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ARTICLE

Characterisation of Phenotypic and Genotypic Antibiotic Resistance Profile of Enterococci from Cheeses in Turkey

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Abstract

The aim of this study was to determine the prevalence of enterococci in cheese samples and to characterize their antimicrobial resistance profiles as well as the associated resistance genes. A total of 139 enterococci were isolated from 99 cheese samples, the isolates were identified as *E. faecalis* (61.2%), *E. faecium* (15.1%), *E. gallinarum* (12.9%), *E. durans* (5.0%), *E. casseliflavis* (2.9%) and *E. avium* (2.9%). The most frequent antimicrobial resistance observed in enterococci isolates was to lincomycin (88.5%), followed by kanamycin (84.2%), gentamycin (low level, 51.1%), rifampin (46.8%) and tetracycline (33.8%). Among the isolates, the frequencies of high level gentamycin and streptomycin resistant enterococci strains were 2.2% and 5.8%, respectively. Apart from the mentioned antibiotics, low levels of resistance to ciprofloxacin, erythromycin and chloramphenicol were found. Moreover no resistance was observed against penicillin and ampicillin. The antimicrobial resistance genes including *tetM*, *tetL*, *ermB*, *cat*, *aph(3')-IIIa*, *ant(6)-Ia* and *aac(6')-Ie-aph(2")-Ia* were found in enterococci from Turkish cheese samples. In the current study, we provided data for antibiotic resistance and the occurrence of resistance genes among enterococci. Regulatory and quality control programs for milk and other dairy products from farms to retail outlets has to be established and strengthened to monitor trends in antimicrobial resistance among emerging food borne pathogens in Turkey.

Keywords: enterococci, cheese, antimicrobial resistance, resistance genes

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Introduction

Enterococci are Gram-positive cocci bacteria that belong to the family enterococcaceae. Enterococci strains are widespread in nature (soil, water and foods) and have been shown to play an important role in contributing to ripening and flavouring processes of certain foods (Foulquie *et al.,* 2006; Franz *et al.,* 1999). In addition to contribution of quality of finished products, some strain of enterococci are believed to be involved in the preservation of foods against food borne pathogens such as *Listeria monocytogenes* with bacteriocin (Ahmadova *et al.,* 2013). Even though enteroccoci were used to be considered as harmless inhabitants of the gut flora of humans and animals, these organisms are one of the leading causes of nosocomial infections (Leavis *et al.,* 2006). *E. feacalis* and *E. faecium* are by far the most prevalent species accounting for over 90% of these infections worldwide (Treitman *et al.,* 2005). Infections associated with enterococci used to be treated successfully with antibiotic treatments, but many antibiotics are currently less effective resulting in longer hospitalization periods, treatment failure and significant financial burdens (DiazGranados *et al.,* 2005). This rapid and global dissemination of multidrug resistant strains were attributed to the imprudent and overuse of antibiotics in human and veterinary practices as well as inappropriate use in animal production (Borgen *et al.,* 2000).

There are a number of well-established international organizations and government agencies such as the Japanese Veterinary Antimicrobial Resistance Monitoring Sys-

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tem and DANMAP to monitor antibiotic resistance in order to perform risk assessment for public health (Harada and Asai, 2010). In recent years, efforts have been made to provide some knowledge on the prevalence of enterococci from foods of animal origin, and their antimicrobial resistance worldwide (Hammad *et al.,* 2015; Jamet *et al.,* 2012; Yilmaz *et al.,* 2016). There is evidence supporting the potential transmission of enterococci via the consumption of contaminated foods of animal origin (Olsen *et al.,* 2012). Based on the importance of the emergence of antimicrobial resistant strains of pathogens in foods of animal origins, some studies have examined the prevalence and the frequency of antimicrobial resistance in Enterococci from different retail samples in Turkey (Çitak *et al.,* 2004; Koluman *et al.,* 2009; Özmen Toğay *et al.,* 2005), however no study has been conducted regarding the genetic mechanisms of resistance to aminoglycosides, erythromycin, tetracycline and vancomycin among enterococci isolated from cheese in Turkey. Therefore, the biodiversity of the enterococcal species isolated from eleven different cheeses and their antibiotic resistance profile was determined in this study. In addition, the presence of several antibiotic resistance genes was also screened.

Materials and Methods

Sample collection

In total, one hundred cheese samples were randomly collected from supermarkets, retail markets and open-air markets between January and July 2014 in Hatay, Turkey. The analysed-cheeses consisted of eleven different types of hard, soft and semi-soft ripened cheeses (White, Kasar, Tulum, Ezine, Antep, Sülk, Lor, Van Otlu, Civil, Orgu and Dil) and manufactured in different geographical areas of Turkey. All these cheese samples were collected in sterile bags and transferred in ice-boxes and investigated immediately after arrival at the laboratory.

Isolation and identification of *Enterococcus* spp.

From each cheese, 25 g of sample was removed and blended for 2 min in a stomacher (BagMixer® 400P, Interscience, France) with 225 mL of buffered peptone water (pH 7.0). Homogenised cheese samples were incubated overnight at 37ºC. After that, an aliquot of 100 μL of enriched suspension was added into enterococcal broth and incubated at 37ºC for 24 h. Post-enrichment, 10 μL of this was streaked on the enterococcal agar with and without vancomycin (6 mg/L). From each agar plates, 1-2 suspected colonies with typical enterococci morphology were picked and plated on the blood agar plates. The isolates were identified biochemically by using VITEK2 System (bioMérieux, Marcy-l'Étoile, France).

Antibiotic susceptibility profiles

The susceptibility pattern determination was performed by disk diffusion method on Mueller Hinton agar with antibiotic disks according to the Clinical Laboratory Standards Institute (CLSI, 2012). Fifteen different antibiotics were used: gentamicin (10 μg/disc and 120 μg/disc), streptomycin (300 μg/disc), kanamycin (30 μg/disc), ciprofloxacin (5 μg/disc), vancomycin (30 μg/disc), teicoplanin (30 μg/disc), linezolid (30 μg/disc), lincomycin (10 μg/disc), erythromycin (15 μg/disc), penicillin (10 μg/disc), ampicillin (10 μg/disc), tetracycline (30 μg/disc), chloramphenicol (30 μg/disc), rifampin (5 μg/disc) and quinopristine/dalfopristine (4.5/10.5 μg/disc). *E. casseliflavis* (ATCC 700327) and *Staphylococcus aureus* (ATCC 29213) strains were used as positive controls. The results of the antimicrobial susceptibilities were interpreted according to the CLSI guidelines.

Analysis of the molecular mechanisms of antibiotic resistance

Genomic DNA was extracted by using GeneMATRIX bacterial & yeast genomic DNA purification kit (EURx ltd. Gdansk, Poland) according to manufacturer's instruction. DNA to be analyzed was stored at -20°C. The identification of the genes (*erm*A, *erm*B, *mef*A/E, *tet*K, *tet*L, *tet*M and *tet*O) that involve in tetracycline and macrolide resistance was performed by a multiplex-PCR approach according to Malhotra-Kumar *et al.* (2005). All vancomycin resistant, including intermediate resistant, enterococci were screened for resistance genes (*vanA, vanB, vanC1/2, vanD, vanE, vanG*) by PCR as previously described (Depardieu *et al.,* 2004). The presence of aminoglycosides resistance associated genes (*aac(6)-Ie-aph(2)-Ia, aph(2)-Ib, aph(2)-Ic, aph(2)-Id, aph(3)-IIIa, ant(4)-Ia* (Vakulenko *et al.,* 2003) and chloramphenicol resistance gene (*cat)* (Aarestrup *et al,.* 2000) were also determined by PCR. PCR reactions were carried out with primers stated by abovementioned references.

Results and Discussion

Although the importance of enterococci in food industry as probiotics and starter cultures is unquestioned, they have commonly been implicated in nosocomial infections worldwide (Arias *et al.,* 2010). Having demonstrated that the virulence traits in enterococci strains from food samples were found to be identical to those obtained from humans suggesting that the consumption of animal foods could be a significant source of enterococcal infections (Olsen *et al.,* 2012). Enterococci can tolerate high concentration of salt and contaminate milk and its products easily due to poor hygienic practices at any point of manufacturing. Hence, the incidence and prevalence of enterococci in foods of animal origin have been examined worldwide. Presently, there are some data on the presence of enterococci at the concentration up to 10^6 CFU/g in cheese samples in Turkey (Aygun *et al.,* 2005; Özmen Toğay *et al.,* 2010). Out of 100 cheese samples, 99 contained enterococci with 61.2% of the isolates identified as *E. faecalis*, 15.1% as *E. faecium*, 12.9% as *E. gallinarum*, 5.0% as *E. durans*, 2.9% as *E. casseliflavis* and 2.9% as *E. avium* in the current study.

In Turkey, the prevalence of enterococci in cheese samples was reported to range between 60 and 100% according to the prior studies (Çitak *et al.,* 2004; Koluman *et al.,* 2009; Özmen Toğay *et al.,* 2010). The most recently observed prevalence of enterococci in dairy products in other countries include 100% in Brazil (Furlaneto-Maia *et al.,* 2014), 90% in Egypt (Hammad *et al.,* 2015), 72% in France (Jamet *et al.,* 2012) and 27% in Italy (Pesavento, 2014). The prevalence and species distribution of enterococci in foods of animal origin varies by country and type of foods. It was also previously shown that known factors in manufacturing process such as pasteurization of milk can influence the abundance and prevalence of enterococci in the final products (Jamet *et al.,* 2012). In this study, *E. faecalis* was found to be the most frequently isolated species which is in accordance to data published in European countries and in Turkey (Jamet *et al.,* 2012; Koluman *et al.,* 2009; Nieto-Arribas *et al.,* 2011; Özmen Toğay *et al.,* 2010). In contrast, there are also some studies reporting *E. faecium* being the most frequently isolated species (Hammad *et al.,* 2015; Tuncer, 2009).

Enterococci are noted for their intrinsic resistance to aminoglycoside (low level) and β-lactam antibiotics. The high level resistance to gentamicin and streptomycin is of significant importance because of their use in the treatment of enterococcal infections (Arias *et al.,* 2010) and characterised by no growth zone around the discs (120 μg for gentamycin and 300 μg for streptomycin) (CLSI, 2012). In the current study, 84.2% (117/139) of enterococci isolates were found to be resistant to kanamycin, 5.8% (8/139) to high level streptomycin and 51.1% (71/139) to gentamycin, whereas high level gentamycin resistance was found in three (2.2%) isolates (two *E. faecalis*, and one *E. gallinarum*) (Table 1). The multiplex-PCR analysis showed that two *E. feacalis* isolates harbor the *aph(3)-IIIa* gene and a single *E. gallinarum* isolate had the *acc6-le-aph(2)-la* gene (Table 2). Recently, we detected the *aph (3')-IIIa*, *ant(6)-Ia* and *aac(6')-Ie-aph(2")-Ia* genes in *E. feacalis* isolates obtained from chicken meat (Yilmaz *et al.,* 2016). Our results are higher than those recently noted in Egypt where only one *E. faecium* strain with aph (3)

Table 1. Antimicrobial susceptibilities of *Enterococci* isolates from cheeses in Turkey

Antibiotics[1]	Number (%) of resistant strains						Total (n=139)
	E. faecalis (n=85)	*E. faecium* (n=21)	*E. gallinarum* (n=18)	*E. casseliflavis* (n=4)	*E. durans* (n=7)	*E. avium* (n=4)	
TEC	9 (10.6)	0	0	0	0	0	9 (6.5)
VA	3 (3.5)	0	0	0	0	0	3 (2.2)
CN (120 μg)	2 (2.4)	0	1 (5.6)	0	0	0	3 (2.2)
CN (10 μg)	61 (71.8)	7 (33.3)	2 (11.1)	0	0	1 (25.0)	71 (51.1)
K	67 (78.8)	21 (100.0)	17 (94.4)	4 (100.0)	7 (100.0)	1 (25.0)	117 (84.2)
RD	46 (54.1)	14 (66.7)	3 (16.7)	1 (25.0)	1 (14.3)	0	65 (46.8)
TE	40 (47.1)	0	5 (27.8)	1 (25.0)	0	1 (25.0)	47 (33.8)
E	8 (9.4)	7 (33.3)	1 (5.6)	0	0	0	16 (11.5)
MY	84 (98.8)	10 (47.6)	18 (100.0)	4 (100.0)	3 (42.9)	4 (100.0)	123 (88.5)
P	0	0	0	0	0	0	0/0
AMP	0	0	0	0	0	0	0/0
LZD	6 (7.1)	0	2 (11.1)	0	0	0	8 (5.8)
QD	55 (64.7)	1 (4.8)	2 (11.1)	0	0	1 (25.0)	59 (42.4)
C	4 (4.7)	0	0	0	0	1 (25.0)	5 (3.6)
S	7 (8.2)	0	1 (5.6)	0	0	0	8 (5.8)
CIP	0	3 (14.3)	1 (5.6)	0	0	0	4 (2.9)

[1]TEC, teicoplanin; VA, vancomycin; CN, gentamicin; K, kanamycin; RD, rifampin; TE, tetracycline; E, erithromycin; MY, lincomycin; P, penicillin; AMP, ampicillin; LZD, linezolid; QD, quinopristine/dalfopristine; C, chloramphenicol; S, streptomycin; CIP, ciprofloxacin.

Table 2. The genotypic and phenotypic characteristics of *Enterococcus* spp. that harboured resistance genes

Species	Antibiotic resistance phenotype	Antibiotic Resistance Genes
E. faecalis	K, TE, E, MY, QD	tetM, ermB
E. faecalis	CN K, TE, MY	tetM
E. faecalis	CN, K, TE, MY, QD	tetM
E. faecalis	CN, K, TE, MY, QD, S	tetM
E. faecalis	CN, K, TE, E, MY, QD, C, S	tetM, ermB, cad
E. faecalis	CN, K, TE, MY	tetM
E. faecalis	CN, K, RD, TE, MY	tetM
E. faecalis	CN, K, RD, TE, MY, QD	tetM
E. faecalis	K, TE, MY,	tetM
E. faecalis	CN, K, RD, TE, MY	tetM
E. faecalis	CN, TE, MY,	tetM
E. faecalis	CN, K, RD, TE, MY	tetM
E. faecalis	CN, K, RD, MY	tetM
E. faecalis	TE, MY, QD	tetM
E. faecalis	TE, MY	tetM
E. faecalis	RD, TE, MY, QD	tetM
E. faecalis	CN, K, TE, E, MY, QD, C, S	tetM, tetL, ermB, cat
E. faecalis	CN, CN (120), K, RD, TE, E, MY, QD, S	tetM, tetL, cat, aph(3)IIIa
E. faecalis	K, RD, TE, MY,	tetM
E. faecalis	CN, K, RD, TE, MY	tetM
E. faecalis	RD, TE, MY	tetM
E. faecalis	TE, MY	tetM
E. faecalis	K, TE, MY, S	tetM
E. faecalis	TE, MY, QD	tetM
E. faecalis	CN, K, TE, MY, QD	tetM
E. faecalis	CN, CN (120), K, MY, QD	acc6-le-aph(2)-la
E. faecalis	CN, K, TE, E, MY, QD, C, S	tetM, ermB, cat
E. faecalis	CN, K, RD, TE, E, MY, QD, C, S	tetM, ermB, cat
E. faecalis	CN, K, RD, TE, MY, QD	tetM
E. faecalis	TEC, CN, K, RD, TE, MY, QD	tetM
E. faecalis	CN, K, RD, TE, MY, LZD, QD	tetM
E. faecalis	TEC, CN, K, RD, TE, MY	tetM
E. faecalis	CN, K, RD, TE, MY	tetM
E. faecalis	CN, K, RD, TE, MY	tetM
E. faecalis	CN, K, RD, TE, MY, QD	tetM
E. faecium	K, RD, E, MY	tetL
E. gallinarum	CN, CN (120), K, TE, E, MY, LZD, QD, S, CIP	tetM, ermB, aph(3)-IIIa
E. avium	CN, K, TE, MY, QD, C	tetM
E. casseliflavis	K, TE, MY	tetM

VA, vancomycin; CN, gentamicin; K, kanamycin; RD, rifampin; TE, tetracycline; E, erithromycin; MY, lincomycin; LZD, linezolid; QD, quinopristine/dalfopristine; C, chloramphenicol; S, streptomycin; CIP, ciprofloxacin.

gene was recorded (Hammad *et al.,* 2015). A recent study from France reported that only 7% of raw cheese samples were contaminated with low level gentamycin of which three strains had the *aph2-aac6* gene (Jamet *et al.,* 2012). In addition, there was no high level gentamycin enterococci found in Spanish cheeses (Nieto-Arribas *et al.,* 2011). The occurrence of high level resistance to gentamicin has previously reported among enterococci isolates from clinical infections (Araoka *et al*., 2011; Dorabat *et al*., 2010), animals (Choi and Woo, 2013; Liu *et al*., 2012) and foods of animal origins (Hammad *et al.,* 2014; Hammad *et al.,* 2015). However, to our best knowledge, this study revealed the presence of *E. feacalis* and *E. gallinarum* with aminoglycoside resistance genes from cheeses in Turkey for the first time.

The glycopeptide group of antibacterial agents (vancomycin and teicoplanin) are recognised as very reliable reserve antibiotics against multi-drug resistance enterococci in humans (Kos *et al.,* 2012). In 1988, vancomycin resistant enterococci (VRE) was first reported in European countries (Leclercq *et al.,* 1988; Uttley *et al.,* 1988), and since then VRE have been frequently isolated worldwide. From a total of 100 cheese samples, only five (5%) samples were found to be contaminated with VRE in this study (Table 1). Of the five VRE isolates, three (3.5%) were found to have full resistance while other two were with intermediate resistance. In 2004, a study carried out by Çitak *et al.* (2004) revealed a high frequency of vancomycin resistance among *E. feacalis* and *E. faecium* from Turkish white cheese as high as 96.8% and 76% respectively. However, a more recent report revealed lower levels (13.1%) of intermediate vancomycin resistance among enterococci isolated from fermented foods including cheese in Turkey (Özmen Toğay *et al.,* 2010). Recently, studies reported vancomycin resistance rates among enterococci from dairy products in Europe ranging between none in France (Jamet *et al.,* 2012), and 2% in Spain (Nieto-Arribas *et al.*, 2011). There have been nine types of glycopeptide resistance (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanI*, *vanM* and *vanN*) characterized and reported in enterococci so far. Despite recent study in which the *vanA* gene was shown to be present in 7% of enterococci obtained from chicken meat in Turkey (Yilmaz *et al.,* 2016), we did not find any glycopeptide resistance genes tested. Similarly, in Egypt, 5% of enterococci isolates from Egyptian raw Karish cheeses were found to be vancomycin resistant but vancomycin resistant genes were not detected in these isolates (Hammad *et al.,* 2015). The results of this study also demonstrate that VRE has decreased dramatically from over 70% in 2004 (Çitak *et al.,* 2004) to 5% in the current study. Because widespread use of avoparcin as a growth promoting agent in animal production led to emergence of vancomycin-resistant enterococci in humans, animal and animal products, this dramatic decrease might be attributed to the ban of antibiotic usage in animal production starting from 2006. When the use of avoparcin in animal production was banned in 1996, the prevalence of VRE decreased markedly in European countries (Borgen *et al.,* 2000; Bortolaia *et al.,* 2015).

Resistance to other class of antibiotics including erythromycin and tetracycline among enterococci obtained from foods of animal origin has been previously described as a common trait (Hammad *et al.,* 2015; Jamet *et al.,* 2012; Koluman *et al.,* 2009; Nieto-Arribas *et al.,* 2011). In our study, the highest resistance was observed for lincomycin (88.5%) (Table 1), which is not surprising as most enterococci were reported to have intrinsic resistance to lincosamides (Klare *et al.,* 2003). About 11.5-34% of the enterococcal isolates were also resistant to tetracycline and erythromycin, respectively. A low level resistance to ciprofloxacin (2.9%) and resistance to chloramphenicol (3.6%) was noticed in this study. Yet, all enterococci strains were sensitive to ampicillin and penicillin. Antibiotic resistance profile we observed in this study was in accordance with those reported for the fermented Turkish foods and chicken meats in turkey (Özmen Toğay *et al.,* 2010; Yilmaz *et al.,* 2015) and cheeses in Europe (Jamet *et al.,* 2012; Nieto-Arribas *et al.,* 2011) and in Egypt (Hammad *et al.,* 2015). The high frequency of resistance to tetracycline among enterococci isolated from animal foods has previously been attributed to its extensive usage in veterinary practice (Hammad *et al.,* 2015). In contrast to our findings, Çitak *et al.* (2004) reported higher levels of resistance among enterococci isolated from Turkish white cheese sample, which may be related to the ban of antibiotic usage in animal production as a growth promoter in 2006. This study found that *E. feacalis* strains were more resistant to antibiotics compared to other species. In a study carried out in Italy, *E. feacalis* strains were found to be resistant to antibiotics more frequently than other species (Pesavento *et al.,* 2014). In addition, the strains of *E. durans* were found to be less antibiotic resistant when compared to other species, which is not surprising as *E. durans* has long been generally used as starter cultures in dairy technology (Litopoulou-Tzanetaki *et al.,* 1993; Pesavento *et al.,* 2014). The gene encoding 23S rRNA methylases, *ermB*, was found in five *E. faecalis* strains and in a single *E. gallinarum* strains (Table 2). Of the six tetracycline resistant genes, *tetM* was present in 26.6% (37/139) of the isolates, followed by *tetL* gene that was present in only 2.2% (3/139) (Table 2). We recently observed *tetM* as the most predominant gene in tetracycline resistant enterococci found in raw minced meat in Turkey (Yilmaz *et al.,* 2015). The five chloramphenicol resistance *E. feacalis* strains (one having intermediate resistance) had *cat* gene, but this gene was not present in *E. avium* strain that was also resistant to choloramphenicol (Table 2). The presence and particular involvement of *tetM*, *tetL*, *ermB* and *cat* genes for resistance phenotypes has already been presented in enterococci isolated from foods of animal origin including cheese around the world (Hammad *et al.,* 2014; Jamet *et al.,* 2012; Nieto-Arribas *et al.,* 2011).

Some antibiotics (quinopristine/dalfopristine, rifampin and linezolid) examined in this study are critically important classes of antibiotics for the treatment of VRE infections evaluated by WHO (2011) and there is no data available for their usage in veterinary medicine in Turkey. In this study, 46.8% of the isolates were found to be resistant to rifampin and 42.4% were resistant to quinopristine/dalfopristine with *E. feacalis* (64.7%; 55/85) showing higher resistance than *E. avium* (25%; 1/4), *E. gallinarum* (11.1%; 2/18) and *E. faecium* (4.8%; 1/21) (Table 1). We found that 5.8% of isolates were resistant to linezolid (six *E. faecalis* and two *E. gallinarum*). Quinopristine/dalfopristine is one of the approved antibiotics for the treatment of vancomycin resistant *E. faecium* infections (Liu *et al.,* 2012) which necessitates the regular monitoring of resistance against these classes of antibiotics among the isolates obtained from foods of animal origin.

As a result, this study does not produce enough evidence to rule out that cheese samples are a definitive vehicle of infection in humans. However, the occurrence of multidrug resistant enterococci in cheese samples highlights a potential source for humans. It seems noteworthy that the presence of antibiotic resistant enterococci in cheese possesses a health risk since vancomycin resistant infections are highly associated with VRE colonization in humans (Kim *et al.,* 2012). In addition, the presence of antibiotic resistance genes detected in enterococci indicates a risk factor for dissemination of genes through food. Taking abovementioned results into consideration, some elementary hygiene principles with together quality management in dairy plants have to be applied urgently in order to reduce the frequency of multidrug resistant strains of enterococci in Turkey.

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