Kocatepe Veterinary Journal

Kocatepe Vet J. (2022) 15(1): 120-127

DOI: 10.30607/kvj.1048982

RESEARCH ARTICLE

Determination of Virulence Factors and Antibiotic Resistances of *Enterococcus* spp. Identified from Different Stages of Ripened (Classical) White Cheese Production

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ABSTRACT

The objective of this research was to determine the presence of virulence genes (*asa1, gelE, cylA, ace, esp, hyl and efaA*), and vancomycin resistance genes (*vanA, vanB, vanC2/C3*) and the resistance to some antibiotics of *Enterococcus* spp. isolates previously identified from different stages of ripened white cheese production. In addition, gelatinase, β-hemolytic and DNase activity, and biofilm formations were examined phenotypically. In this study, *efaA* in 95.9%, *asa1* in 89%, *ace* in 68.5%, *esp* in 52.1%, *gelE* in 78.1%, *cylA* in 16.4% and *hyl* in 23.3% of isolates were detected. Also, *vanA* in 31.5%, *vanB* in 8.2%, and in *vanC2/C3* 23.3% resistance genes were determined. β-hemolytic and DNase activity were detected in 23.2% and 16.4% of the isolates, while gelatinase activity and biofilm formation could not be detected phenotypically. Moreover, streptomycin and erythromycin resistances were found in 73.9% and in %43.8 of isolates. As a result, it was concluded that *Enterococcus* spp. may pose a risk for public health and food safety in terms of their virulence factors and antibiotic resistance. For this reason, it was suggested that the strains to be selected as starter cultures should be used after evaluating their virulence factors and resistance to antibiotics.

Keywords: Antibiotic Resistance, *Enterococcus* spp., Ripened White Cheese, Virulence Factor.

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Olgunlaştırılmış (Klasik) Beyaz Peynir Üretiminin Farklı Aşamalarından İzole Edilen *Enterococcus* spp.'nin Virülens Faktörlerinin ve Antibiyotik Dirençliliğinin Belirlenmesi

ÖZ

Bu çalışmada, daha önce klasik beyaz peynir üretiminin farklı aşamalarından identifiye edilen *Enterococcus* spp. izolatlarının virülens genleri (*asa1, gelE, cylA, ace, esp, hyl ve efaA*) ve vankomisin direnç genlerinin (*vanA, vanB, vanC2/C3*) varlığı ile bazı antibiyotiklere dirençliliklerinin belirlenmesi amaçlandı. Ayrıca jelatinaz, β-hemolitik ve DNase aktiviteleri ile biyofilm oluşumları fenotipik olarak ise incelendi. İzolatların %95,9'unda *efaA*, %89'unda *asa1*, %68,4'ünde *ace*, %52,1'inde *esp*, %78,1'inde *gelE*, %16,4'ünde *cylA* ve %23,3'ünde *hyl* virülens genleri tespit edildi. Ayrıca %31,5'inde *vanA*, %8,2'sinde *vanB* ve %23,3'ünde *vanC2/C3* direnç genleri belirlendi. Fenotipik olarak ise izolatların sırasıyla %23,2'sinde ve %16,4'ünde β-hemolitik aktivite ve DNase aktivitesi tespit edilirken, jelatinaz aktivitesi ve biyofilm oluşumu tespit edilememiştir. Bunun yanı sıra izolatların %73,9'unda streptomycin ve %43,8'inde erythromycin direnci saptandı. Sonuç olarak, *Enterococcus* spp.'nin virülans faktörleri ve antibiyotik direnci açısından halk sağlığı ve gıda güvenliği için risk oluşturabileceği kanaatine varıldı. Bu nedenle starter kültür olarak seçilecek suşların virülens faktörleri ve antibiyotiklere dirençlilik bakımından değerlendirildikten sonra kullanılmaları önerildi.

Anahtar kelimeler: Antibiyotik Direnci, *Enterococcus* spp., Klasik Beyaz Peynir, Virülens Faktör.

To cite this article: Gökmen M,. Ektik N., Determination of Virulence Factors and Antibiotic Resistances of Enterococcus spp. *Identified from Different Stages of Ripened (Classical) White Cheese Production*
Kocatepe Vet J. (2022) 15(1): 120-127
Submission: 27.12.2021 **Accepted**: 28.02.2022 **Published Online**: 01.03.2022
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INTRODUCTION

Enterococcus spp. is a group of bacteria which are Gram-positive, facultative anaerobic, catalase and oxidase negative, motile and non-spore forming. There are 58 described species in this group (García-Solache and Rice 2019) and some of these have ability to growth in environmental conditions, including wide temperature (10-45 °C), wide pH range (4.5-10) and salt concentrations up to 9.6 (Arias and Murray 2012). Natural habitat of *Enterococcus* spp. is primarily the gastrointestinal system of both humans and warm blooded animals. They are also widely found in soil, water and sewage due to their high adaptability to different environments. Therefore, they can easily contaminate foods like dairy products and milk, meat, and vegetables from these sources (Giraffa 2002, Giraffa 2003). *Enterococcus* spp., which is used as an indicator of fecal contamination, is not considered "generally recognized as safe (GRAS)" (Ogier and Serror 2008) due to biogenic amine production and its frequent association with foodborne illnesses (Riboldi et al. 2009). However, some *Enterococcus* spp. can be used as starter culture in the food industry due to their lipolytic activities, use of citrate and production of aroma compounds. On the other hand, it has been reported that *Enterococcus* spp. found in the normal microflora of various cheeses positively affect the taste, texture and general sensory profile (Giraffa 2003). In various studies it has been stated that, some species of *Enterococcus* spp. such as mainly *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus casseliflavus* and *Enterococcus durans* were detected in dairy products (Settanni et al. 2012, Gaglio et al. 2016).

The main reason of serious infections, especially nosocomial infections, caused by *Enterococcus spp*, is related to the virulence factors encoded by their virulence genes. Virulence factors are such as gelatinase (*gelE*), cytolysin (*cyl*), collagen binding protein (*ace*), aggregation factor (*asa1*), enterococcal surface protein (*esp*), hyaluranidase (*hyl*) and endocarditis antigen (*efaA*) that bacteria have (Chajęcka -Wierzchowska et al. 2017). The biofilm-forming properties of *Enterococcus* spp. both increase their resistance and facilitate contamination in the environment and food plants. At the same time, biofilms can serve as a reservoir for the antibiotic resistance genes of these species (Ch'ng et al. 2019).

Today, the development of antimicrobial resistance in bacteria causes an important problem in terms of both public health and food safety. In this context, it has been reported that genetic elements such as plasmids in their structures and chromosomal changes have a role in the development of antibiotic resistance in *Enterococcus* spp. (Hegstad et al. 2010). Because of these features, they are considered as reservoirs of antimicrobial resistance genes, as they have the potential to transmit these genes to other bacteria in food environments (Gaglio et al. 2016). The objective of this research was to determine some virulence factors and antibiotic resistance, especially vancomycin resistance of *Enterococcus* spp. isolates previously identified from different stages of ripened white cheese production.

MATERIAL AND METHOD

Enterococcus spp. Isolates

In the study, 73 *Enterococcus* spp. isolates, which were previously isolated (Pesavento et al. 2014) and identified by MALDI-TOF MS method, from different stages of ripened white cheese production between March 2020 and December 2020 were used. Fourteen of these isolates were obtained from raw milk, 11 of them from pre-ripened cheese and 48 of them from different stages (17 from 30th day of ripening, 15 from 60th day of ripening, 10 from 90th day of ripening, and 6 from 120th day of ripening) of ripening. The species of these isolates are as follows: 37 *Enterococcus faecalis*, 27 *Enterococcus faecium*, 7 Enterococcus gallinarium, 1 *Enterococcus casseliflavus* and 1 *Enterococcus durans* (Table 1). The isolates were stored in sterile glycerol (20%) at -80°C. Before use, all Enterooccus spp. isolates were activated by incubating in Tryptic Soy Broth (TSB, Merck 105459, Germany) for 24 hours at 37°C.

DNA extraction

The DNA was extracted from overnight cultures grown in TSB using the DNA extraction kit (PureLink™ Genomic DNA Mini Kit, Thermo Fisher Scientific, K1820-02, USA) following the protocol provided by the manufacturer.

Detection of Virulence Genes and Vancomycin Resistance Genes in *Enterococcus* spp. by Multiplex PCR

The presence of *asa1*, *gelE*, *cyl*A, *ace*, *esp, hyl* and *efaA* virulence genes and *vanA*, *vanB* and *vanC2/C3* vancomycin resistance genes of *Enterococcus* spp. were determined by multiplex polymerase chain reaction (multiplex PCR) according to a previously described method (Vankerckhoven et al. 2004) (Table 2). Conditions of amplification were an initial denaturing step at 94°C for 10 min, followed by 35 cycles of denaturation step at 94°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis after stained with ethidium bromide (1%) and photographed under ultraviolet illumination. (Vilber Lourmat, France).

Table 1. Distribution of samples from which *Enterococcus* spp. was obtained

Sample	*Enterococcus* spp.	*E. faecalis*	*E. faecium*	*E. gallinarium*	*E. casseliflavus*	*E. durans*
Raw milk	14	13	1	-	-	-
Pre-ripened cheese	11	5	6	-	-	-
30th day of ripening	17	6	7	2	1	1
60th day of ripening	15	4	9	2	-	-
90th day of ripening	10	7	3	-	-	-
120th day of ripening	6	2	1	3	-	-
Total	73	37	27	7	1	1

Table 2. PCR primers used for the detection of virulence and vancomycin resistance genes in *Enterococcus* spp.

Gene	Targeting	Primers (5'-3')	bp	Reference
ASA1 1	*asa1*	GCACGCTATTACGAACTATGA	375	Vankerckhoven et al. (2004)
ASA1 2		TAAGAAAGAACATCACCACGA		
GEL 11	*gelE*	TATGACAATGCTTTTTGGGAT	213	Vankerckhoven et al. (2004)
GEL 12		AGATGCACCCGAAATAATATA		
CYT I	*cylA*	ACTCGGGGATTGATAGGC	688	Vankerckhoven et al. (2004)
CYT IIb		GCTGCTAAAGCTGCGCTT		
ACE-F	*ace*	GAATTGAGCAAAAGTTCAATCG	1008	Ben Omar et. al. (2004)
ACE-R		GTCTGTCTTTTCACTTGTTTC		
EFA-A-F	*efaA*	GCCAATTGGGACAGACCCTC	688	Creti et. al. (2004)
EFA-A-R		CGCCTTCTGTTCCTTCTTTGGC		
ESP 14F	*esp*	AGATTTCATCTTTGATTCTTGG	510	Vankerckhoven et al. (2004)
ESP 12R		AATTGATTCTTTAGCATCTGG		
HYL n1	*hyl*	ACAGAAGAGCTGCAGGAAATG	276	Vankerckhoven et al. (2004)
HYL n2		GACTGACGTCCAAGTTTCCAA		
Van-A-F	*vanA*	CATGAATAGAATAAAAGTTGCAATA	1030	Kariyama et al. (2000)
Van-A-R		CCCCTTTAACGCTAATACGATCAA		
Van-B-F	*vanB*	AAGCTATGCAAGAAGCCATG	536	Elsayed et al. (2001)
Van-B-R		CCGACAATCAAATCATCCTC		
Van-C2/C3-F	*vanC*	CGGGGAAGATGGCAGTAT	484	Kariyama et al. (2000)
Van-C2/C3-R		CGCAGGGACGGTGATTTT		

Reference Strains

In this study, *E. faecalis* ATCC 51299, *E. faecalis* ATCC 29212, *E. faecium* ATCC 6057 reference strains were used (Table 3).

Phenotypical Assessment of Virulence Factors Gelatinase Production, Hemolytic and DNase Activity, Biofilm Forming

Enterococcus spp. cultures were streaked on the surf*ace* of Brain Heart Infusion Agar (BHI, Merck 103870, Germany) plates containing 3% gelatin. Plates were incubated for 2-3 days at 37°C and then kept 4 hours at 4°C. Production of gelatinase was determined the appearance of a turbid halo or zone around the colonies (Perin et al. 2014).

Hemolytic activity of isolates was evaluated by culturing of fresh overnight cultures on Blood Agar (Merck 110886, Germany) containing 5% ml defibrinated sheep blood. The plates were incubated for 24-48 h at 37°C. After incubation, appearance of a clear hydrolysis zone formed around the colonies was evaluated as β-hemolysis (Gaspar et al. 2009).

Each *Enterococcus* spp. strain was spread onto the surf*ace* of DNase Agar (Merck 110449, Germany) and the plates incubated for 2 days at 37°C. After incubation 1 N HCl was flooded on the plates and colonies with clear zones were considered as DNase activity positive (Perin et al. 2014).

Table 3. PCR primers used for the detection *Enterococcus* spp. reference strains

Isolate	Primer	Sekans (5'-3')	bp	Reference
Enterococcus spp.	E1	TCAACCGGGGAGGGT	733	Deasy et al. (2000)
	E2	ATTACTAGCGATTCCGG		
E. faecalis	FL1	ACTTATGTGACTAACTTAACC	360	Jackson et al. (2004)
	FL2	TAATGGTGAATCTTGGTTTGG		
E. faecium	FM1	GAAAAAACAATAGAAGAATTAT	215	Jackson et al. (2004)
	FM2	TGCTTTTTTGAATTCTTCTTTA		
E. durans	DU1	CCTACTGATATTAAGACAGCG	295	Jackson et al. (2004)
	DU2	TAATCCTAAGATAGGTGTTTG		

The ability of the strains to form biofilms was checked using of a specially prepared medium composed of Brain Heart Infusion Broth (BHI, Merck 110493, Germany) (37 gm/l), agar number 1 (10 gm/l), Congo red dye (0.8 gm/l) and sucrose (5 gm/l). Isolates were inoculated on the medium and incubated for 24 to 48 hours at 37°C. Biofilm production was indicated by black colonies with a dry crystalline consistency (Freeman et al. 1989).

Assessment of Antibiotic Resistance

Antibiotic Resistance tests were performed by the standard disc diffusion method of Bauer et al. (1966) on Mueller-HintonAgar (Oxoid CM0337, UK). Antibiotic discs used were (all from Thermo Fisher Scientific, Oxoid, UK) ampicillin (10 mg), gentamicin (10 mg), streptomycin (10 mg), chloramphenicol (30 mg), erythromycin (5 mg), tetracycline (30 mg) and ciprofloxacin (5 mg). The diameters of the inhibition zones were measured after 18±2 h incubation at 35±1°C. The results were reported as susceptible, intermediate resistant or resistant based on the Clinical and Laboratory Standards Institute indications (CLSI, M100-ED31:2021).

RESULTS

In this study, virulence factors and antibiotic resistance of the strains of 73 *Enterococcus* spp. (37 *E. faecalis*, 27 *E. faecium*, 7 *E. gallinarium*, 1 *E. casseliflavus* and 1 *E. durans*) which are isolated from different stages of production of ripened white cheese were determined. The following virulence genes were detected from these 73 *Enterococcus* spp. isolates: 89% *asa1*, 78.1% *gelE*, 16.4% *cyl*A , 68.5% *ace* , 52.1% *esp* , 23.3% *hyl* and 95.9% *efaA*. Also from same isolates; 31.5% *vanA* , 8.2% *vanB* and 23.3% *vanC2/C3* resistance genes were determined (Table 4).

Table 4. Distribution of virulence and vancomycin resistance genes in *Enterococcus* spp. isolates

	E. faecalis **(n=37)**	***E. faecium*** **(n=27)**	***E. gallinarium*** **(n=7)**	***E. casseliflavus*** **(n=1)**	***E. durans*** **(n=1)**	***Enterococcus*** spp. **(n=73)**
Virulence genes	**%(n)**	**%(n)**	**%(n)**	**%(n)**	**%(n)**	**%(n)**
asa1	78.4(29)	100(27)	100(7)	100(1)	100(1)	89(65)
gelE	67.6(25)	85.2(23)	100(7)	100(1)	100(1)	78.1(57)
cylA	13.5(5)	22.2(6)	-	100(1)	-	16.4(12)
ace	67.6(25)	77.8(21)	28.6(2)	100(1)	100(1)	68.5(50)
esp	51.4(19)	59.3(16)	28.6(2)	-	100(1)	52.1(38)
hylA	13.5(5)	40.7(11)	-	-	100(1)	23.3(17)
efaA	94.6(35)	96.3(26)	100(7)	100(1)	100(1)	95.9(70)
Vancomycin resistance genes						
vanA	24.3(9)	40.7(11)	42.9(3)	-	-	31.5(23)
vanB	13.5(5)	3.7(1)	-	-	-	8.2(6)
vanC2/C3	8.1(3)	40.7(11)	28.6(2)	100(1)	-	23.3(17)

According to phenotypic tests, gelatinase activity could not be detected in the isolates. On the other hand, β-hemolytic activity was detected in 23.2% of the isolates. The distribution of β-hemolytic activity at the species level is; 18% of *E. faecalis*, 25% of *E. faecium*, 28% of *E. gallinarium* and 100% of *E. casseliflavus*. DNase activity was found positive in 16.4% of the isolates. The distribution of DNase activity at the species level is; 13.5% of *E. faecalis*, 22.2% of *E. faecium* and *E. durans* in 100%. However, it was determined that none of the isolates formed a biofilm.

According to the antibiotic resistance test results, it is determined that 33 of the *E. faecalis* isolates were resistant to streptomycin, 17 to ciprofloxacin and 15 to tetracycline. It is also found that 13 of the *E. faecium* isolates to streptomycin and erythromycin and 7 of the *E. gallinarium* were found to be resistant to streptomycin and erythromycin 13 of the *E. faecium* isolates had streptomycin and erythromycin; seven of the *E. gallinarium* isolates were found to be resistant to streptomycin and erythromycin. While *E. casseliflavus* isolate did not show resistance to any antibiotic, *E. durans* was found resistant to streptomycin, tetracycline and ciprofloxacin antibiotics (Table 5).

Table 5. Distribution of antibiotic resistance in *Enterococcus* spp. isolates

Antibiotic	*E. faecalis* n(%)			*E. faecium* n(%)			*E. gallinarium* n(%)			*E. casseliflavus* n			*E. durans* n		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
AMP	35(94.6)	-	2(5.4)	25(92.6)	-	2(7.4)	5(71.4)		2(28.6)	1		-	1		-
CN	27(73)	1(2.7)	9(24.3)	24(88.9)	-	3(11.1)	2(28.6)	-	5(71.4)	1	-	-	1	-	-
S	3(8.1)	1(2.7)	33(89.2)	14(51.9)	-	13(48.1)	-	-	7(100)	1	-	-	-	-	1
C	19(51.3)	17(46)	1(2.7)	24(88.9)	3(11.1)	-	4(57.1)	3(42.9)	-	1	-	-	-	1	-
E	6(16.2)	19(51.3)	12(32.4)	6(22.2)	8(29.6)	13(48.1)	-	-	7(100)	1		-	-	1	-
TE	13(35.1)	9(24.3)	15(40.5)	14(51.9)	6(22.2)	7(25.9)	-	2(28.6)	5(71.4)	-	1	-	-	-	1
CIP	7(18.9)	13(35.1)	17(45.9)	10(37)	12(44.4)	5(18.5)	2(28.6)	4(57.1)	1(14.3)	-	1	-	-	-	1

(S: Susceptible, I: Intermediate resistant, R: Resistant, AMP: ampicillin, CN: gentamicin, TE: tetracycline, E: Erythromycin, CIP: ciprofloxacin, S: streptomycin, C: chloramphenicol)

DISCUSSION

It has been reported that *Enterococcus* spp. has a role in the ripening of many cheese varieties produced by traditional methods, especially in Mediterranean countries, and contributes to the formation of typical tastes and flavors (Foulquie Moreno et al. 2006). On the other hand, the virulence genes of these bacteria and their antibiotic resistance pose a significant threat to public health (Gaglio et al. 2016). In present study, virulence factors and antibiotic resistance of the 73 *Enterococcus* spp. strains which are isolated from different stages of production of ripened white cheese were determined.

The general distribution of virulence and vancomycin resistance genes of the detected *Enterococcus* spp. isolates is presented in Table 4. In this study, *efaA*, *asa1*, *ace*, *esp* virulence genes supporting host colonization of *Enterococcus* spp. were detected in 95.9%, 89%, 68.5% and 52.1% of isolates, respectively. Domingos-Lopes et al. (2017) detected *efaA*, *ace*, *asa1*, and *esp* virulence genes of *Enterococcus* spp. isolates obtained from cheese at a rate of 33-99%. Câmara et al. (2020) reported that 100% of their *Enterococcus* spp. isolates which detected from cheeses carried *efaA*, 71% *ace*, 46% esp, and 43% *asa1* virulence genes. It is seen that our study's results are compatible with the results of these researchers. Chajęcka-Wierzchowska et al. (2017) reported the *efaA*, *asa1* and *ace* virulence genes play an important role in the attachment of *Enterococcus* spp. to host cells.

After colonization, *Enterococcus* spp. pathogen strains release toxic substances that damage host tissues. The virulence factors secreted by *Enterococcus* spp. and affecting the tissues are gelatinase (*gelE*), cytolysin (*cyl*) and hyaluronidase (*hyl*) (Chajęcka-Wierzchowska et al. 2017). By hydrolyzing gelatin and collagen, gelatinase enzyme (*gelE*) can facilitate the penetration of bacteria into the host's tissues by participating in biofilm formation (Anderson et al. 2016). Additionally, these genes cause the initiation and progression of inflammatory processes associated with *Enterococcus* spp. (Domingos-Lopes et al. 2017). In the study, *gelE* was detected in 78.1% of the isolates genotypically, although gelatinase activity was not observed phenotypically in this study. Templer and Baumgartner (2007) stated that in their *Enterococcus* spp. isolates from traditional cheese, gelatinase activity was not observed phenotypically but they detected the *gelE* gene in 76% of them genotypically. Domingos-Lopes et al. (2017) also determined 77% and İspirli et al. (2016) 75% *gelE* gene in their isolates obtained from cheeses. The results of these researchers show similarity to our study's results. Lopes et al. (2006) reported that the detection of the *gelE* gene is not sufficient for gelatinase activity in bacteria, and full expression of the fsr operon is required. However, they also reported that the fsr operon could not be detected in the laboratory as it is easily damaged during cell freezing. Contrary to the results of our study; some researchers (Fuka et al. 2017, Câmara et al. 2020, Hammad et al. 2021) stated that they detected *gelE* gene at lower rates (16.6-54%)

in their *Enterococcus* spp. isolates. Eaton and Gasson (2001) reported that genes may be lost due to the negative effects of *gelE* expression of isolates during laboratory studies and culture conditions, and in vitro tests this may cause loss of gelatinase activity.

Cytolysin (*cyl*) has a bactericidal effect against gram-negative bacteria and a toxic effect (β-hemolysis) against macrophages, erythrocytes and leukocytes (De Vuyst et al. 2003). In the present study, the *cyl*A gene was found in 16.4% of the isolates (Table 4). Fuka et al. (2017) found *cyl*A gene in 19.8% of *Enterococcus* spp. isolates and Câmara et al. (2020) in 21% of their isolates. The findings of this study are consistent with the above researchers. Domingos-Lopes et al. (2017) detected the *cyl*A gene in 32% of the isolates in their study. Avcı and Ozden Tuncer (2017) also found *cyl*A gene in 45.5% of *Enterococcus* spp. strains isolated from traditional Turkish cheeses. On the other hand, Templer and Baumgartner (2007) reported that they detected the *cyl*A gene in 6% of their *Enterococcus* spp. isolates. *cyl*A gene has been detected in *Enterococcus* spp. strains both isolated from infections and forming the commensal microbiota. It has also been reported that it is frequently detected from isolates obtained from foods (Trivedi et al. 2011).

By degrading mucopolysaccharides in connective tissue, the hyaluronidase enzyme (*hyl*) aids the transmission of bacteria and their toxins in the host (Vankerckhoven et al. 2004). In our study, hyl gene was found in 23.3% of the isolates. In species, the hyl gene was detected in *E. faecium*, *E. faecalis*, *E. casseliflavus* and *E. durans*. It has been mentioned that the *hyl* gene is commonly found in *E. faecium* and very seldom in *E. faecalis* from clinical strains (Vankerckhoven et al. 2004). Trivedi et al. (2011) determined that *E. casseliflavus* and *E. durans* species isolated from food also have the *hyl* gene. Yuksekdag et al. (2021) reported that 12% of *E. faecium* isolates obtained from white cheese samples were positive for the *hyl* gene. Vancomycin-resistant *Enterococcus* spp. (VRE) has become one of the most challenging pathogens with the rapidly spread of multidrug resistant strains with limited therapeutic options. *vanA* is the most common of them and it is dominantly found in *E. faecium* and *E. faecalis*, the species responsible for human infections (Cetinkaya et al. 2000). Also Ahmed and Baptiste (2018) reported that *vanC* genes are naturally found in *E. casseliflavus* and E. gallinarum. In this study, *vanA*, *vanB* and *vanC2/C3* genes were detected in 31.5%, 8.2% and 23.3% of *Enterococcus* spp. isolates, respectively. As well Hammad et al. (2021) stated that they have found *vanB* and *vanC* genes in 29.1% and 20.8% of milk isolates, respectively. Oruc et al. (2021) reported that while 13.63% of *Enterococcus* spp. strains isolated from traditional white cheeses harbored *vanA* gene, *vanB* gene observed only in the 31.82%. Also, Yuksekdag et al (2021) informed that 59% of *E. faecium* isolates were positive for the *vanB* gene and 6% was positive for *vanA* gene. On the contrary of our study, Domingos-Lopes et al. (2017) and Jurkovič et al. (2006) reported that a small number (<5%) of Enterococcus isolates harbored vancomycin (*vanA*, *vanB*) resistance genes.

In this study, β-hemolytic activity was detected phenotypically in 23.2% of the *Enterococcus spp* isolates. Different researchers (Domingos-Lopes et al. 2017, Adifon and Tuncer 2019, Ayhan et al. 2020, Câmara et al. 2020, Margalho et al. 2020) determined that 2.3-11.3% of the *Enterococcus* spp. isolates from cheeses showed β-hemolytic activity. β-hemolytic activity rates of these studies are lower than the rates of this study. Hemolytic activity is the least detected virulence factor in food sourced *Enterococcus* spp. (Chajęcka-Wierzchowska et al. 2017).

In present study, DNase activity was detected in 16.4% of the isolates. It has been reported that this virulence factor, which is typically researched in clinical isolates, is found at high rates in *Enterococcus* spp. and is frequently linked to the competitive characteristics of pathogenic strains (Semedo et al. 2003)

It was determined that 33 of the *E. faecalis* isolates were resistant to streptomycin, 17 to ciprofloxacin and 15 to tetracycline. Thirteen of the *E. faecium* isolates had streptomycin and erythromycin; 7 of the *E. gallinarium* isolates were found to be resistant to streptomycin and erythromycin. Also *E. durans* was found resistant to streptomycin, tetracycline, and ciprofloxacin antibiotics. De Paula et al. (2020) reported that they detected erythromycin resistance in 16.6% of *E. faecium* isolates from raw milk and traditional cheese. Templer and Baumgartner (2007) found that 30% of *Enterococcus* spp. isolates from traditional cheese made from raw milk were resistant to chloramphenicol, 50% were resistant to erythromycin, and 68% were resistant to tetracycline. According to Hammad et al. (2021); *Enterococcus* spp. identified from raw cow's milk was resistant to erythromycin (87.5%), and tetracycline (29.1%). Gaglio et al. (2016) also found that *Enterococcus* spp. isolates from artisanal cheeses were resistant to erythromycine (52.5%), ciprofloxacine (35.0%) and tetracycline (17.5%). One reason that may explain this finding is the widespread use of antibiotics in agriculture.

CONCLUSION

There is no evidence that *Enterococcus* spp. isolated from food causes infection in humans. However, there is a risk of genetically transferring virulence and antibiotic resistance genes of *Enterococcus* spp. to pathogenic bacteria. Therefore, the presence of these bacteria in foods is still debated today and is a matter

of public health concern. Especially in immunocompromised individuals, such strains are important as they pose a risk of disease. In this study, it has been revealed that *Enterococcus* spp. isolates could be a source of the transmission and spread of virulence and antimicrobial resistance genes among bacteria. This situation also reveals the necessity for a more careful valuation of strains to be used as starter cultures in the dairy industry, especially relating to their virulence characteristics and antibiotic resistance. Even if the aforementioned isolates contribute to the formation of aroma and texture in cheese production, it is considered more appropriate to use isolates that do not contain virulence genes and are sensitive to antibiotics as starter cultures.

Conflict of interest: The authors declared that there is no conflict of interest.

Ethical Approval: This study does not present any ethical concerns. The authors declared that they comply with the Research and Publication Ethics.

Financial support: --

Acknowledgment: --

Description: --

Authors Contribution Rate: The contribution rate of the authors is equally.

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