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 enterococci were used to be consid-
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; Ozmen 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 37°C. After that, an aliquot of 100 µL of enriched suspension was added into enterococcal broth and incubated at 37°C 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 quinupristine/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 (*ermA*, *ermB*, *mefA/E*, *tetK*, *tetL*, *tetM* and *terO*) 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 above-mentioned 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 10^8 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. casseliflavus 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 gentamicin, whereas high level gentamicin 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. faecalis 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 acc(6')-le-aph(2')-la genes in E. faecalis 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)
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. faecalis 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 Euro-

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, MY, QD, C, S         | tetM, ermB, cad             |
| E. faecalis     | CN, K, TE, MY                   | tetM                        |
| E. faecalis     | CN, RD, TE, MY                  | tetM                        |
| E. faecalis     | CN, RD, TE, 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, 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     | CN, K, RD, TE, MY               | tetM                        |
| E. faecalis     | RD, TE, MY                      | tetM                        |
| E. faecalis     | TE, MY                          | tetM                        |
| E. faecalis     | K, TE, MY                       | 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)-Ia           |
| 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               | tetM                        |
| E. faecalis     | TEC, CN, K, RD, TE, MY, QD      | tetM                        |
| E. faecalis     | TEC, CN, K, RD, TE, MY, LZD, QD | tetM                        |
| E. faecalis     | CN, K, RD, TE, MY               | tetM                        |
| E. faecalis     | CN, RD, TE, MY                  | tetM                        |
| E. faecalis     | CN, RD, TE, MY                  | tetM                        |
| E. faecalis     | CN, RD, TE, MY                  | tetM                        |
| E. faecalis     | CN, RD, TE, MY                  | tetM                        |
| E. faecalis     | CN, K, TE, MY, QD               | tetM                        |
| E. faecalis     | CN, K, RD, TE, MY               | tetM                        |
| E. faecalis     | CN, K, RD, TE, MY, QD, S        | tetM, ermB, aph(3)-IIIa     |
| E. gallinarum   | CN, CN (120), K, TE, E, MY, LZD, QD, S, CIP | tetM                        |
| 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.
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*, *vanL*, *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. faecalis* 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 than 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* strain (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. faecalis* strains (one having intermediate resistance) had *cat* gene, but this gene was not present in *E. avium* strain that was also resistant to chloramphenicol (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 (quinupristine/dalopristine, 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.
this study, 46.8% of the isolates were found to be resistant to rifampin and 42.4% were resistant to quinupristine/dalfopristine with Enterococcus faecalis (64.7%; 55/85) showing higher resistance than Enterococcus avium (25%; 1/4), Enterococcus gallinarum (11.1%; 2/18) and Enterococcus 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). Quinupristine/dalfopristine is one of the approved antibiotics for the treatment of vancomycin resistant Enterococcus 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 aforementioned 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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