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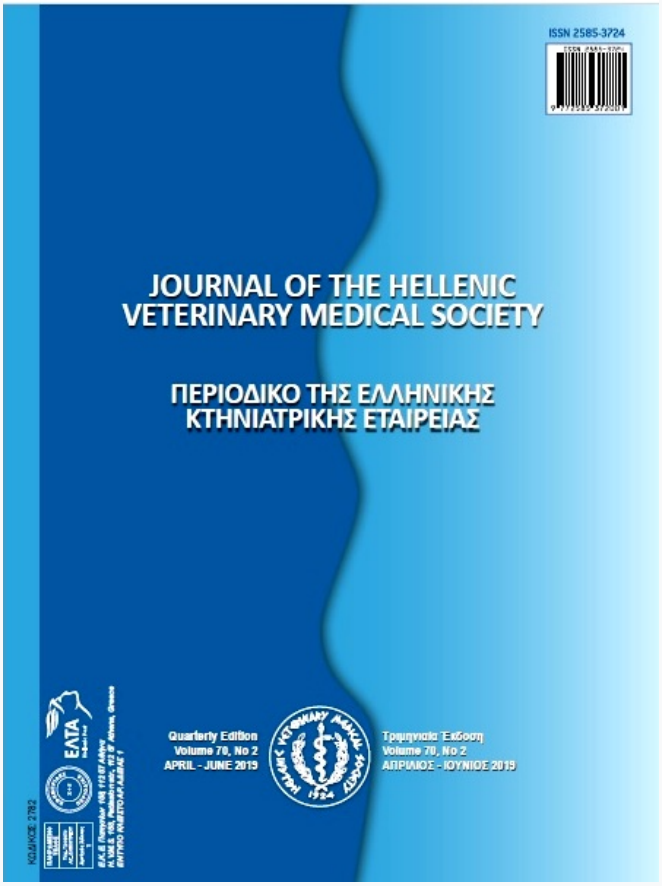
Journal of the Hellenic Veterinary Medical Society

Vol 70, No 2 (2019)



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doi: 10.12681/jhvms.20819

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To cite this article:

MUS, T. E., CETINKAYA, F., CIBIK, R., DEGIRMENCI, G., & DILER, F. B. (2019). Molecular Identification of Vancomycin Resistance and Virulence Genes in Foodborne Enterococci. *Journal of the Hellenic Veterinary Medical Society*, *70*(2), 1487–1494. https://doi.org/10.12681/jhvms.20819

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J HELLENIC VET MED SOC 2019, 70(2): 1487-1494
ΠΕΚΕ 2019, 70(2): 1487-1494

Research article
Ερευνητικό άρθρο

Molecular Identification of Vancomycin Resistance and Virulence Genes in Foodborne Enterococci

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ABSTRACT. The study was performed to determine the presence of vancomycin phenotyping genes and some virulence traits in enterococci species. For this purpose, a total of 42 enterococci including 6 vancomycin-resistant and 36 vancomycin-susceptible strains originated from meat/meat products and milk/dairy products were assessed for the *van*A, *van*B and *van*C genes and *agg, esp, gel*E, *ace* and *efa*A virulence genes by using polymerase chain reaction or multiplex polymerase chain reaction. The *van*A gene was found in 12% (n=5) of the strains and *van*C gene in 50% (n=21). From these, three *van*A- (*E. faecalis, E. durans, E. casseliflavus*) and two *van*C-positive (*E. durans*) strains had a minimum inhibitory concentration of > 256 μg/ml as previously determined with the E-test. The strains expressing vancomycin susceptibility originating from ready-to-eat food were found to carry *van*A (n=1) and *van*C (n=5) genes. On the other hand, the *van*B gene was not detected among strains. Moreover, no strain was found to harbor virulence traits studied. Our results indicated that resistant or susceptible enterococci from foods of animal origin can be a possible reservoir for resistance genes and may have a potential role for transfer of genetic elements among enterococci or to other bacteria. Furthermore, to develop epidemiological surveillance systems for foodborne antibiotic resistant pathogens as vancomycin-resistant enterococci and their genes responsible for resistance, primarily *van*A, *van*B, continues to be an essential issue all around the world. The present work provides data for foodborne enterococci isolates harboring *van*A gene from Turkey.

Keywords: enterococcus, food, *van*A, *van*C, vancomycin

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Date of initial submission: 27-06-2018
Date of revised submission: 25-10-2018
Date of acceptance: 31-10-2018

INTRODUCTION

Antibiotic resistance among the microorganisms and emergence of resistance is an ancient phenomenon. Glycopeptide (vancomycin) resistance gene *van*A was detected from 30 000 years old permafrost sample in the Yukon (Canada) and its similarity to modern variants was clearly evidenced. As a result, antibiotic resistance is accepted as a natural phenomenon (D'Costa *et al.*, 2011). In recent times, antibiotic resistant pathogens have become significant public health threat worldwide. The occurrence and spread of vancomycin resistant enterococci (VRE) is another concern because enterococci species are responsible for most of nosocomial infections (Oravcova *et al.*, 2016). In particular, *E. faecalis* and *E. faecium* are the third and fourth prevalent hospital environment acquired pathogens all around the world. Enterococci demonstrate resistance to many different antibiotics, most particularly resistance to glycopeptides. Vancomycin is more important than other antibiotics since it is more frequently used to treat most of Gram-positive bacterial infections. Nine vancomycin resistance genotypes were detected in enterococci (*van*A, *van*B, *van*C, *van*D, *van*E, *van*G, *van*L, *van*M and *van*N) (Werner, 2012). *E. gallinarum* and *E. casseliflavus* strains have *van*C genotype associated with intrinsic vancomycin resistance (Gousia *et al.*, 2015). Strikingly, *van*A, *van*B, *van*G, *van*N and *van*M genotypes are genetically located on plasmid or chromosome and they can be transferred to other species and/or bacteria (Cattoir and Leclercq, 2013). VRE infections, especially caused by high-level resistant enterococci carrying *van*A and/or *van*B genotypes, can only be treated with a few numbers of effective medical agents. Therefore, they are accepted as one of the clinically important antimicrobial resistant pathogens. The *van*A-type vancomycin resistance is very common among the enterococci and the encoding gene has been primarily identified in *E. faecalis*, *E. faecium* and secondly *E. durans*, *E. hirae*, *E. gallinarum*, *E. casseliflavus*, *E. raffinosus*, *E. avium*, *E. mundtii*, *E. cecorum* (Harada *et al.*, 2012).

Together with *E. faecium* and *E. faecalis,* the incidence of other enterococci species isolated from patients display an alarming increase. This is mainly correlated with their increased putative virulence traits and multiple antibiotic resistances (Biswas *et al.*, 2016). The presence of virulence factors in enterococci gives them different roles both as commensal and as pathogen bacteria for human health (Farahani, 2016). Enterococcal virulence factors are divided into two groups; promoting colonization traits such as aggregation substance (*aga*), collagen binding protein (*ace*), endocarditis specific antigen (*efa*A), surface protein (*esp*) and affecting tissues such as cytolysin (*cyl*), gelatinase (*gel*E), hyaluronidase (*hyl*). In addition to these, sex pheromone genes (*cpd*, *cob*, *ccf*, *cad*) work together with other virulence genes help to trigger infection reactions (Chajecka-Wierzchowska *et al.*, 2017). Animal originated food isolates of enterococci may harbor many of above mentioned virulence genes thus, these foods may play important role as potential source for human infections (Yilmaz *et al.*, 2016).

The goals of this study were to investigate the vancomycin resistance profile, to determine the presence of virulence genes in vancomycin-resistant/susceptible enterococci from food of animal origin, and to raise public awareness about the possible health risks.

MATERIALS AND METHODS

Strains

Bacterial strains were from the Food Hygiene and Technology Department collection, Veterinary Faculty. A total of 42 strains consisting of 36 *E. faecium*, 4 *E. avium* and 2 *E. gallinarum* were selected among enterococcci collected from foods of animal origin between September and December 2011 from different cities (Istanbul, Bursa, Yalova, Balikesir) in Marmara Region. API identification and vancomycin/teicoplanin MIC's results (Cetinkaya *et al*., 2013) of the selected strains are summarized in Table 1. Stock cultures were kept frozen (-20ºC) in Brain Heart Infusion broth (Oxoid CM1135, England) containing 20% (v/v) glycerol. The cultures were activated in Brain Heart Infusion broth at 37ºC.

PCR confirmation of strains and Determination of vancomycin resistance and virulence genes

Total DNA from bacterial strains was extracted by using Chelex 100 (Sigma Aldrich, USA). The PCR was processed in a ThermoCycler (Runik, SCM 96G). Each 25 µl reaction mixture consisted of 1 µl template DNA, 1.25 U of Hot Start Taq DNA polymerase (Bioron, Germany), 10 mM of Tris-HCl pH 8.9, 22 mM of KCl, 1.8 mM of $MgCl_2$ (Fermentas, USA), 200 µM of dNTPs (Biolabs, UK) and 0.5 mM of each primers (Sentegen, Turkey). The PCR method was used to confirm previously API identified strains at the genetic level by using species-specific primers (*E. faecium*, *E. faecalis*, *E. durans*, *E. gallinarum*, *E. casseliflavus*, *E. avium*) and to investigate the pres-

ence of *van*A, *van*B and *van*C resistance genes, and the virulence trait genes *agg*, *esp*, *gel*E, *ace* in all strains as well as *efa*A_{fs}, *efa*A_{fm} in *E. faecalis* and *E. faecium*. The primers, their sequences, products sizes and amplification procedures for PCR conditions are presented in Table 2. The PCR products were electrophoresed (Thermo Scientific EC300XL, USA) on 3% agarose gel (Biomax, Dubuque, USA) and visualized (BioRad Gel DocXR+, USA) by ethidium bromide staining.

Table 1. Description of the strains used in this study and the results of screening for vancomycin resistance genes.

No.	Source	API Identification[a]	PCR Identification	Vancomycin MIC's[a] (μg/ml)	Teicoplanin MIC's[a] (μg/ml)	Genes
1	Meatball	*E. gallinarum*	*E. casseliflavus*	> 256	> 256	*van*A
2	Meatball	*E. avium*	*E. durans*	> 256	> 256	*van*A
3	Meatball	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
4	Meatball	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
5	Meatball	*E. avium*	*E. durans*	> 256	> 256	*van*C
6	Meatball	*E. avium*	*E. durans*	> 256	> 256	*van*C
7	Meatball	*E. feacium*	*E. faecalis*	> 256	> 256	not detected
8	Minced meat	*E. faecium*	*E. faecium*	4	≤ 8	*van*A
9	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
10	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
11	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
12	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
13	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
14	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
15	Beef	*E. avium*	*E. faecalis*	> 256	> 256	*van*A
16	Beef	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
17	Beef	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
18	Beef	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
19	Lamb	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
20	Lamb	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
21	Salami	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
22	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
23	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
24	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
25	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
26	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
27	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
28	Raw goat's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
29	Raw goat's milk	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
30	Village cheese	*E. faecium*	*E. faecium*	<4	≤ 8	*van*A
31	Urfa cheese	*E. gallinarum*	*E. gallinarum*	<4	≤ 8	*van*C
32	Kashar cheese	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
33	Cottage cheese	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
34	Mihalic cheese	*E. faecium*	*E. faecalis*	<4	≤ 8	not detected
35	Antep cheese	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
36	Cottage cheese	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
37	Kashar cheese	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
38	Cottage cheese	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
39	White cheese	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
40	Butter	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
41	Butter	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
42	Butter cream	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C

[a]published in elsewhere (Cetinkaya *et al*., 2013)

Table 2. Oligonucleotide primer sequences and amplification conditions.

Gene	Oligonucleotid sequences (5´-3´)	Product size (bp)	Amplification procedure
fcm	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	215	An initial cycle of: 95°C for 4 min, followed by 30 cycles of: 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and final cycle 72°C for 7 min (Jackson *et al*., 2004)
fls	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360	
dur	CCTACTGATATTAAGACAGCG TAATCCTAAGATAGGTGTTTG	295	
gal	TTACTTGCTGATTTTGATTCG TGAATTCTTCTTTGAAATCAG	173	
cas	TCCTGAATTAGGTGAAAAAAC GCTAGTTTACCGTCTTTAACG	288	
avi	GCTGCGATTGAAAAATATCCG AAGCCAATGATCGGTGTTTTT	368	
*van*A	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1030	An initial cycle of: 94°C for 5 min, followed by 30 cycles of: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and final cycle 72°C for 10 min (Evers *et al*., 1993)
*van*B	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	433	An initial cycle of: 94°C for 5 min, followed by 30 cycles of: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and final cycle 72°C for 10 min (Handwerger *et al*., 1992)
*van*C	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822	An initial cycle of: 94°C for 5 min, followed by 30 cycles of: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and final cycle 72°C for 10 min (Dutka-Malen *et al*., 1995)
esp	TTACCAAGATGGTTCTGTAGGCAC CCAAGTATACTTAGCATCTTTTGG	432	30 cycles of: 94°C for 30 s, 58°C for 30 s, 72°C for 30 s (Shankar *et al*., 1999)
ace	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCG	320	33 cycles of: 94°C for 1 min, 56°C for 1 min, 72°C for 1 min (Mannu *et al*., 2003)
*gel*E	AGTTCATGTCTATTTTCTTCAC CTTCATTATTTACACGTTTG	402	30 cycles of: 94°C for 30 s, 56°C for 30 s, 72°C for 30 s (Mannu *et al*., 2003)
agg	AAGAAAAAGAAGTAGACCAAC AAACGGCAAGACAAGTAAATA	1553	30 cycles of: 94ºC for 30 s, 58ºC for 30 s, 72ºC for 30 s (Eaton and Gasson 2001)
efaA_{fs}	GACAGACCCTCACGAATA AGTTCATCATGCTGTAGTA	705	An initial cycle of: 94ºC for 2 min, 52ºC for 2 min, 72ºC for 2 min, followed by 27 cycles of: 94ºC for 15 s, 52ºC for 15 s, 72ºC for 15 s (Eaton and Gasson 2001)
efaA_{fm}	AACAGATCCGCATGAATA CATTTCATCATCTGATAGTA	735	An initial cycle of: 94ºC for 2 min, 52ºC for 2 min, 72ºC for 2 min, followed by 27 cycles of: 94ºC for 15 s, 52ºC for 15 s, 72ºC for 15 s (Eaton and Gasson 2001)

Table 3. Distribution of vancomycin resistance genes in enterococci (n=42) and their MIC's.

Gene	Numbers of *van*A, *van*B and *van*C positive strains (%)	Strains	Vancomycin/teicoplanin MIC's (μg/ml)
*van*A	5 (11.9%)	*E. casseliflavus* (n=1)	> 256 / > 256
		E. durans (n=1)	> 256 / > 256
		E. faecalis (n=1)	> 256 / > 256
		E. faecium (n=2)	≤ 4 / ≤ 8
*van*B	0		
*van*C	21 (50%)	*E. durans* (n=2)	> 256 / > 256
		E. faecium (n=18)	< 4 / ≤ 8
		E. gallinarum (n=1)	< 4 / ≤ 8
Not detected	16 (38.1%)	*E. faecalis* (n=1)	> 256 / > 256
		E. faecalis (n=1)	< 4 / ≤ 8
		E. faecium (n=14)	< 4 / ≤ 8

RESULTS

PCR identification of tested strains evidenced differences from previous API results. According to PCR, three *E. avium* were identified as *E. durans*; two *E. faecium* as *E. faecalis*, one *E. avium* as *E.faecalis*, and one *E. gallinarum* as *E. casseliflavus* (Table 1).

Among the tested strains *van*A and *van*C genes were found in five (12%) and 21 strains (50%) respectively, meanwhile *van*B gene was not detected. Strains carrying *van*A genes were from cheeses (*E. faecium*), meatballs (*E. durans* and *E. casseliflavus*), minced meat (*E. faecium*) and beef (*E. faecalis*). *Van*C gene was determined in strains derived from five different ready-to-eat dairy products (three cheeses, butter and butter cream). The prevalence of *van*C gene was more common among *E. faecium* (n=18) compared to *E. durans* (n=2) and *E. gallinarum* (n=1). Interestingly, an important percentage (38%) of *E. faecium* (14 strains) and *E. faecalis* (2 strains) did not give any band for these genes.

Data related to the distribution of vancomycin resistance genes in enterococci and their respective minimum inhibitory concentrations (MIC) values for vancomycin/teicoplanin antibiotics is shown in Table 3. Three of five *van*A-positive strains exhibited high MICs to vancomycin/teicoplanin. Among the *van*C gene positive strains, only two *E. durans* had MIC values higher than 256 μg/ml for vancomycin/teicoplanin.

The strains were also screened for the presence some virulence factors such as *agg*, *esp*, *gel*E, *ace* and *efa*A, nonetheless the searched virulence genes were not detected in any tested strain.

DISCUSSION

Different types of acquired vancomycin resistance are known in enterococci, meanwhile the *van*A followed by *van*B are the most prevalent resistance genotype (Werner, 2012). In this study we examined acquired resistance genes including *van*A, *van*B, and *van*C responsible for intrinsic resistance in vancomycin-resistant/susceptible enterococci isolates from animal originated food. The results indicated that strains belonged to *E. faecalis*, *E. faecium*, *E. durans* and *E. casseliflavus* species carried *van*A gene with a prevalence of 11.9% (5 strains). Among these strains an *E. faecium* isolated from ready-to-eat food (village cheese) showed susceptibility to vancomycin (MIC, ≤4 μg/ml).

Several studies from different countries reported the presence of *van*A gene in foodborne enterococci. Lopez *et al*. (2009) reported the prevalence of *van*A gene as 22.6% (two *E. faecium*, three *E. durans* and two *E. hirae*) in 31 VRE isolates. Likewise, Gou-

sia *et al.* (2015) stated that 22 *E. faecium* (15.6%) among 141 enterococci carried *van*A gene. Relatively lower prevalence (2.4% of enterococci from meat and poultry) was reported by Yilmaz *et al*. (2016). Another work revealed the presence of *van*A gene in three vancomycin-susceptible *E. faecalis* isolates and one *E. hirae* isolate (Perin *et al*. 2014). Osman *et al*. (2016) and Harada *et al*. (2012) detected *van*A gene in one *E. faecalis* strain from fish and one *E. cecorum* strain from poultry samples**,** respectively. Contrary results were reported by Kasımoğlu-Doğru *et al.* (2010) and Chajecka-Wierzchowska *et al.* (2016). Contrary results were given by Kasımoğlu-Doğru et al. (2010) and Chajecka-Wierzchowska et al. (2016) suggesting that the strains from food and livestock samples did not harbor *van*A gene. In our study, tested strains had negative results for *van*B gene. These results are similar to those obtained by Kasımoğlu-Doğru *et al.* (2010), Perin *et al.* (2014), Yılmaz *et al.* (2016) and Chajecka-Wierzchowska *et al*. (2016). Nevertheless, Gousia *et al*. (2015) and Lopez *et al*. (2009) reported the presence of *van*B gene in *E. faecium* (1.4%) and *E. faecium* (6.4%) respectively. Another study conducted by Perin et al. (2014) indicated the presence of both *van*A and *van*B genes at seven *E. faecalis* strains.

Many species of enterococci, as stated for *Corynebacterium* spp., *Arcanobacterium haemolyticum* and *Lactococcus* spp. were reported to harbor *van*A ligase gene while *van*B has been primarily determined in *E. faecium* and *E. faecalis*. The difference observed in the dissemination of *van*A and *van*B resistance genes may be attributed to the fact that *van*A gene is mostly located on transposon, a mobile genetic element, in comparison to *van*B gene cluster (Cetinkaya *et al.* 2000). *Van*A gene cluster responds to both vancomycin and teicoplanin resistance but *van*B gene cluster is responsible for resistant to vancomycin but not for teicoplanin (Lefort *et al*., 2004). In our study, strains resistant to vancomycin were also resistant to teicoplanin and they were not carrying *van*B gene.

The presence of *van*C gene in enterococi has been characterized as the intrinsic resistance (Gousia *et al.*, 2015). As seen in Table 1, *van*C gene was found in 10 (eight *E. faecium*, two *E. durans*) meat/meat products and 11 (ten *E. faecium*, one *E. gallinarum*) milk/dairy originated strains. Among the strains carrying *van*C gene, only two *E durans* isolated from meatball samples had vancomycin-resistance with a MIC value of > 256 μg/ml while the others were vancomycin-susceptible (MICs, < 4 μg/ml). Previously, the presence of *van*C1 and *van*C2/3 genes in vancomycin-susceptible *E. faecalis* isolated from broilers in Brazil (De Moura *et al.*, 2013) and *van*C gene in *E. gallinarum* in Canada (Diarra *et al.*, 2010) was published. Chajecka-Wierzchowska *et al.* (2016) also reported the presence of *van*C2/3 genes in *E. casseliflavus* isolates from ready-to-eat meat products but not *van*C1 gene. Moreover, a study in Egypt demonstrated *van*C gene carrying *E. gallinarum* and *E. faecalis* strains from fish samples (Osman *et al.*, 2016). Conversely, lack of *van*C gene in animal originated food was recently reported by Yilmaz *et al*. (2016) in Turkey and by Gousia *et al*. (2015) in Greece.

Enterococci strains carrying virulence factor genes cause more severe infections than the strains lacking these pathogenicity traits. Virulence genes have been frequently observed in *E. faecalis* strains (Chajecka-Wierzchowska *et al.*, 2017). In our study, virulence traits (*agg*, *esp*, *gel*E, *ace* and *efa*A) were not found in any of the tested strains. This can be explained by the limited number of *E. faecalis* tested in the study. In contrast to our results, virulence genes *gel*E, *esp*, *ace*, *asa*1, *efa*A and *hyl* in *E. faecalis*, *E.faecium*, *E. durans* and *E. hirae* isolates from milk and dairy products were observed by Perin *et al*. (2014), Hammad *et al*. (2015) and Gaglio *et al*. (2016). A study performed by Klibi *et al*. (2013) in Tunisia indicated the location of *hyl*, *esp* and *gel*E genes in meat isolates of enterococci species (*E. faecalis*, *E. faecium, E. gallinarum*). Another report revealed the presence of *efa*A, *agg*, *esp*, *gel*E, *cyl*, *cop*, *cpd*, *ccf* genes in *E. faecalis* and *E. faecium* isolated from ready-to eat fermented foods in Turkey (Toğay *et al.*, 2010).

CONCLUSIONS

The presence and prevalence of *van*A and *van*C genes and the absence of *van*B and virulence trait genes in vancomycin-resistant/susceptible enterococci strains were proved. Our findings may be evaluated from two different points: firstly, detection of *van*A gene in VRE strains and particularly in one ready-to-eat food isolate is a matter of interest. These strains can be a part of transmission the high level vancomycin resistance to other strains and/or bacteria. Secondly, the role of food isolates on the spread of pathogenicity genes continues to raise public health concerns. Thus, the lack of any investigated virulence genes in the strains constitutes positive part of the study. Furthermore, monitoring the presence of virulence and *van* genes in different food isolates is essential

to evidence their spreading speed and possible public health risks all over the world.

ACKNOWLEDGEMENTS

The present study was financially supported by Bursa Uludag University Scientific Research Centre [Project number: KUAP (V)-2013/81].

CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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