Ent. faecium*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, K_R	1
P_{7-1}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_I, K_R	5
P_{7-2}	Cheese	*Ent. faecalis*	*efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	TE_R, C_I	3
P_{7-3}	Cheese	*Ent. faecium*	*agg*$_2$ *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I	3
P_{7-5}	Cheese	*Ent. faecalis*	*efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, TE_R, K_I, C_I	5
P_{9-1}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*		1
P_{9-2}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*		1
P_{9-3}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*		1
P_{9-4}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*		1
P_{9-5}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*		1
P_{10-1}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	TE_R	1
P_{10-2}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, TE_R	1
P_{10-3}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_I, TE_R	1
P_{10-4}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, TE_R	1
P_{10-5}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_I, TE_R	1
P_{11-1}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, C_I	1
P_{11-2}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*	E_I	1
P_{11-3}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *espfs*, *espfm*	E_I	1
P_{11-4}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *espfs*, *espfm*	E_I, C_I	1
P_{12-1}	Cheese	*Ent. faecalis*	*efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*, *espfm*	E_I, TE_R	1
P_{12-2}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *espfs*, *espfm*	E_I, TE_R	1
P_{12-3}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *espfs*, *espfm*	E_I	1
P_{13-1}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*	TE_R	3
P_{13-2}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*	TE_R	3
P_{13-3}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*	TE_R, C_R	1
P_{14-2}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylA*, *espfs*, *espfm*	TE_R	1

Table 2 (*Continued*)

Isolate	Source	Species	Virulence genes	Antibiotic resistance	Plasmid contents
P_{14-3}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylA*, *espfs*, *espfm*	TE_R	1
P_{14-4}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*	E_I	1
P_{14-5}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*	TE_R, C_I	1
P_{15-3}	Cheese	*Ent. faecium*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*	E_I, K_I	1
P_{17-1}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylA*, *espfs*, *espfm*		3
P_{17-2}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylA*, *espfs*, *espfm*		1
P_{17-3}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*		1
P_{17-4}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*		1
P_{17-5}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*		1
P_{18-1}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*	E_I	1
P_{18-2}	Cheese	*Ent. faecalis*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*	E_R, TE_R, C_I	1
P_{18-3}	Cheese	*Ent. faecalis*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*	E_R, TE_R	4
P_{18-4}	Cheese	*Ent. faecalis*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*	E_I	2
P_{18-5}	Cheese	*Ent. faecalis*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*	E_R, TE_R	4
P_{19-1}	Cheese	*Ent. faecalis*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylA*	E_I	5
P_{19-2}	Cheese	*Ent. faecalis*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylA*, *espfs*	E_I	4
P_{19-3}	Cheese	*Ent. faecalis*	*cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *espfs*, *espfm*		1
P_{19-4}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*	E_I	2
P_{19-5}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *espfs*, *espfm*	E_I	5
P_{20-1}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*	E_I, K_I	1
P_{20-2}	Cheese	*Ent. faecium*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, VA_I, K_R	2
P_{20-3}	Cheese	*Ent. faecium*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *espfs*	E_I, VA_I, K_I	2
P_{20-4}	Cheese	*Ent. faecium*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *espfs*	E_I, VA_I, K_R	2
P_{20-5}	Cheese	*Ent. faecalis*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *espfs*, *espfm*	E_I	1
Z_{11-1}	Olive	*Ent. faecium*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylA*, *espfs*	E_R, K_R	4
Z_{11-2}	Olive	*Ent. faecium*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylA*	E_R, K_R	4
Z_{11-3}	Olive	*Ent. faecium*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*	E_R, K_R	4
Z_{11-4}	Olive	*Ent. faecium*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *espfs*	E_R, TE_I, K_R	4
Z_{11-5}	Olive	*Ent. faecium*	*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *espfs*	E_R, TE_I, K_R	4
Z_{20-1}	Olive	*Ent. faecium*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *espfs*	E_I, K_R	1
Z_{20-2}	Olive	*Ent. faecium*	*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylB*, *cylA*, *espfm*	E_I, K_R	1
Ent. faecalis ATCC 29212			*agg*$_2$, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylA*, *espfs*, *espfm*	E_I, TE_R	3
Ent. faecium probiotic control strain			*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylA*, *espfs*	E_R, VA_I, K_R	4
Ent. faecalis NCIMB 700584			*agg*$_2$, *efaAfm*, *cpd*, *cop*, *ccf*, *cad*, *efaAfs*, *gelE*, *cylM*, *cylB*, *cylA*, *espfs*, *espfm*	E_I, K_R	ND

CN_R, Gentamycin resistance; 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; ND, not determined.

Screening for antibiotic resistance and *vanA* and *vanB* genes

The majority of the isolates were found to be susceptible to the tested antibiotics (Table 2). However, some tested enterococcal strains were highly resistant to erythromycin (14%), tetracycline (32%), gentamycin (6%), kanamycin (24%) and chloramphenicol (3%). The number of antibiotic resistant strains of *Ent. faecalis* was higher than that of *Ent. faecium* strains. An intermediate level of vancomycin resistance was detected in three *Ent. faecium* (3%) and eight *Ent. faecalis* (9%) strains. Although the *vanB* gene was not detected in any isolate, the *vanA* gene was found in two of the *Ent. faecalis* strains isolated from sausage (S_{6-4}) and cheese (P_{3-1}). The *Ent. faecalis* S_{6-4} strain did not show vancomycin resistance; however, the *Ent. faecalis* P_{3-1} strain showed intermediate-level resistance to vancomycin (Table 2). In addition, eight *Ent. faecium* (9%) and ten *Ent. faecalis* (11%) isolates had multiple antibiotic resistance.

Plasmid profiles

The plasmid contents of *Ent. faecium* and *Ent. faecalis* isolates and the plasmid profiles of some *Ent. faecium* and *Ent. faecalis* isolates are shown in Table 2 and Figs 1 and 2, respectively. All tested *Ent. faecalis* and *Ent. faecium* strains carried a certain number of plasmids with

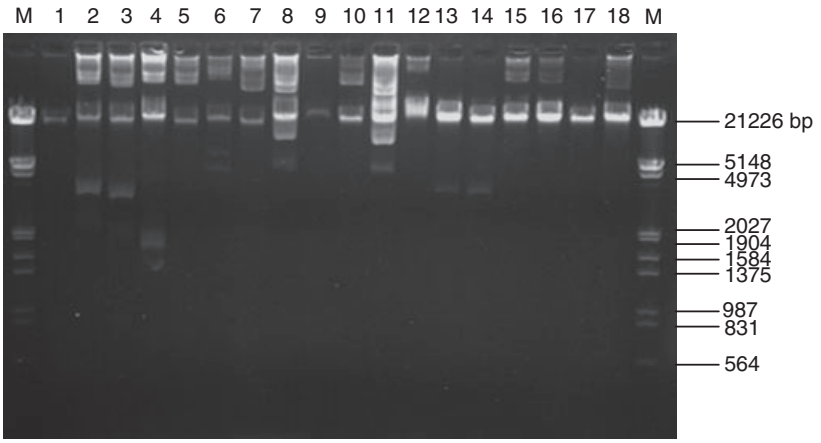

Figure 1 Plasmid profiles of some *Enterococcus faecalis* isolates. [1. $S_{1\text{-}1}$, 2. $S_{2\text{-}1}$, 3. $S_{2\text{-}5}$, 4. $S_{3\text{-}3}$, 5. $S_{6\text{-}4}$, 6. $S_{6\text{-}5}$, 7. $P_{1\text{-}1}$, 8. $P_{1\text{-}2}$, 9. $P_{1\text{-}3}$, 10. $P_{1\text{-}4}$, 11. $P_{1\text{-}5}$, 12. $P_{3\text{-}1}$, 13. $P_{3\text{-}2}$, 14. $P_{3\text{-}3}$, 15. $P_{3\text{-}4}$, 16. $P_{3\text{-}5}$, 17. $P_{4\text{-}1}$, 18. *Ent. faecalis* ATCC 29212 reference strain, M: Lambda DNA *Eco*RI + *Hin*dIII marker. (Number of 1–6 strains isolated from sausage samples, number of 7–17 strains isolated from cheese samples)].

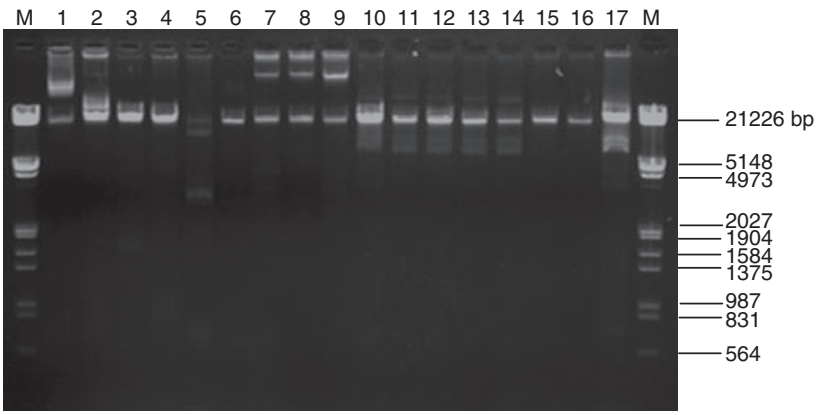

Figure 2 Plasmid profiles of some *Enterococcus faecium* isolates. [1. $S_{1\text{-}3}$, 2. $S_{1\text{-}5}$, 3. $P_{4\text{-}3}$, 4. $P_{5\text{-}5}$, 5. $P_{7\text{-}3}$, 6. $P_{15\text{-}3}$, 7. $P_{20\text{-}2}$, 8. $P_{20\text{-}3}$, 9. $P_{20\text{-}4}$, 10. $Z_{11\text{-}1}$, 11. $Z_{11\text{-}2}$, 12. $Z_{11\text{-}3}$, 13. $Z_{11\text{-}4}$, 14. $Z_{11\text{-}5}$, 15. $Z_{20\text{-}1}$, 16. $Z_{20\text{-}2}$, 17. *Ent. faecium* probiotic control strain, M: Lambda DNA *Eco*RI + *Hin*dIII marker. (Number of 1 and 2 strains isolated from sausage samples, number of 3–9 strains isolated from cheese samples and number of 10–16 strains isolated from olive samples)].

different molecular sizes. The number of plasmids varied between one and six. The strains of *Ent. faecalis* $S_{6\text{-}4}$ and *Ent. faecalis* $P_{3\text{-}1}$ that carried the *vanA* gene had three and two plasmids, respectively of 21 kb and larger molecular size.

Discussion

Enterococci are resistant to inappropriate environmental conditions, such as high temperature, low pH. Therefore, they can be isolated, particularly from traditional fermented cheese and meat products. Some foods, such as meat products, can be spoiled by these bacteria. Enterococci are also considered as an indicator of inappropriate sanitary conditions in food processing (Giraffa 2002). The counts of enterococci in the cheese samples of this study were higher than those reported by Temelli *et al.* (2006) in Turkish white cheeses (3·78 log CFU g^{-1}). *Enterococcus faecalis* and *Ent. faecium* strains were predominantly isolated from the cheese samples in this study. Similar results were reported by Citak *et al.* (2004). The enterococcal load of the sausage samples in this study (2–8 log CFU g^{-1}) was lower than in a study by Sırıken *et al.* (2006). All of these results indicate the importance of enterococcal contamination in terms of the quality of naturally fermented Turkish cheeses and sausages.

Molecular-based studies such as protein profile studies and RAPD–PCR studies continue to be made with the aim of finding out whether the strains isolated from the same food sample have the same phylogenetic structure or not.

The genes coding for enterococcal surface protein and cell wall adhesin (*espfs*, *espfm*, *efaA*fm and *efaAfs*) in *Ent. faecium* and *Ent. faecalis* strains were found to be higher in this study than those reported in previous studies (Eaton and Gasson 2001; Franz *et al.* 2001; Semedo *et al.* 2003; Reviriego *et al.* 2005). Martin *et al.* (2005) stated that all *Ent. faecalis* and *Ent. faecium* strains isolated from fermented sausages carried *efaAfs* and *efaAfm* genes, respectively. Semedo *et al.* (2003) also reported that the *efaAfs* gene occurred widely in enterococci, irrespectively of species. This finding was supported by the results of this study.

The genes *cpd*, *cob*, *ccf*, and *cad* were identified in a large number of the strains. Eaton and Gasson (2001) reported that sex pheromone determinants were detected only in *Ent. faecalis* strains while all of the *Ent. faecium* strains were clear of the *agg* and sex pheromone determinants. However, Semedo *et al.* (2003) indicated that the *agg* gene was detected in all isolates from food, clinical origin and reference strains, including *Ent. faecium*. The results of this study agreed with the findings of Semedo *et al.* (2003). Eaton and Gasson (2001) also stated that pheromone determinants sometimes occurred with and sometimes without the *agg* virulence gene; however, the *agg* virulence gene was always associated with the presence of pheromone determinants. This study produced similar results.

Although beta-haemolytic activity was not present in any of the tested isolates, some isolates carried haemolysin-related genes (*cylM*, *cylB*, *cylA*). It was thought that the cytolysin determinants (*cylM*, *cylB*, *cylA*) behaved as silent genes in most nonhaemolytic isolates (Eaton and Gasson 2001; Semedo *et al.* 2003).

Antibiotic resistance is an important characteristic of enterococcal strains. This characteristic may be transferred to pathogens in the food environment, such as *Listeria monocytogenes* and *Staphylococcus aureus* by plasmids (Franz *et al.* 1999). Similar to the results of this study, erythromycin, gentamycin, tetracycline and chloramphenicol resistance were also reported by Citak *et al.* (2004) in enterococci species isolated from Turkish white cheese.

This study identified intermediate-level vancomycin resistance in certain strains and the *vanA* gene in two *Ent. faecalis* isolates. These strains also had large plasmids, with molecular size of 21 kb or more. Eaton and Gasson (2001) emphasized that the gene transfer system of *Enterococcus* was associated with acquired antibiotic resistance. Acquired antibiotic resistance was generally linked with the VanA phenotype and was located on large plasmids. As with the results of this study, Giraffa *et al.* (2000) and Cariolato *et al.* (2008) also reported that cheese-originated *Ent. faecium* strains carried the *vanA* gene and showed resistance to vancomycin in phenotype. Vancomycin resistance in foodborne enterococci was also reported by other researchers (Robredo *et al.* 2000; Citak *et al.* 2004, 2005).

In this study, multiple-antibiotic-resistant *Ent. faecalis* and *Ent. faecium* isolates contained between 1 and 4 plasmids usually with a molecular size of 21226–5148 bp or larger. Coleri *et al.* (2004) determined that clinical enterococci isolates carried between 1 and 11 plasmids, ranging in size from 2·08 to 56·15 kb. They also reported plasmid-mediated antibiotic resistance in enterococci. Abriouel *et al.* (2006) reported that most food-sourced enterococci isolates had different plasmids with estimated sizes from 2·5 to 53 kb. They also indicated that virulence determinants and antibiotic resistance traits of enterococci may be plasmid-borne; therefore, their potential risk in food applications needed to be carefully evaluated. Several previous studies examined the antibiotic resistance of enterococci isolated from foods in Turkey (Citak *et al.* 2004, 2005; Koluman *et al.* 2009), but prior studies on the virulence determinants and *vanA* and *vanB* genes in Turkish foods could not be found.

The results of this study indicated that the *Ent. faecium* probiotic control strain carried 11 of 13 tested virulence genes. This strain was also resistant to erythromycin and kanamycin. Temmerman *et al.* (2003) defined 29 of *Ent. faecium* strains isolated from probiotic products, which were resistant to kanamycin (90%), tetracycline (24%), penicillin G (41%), erythromycin (97%), chloramphenicol (34%) and vancomycin (38%). They suggested that continuous attention should be paid to the selection of probiotic strains that are free of transferable antibiotic resistance. Eaton and Gasson (2001) reported two *Ent. faecalis* starter strains that carried multiple virulence determinants and demonstrated that starter strains acquired additional virulence determinants from medical strains. They suggested that the use of *Enterococcus* spp. in foods required careful safety evaluation.

In conclusion, the results of this study indicated that *Ent. faecium* and *Ent. faecalis* strains isolated from Turkish white cheese, sausage and olive samples carried most of the virulence genes tested and some antibiotic resistance traits. Certain strains also had the *vanA* gene and some isolates showed intermediate-level vancomycin resistance in phenotype. Most tested strains contained large plasmids, which might harbour acquired antibiotic resistance. The findings of this study suggest that food origin strains of *Ent. faecium* and *Ent. faecalis* may be potential risk factors for consumer health in terms of virulence genes and antibiotic resistance. More detailed studies should be performed on the selection of appropriate enterococcal starter, probiotic and cocultures in food applications. Further investigations are also needed for the determination of virulence gene expressions in phenotype and possibility of gene transfer to other bacteria in the food environment.

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