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Identification of enterococci by MALDI-TOF-MS & 16S rRNA sequencing isolated from squeezed cheeses and evaluation of antibiotic susceptibility and antibacterial activity

Sıkma peynirlerden izole edilen enterokokların MALDI-TOF-MS ve 16S rRNA sekanslama ile tanımlanması ve antibiyotik dirençlilikleri ile antibakteriyal aktivitelerinin değerlendirilmesi

Furkan AYDIN[1], Halil İbrahim KAHVE[1], Mustafa ARDIÇ[1], İbrahim ÇAKIR[2]

ABSTRACT

Objective: This study aims to identify enterococci isolated from squeezed cheeses by MALDI-TOF-MS and 16S rRNA sequence analysis and to evaluate antibiotic resistance and antibacterial effects of these isolates against some food pathogens.

Methods: Identification of 84 *Enterococcus* isolates obtained from squeezed cheese was carried out using MALDI-TOF-MS and 16S rRNA gene sequencing. The isolates were tested for resistance to 14 different antibiotics by the disc diffusion method. The antimicrobial effects of isolates against various food pathogens were determined by the agar spot test. SPSS 22.0.0 software (SPSS Inc., Chicago, USA) was used for the assessment of the correlation between variance analysis (ANOVA, F test) and antibacterial results.

Results: As a result of sequence analysis; 33 (39.3%) were described to *E. faecalis*, 29 (34.5%) to *E. faecium*, 14 (16.7%) to *E. durans*, 4 (4.8%) to *E. gallinarum*, 3 (3.5%) to *E. casseliflavus* and 1 (1.2%) to *E. thailandicus*. Eighty-one isolates gave the same identification result as the MALDI-TOF-MS method (96.43%). The difference

ÖZET

Amaç: Bu çalışmanın amacı, sıkma peynirlerden izole edilen enterokokların MALDI-TOF-MS ve 16S rRNA sekans analizleri kullanılarak tanımlanması ve bu izolatların antibiyotik dirençleri ile bazı gıda patojenlerine karşı antibakteriyel etkilerinin değerlendirilmesidir.

Yöntem: Sıkma peynirlerden elde edilen 84 Enterococcus izolatının MALDI-TOF-MS yöntemi ile tanımlanması yapılmış ve 16S rRNA bölgeleri çoğaltılarak sekanslanmıştır. İzolatların 14 farklı antibiyotiğe karşı dirençlikleri disk difüzyon yöntemiyle incelenmiştir. Ayrıca izolatların çeşitli gıda patojenlerine karşı gösterdikleri antibakteriyel etkileri agar spot testi ile belirlenmiştir. İstatistiksel değerlendirme için varyans analizi (ANOVA, F testi) ve antibakteriyel sonuçlar arasındaki korelasyonun tespiti için SPSS 22.0.0 yazılımı (SPSS Inc., Chicago, ABD) kullanılmışır.

Bulgular: Seksen-dört izolat 16S rRNA sekans analizi sonucunda; 33 (%39.3) *E. faecalis*, 29 (%34.5) *E. faecium*, 14 (%16.7) *E. durans*, 4 (%4.8) *E. gallinarum*, 3 (%3.5) *E. casseliflavus* ve 1 (%1.2) *E. thailandicus* olarak tanımlanmıştır. Seksen bir izolat MALDI-TOF-MS yöntemi ile aynı tanımlama sonucunu vermiştir (%96.43). İzolatların

[1]Aksaray University, Faculty of Engineering, Food Engineering Department, Aksaray
[2]Bolu Abant İzzet Baysal University, Faculty of Engineering, Food Engineering Department, Bolu

İletişim / Corresponding Author : Halil İbrahim KAHVE
Aksaray University, Food Engineering Department 68100 Aksaray - Türkiye
E-posta / E-mail : hibrahimkahve@gmail.com

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between two identification methods has not been found to be statistically significant (p>0.05); however, MALDI-TOF-MS has some advantages over 16S rRNA sequencing, such as being less labor-intensive, more economical and faster. In total, 83.3% of the strains exhibited multidrug-resistant phenotypes. A high incidence of resistance was detected for nalidixic acid, oxacillin, and streptomycin. *E. faecalis* isolates were found to show lower sensitivity to antibiotics tested than *E. faecium* (p <0.05). The anti-listerial effect of *E. faecalis* was determined among all enterococcal isolates (p <0.05). Along with this, a strong correlation was found between *L. innocua* and *L. monocytogenes* inhibition. The results of antibacterial activity revealed that enterococci are more effective for the inhibition of Gram-positive food pathogens.

Conclusion: The correlation between the results from the two identification methods showed that MALDI-TOF-MS is a fast, economical, robust, and reliable method for the characterization of enterococci. When the results were examined in terms of food safety, it was observed that squeezed cheese produced from raw milk without using starter culture was reservoirs of *Enterecoccus* spp. with multiple antibiotic resistance. Enterococci, which does not carry multiple antibiotic resistance, can be used in starter culture combinations and in hurdle technology to prevent the growth of Gram-positive food pathogens; however, to this end, virulence determinants must be determined to achieve these goals.

Key Words: Food, *Enterococcus*, MALDI, sequencing, antibiotic resistance

tanımlanmasında kullanılan iki tanımlama yönteminin arasında anlamlı bir fark bulunmamıştır (p>0.05). Ancak MALDI-TOF-MS yönteminin daha az iş gücü gerektirmesi, ekonomik ve hızlı olması gibi bazı avantajlarının olduğu görülmüştür. İzolatların %83.3'ünün çoklu antibiyotik direnci taşıdığı tespit edilmiştir. İzolatların nalidiksik asit, oksasilin ve streptomisine karşı dirençliklerinin yüksek olduğu saptanmıştır. *E. faecalis* suşlarının kullanılan antibiyotiklere karşı *E. faecium*'dan daha düşük duyarlılık gösterdiği bulunmuştur (p<0.05). Tüm enterokok izolatları içerisinde *E. faecalis*'in anti-listerial etkisi belirlenmiştir (p<0.05). Bununla birlikte, *L. innocua* ve *L. monocytogenes* inhibisyonu arasında güçlü bir korelasyon saptanmıştır. Antibakteriyel aktivitenin sonuçları incelendiğinde, izolatların gıda patojeni olan Gram-pozitif bakterilere karşı inhibasyon etkilerinin daha yüksek olduğu görülmüştür.

Sonuç: İki tanımlama yönteminden elde edilen sonuçlar arasındaki korelasyon MALDI-TOF-MS'nin enterokokların karakterizasyonu için hızlı, ekonomik, sağlam ve güvenilir bir yöntem olduğunu göstermiştir. Sonuçlar gıda güvenliği açısından incelendiğinde, starter kültür kullanılmadan direkt olarak çiğ sütten üretilen sıkma peynirlerin çoklu antibiyotik direnci taşıyan enterokok rezervuarları olduğu görülmüştür. Çoklu antibiyotik direnci taşımayan enterokoklar, peynir üretim teknolojisindeki başlangıç kültürü kombinasyonlarında, engel teknolojileri bağlamında ise Gram-pozitif gıda patojenlerinin gelişmesini engellemek için kullanılabilir, ancak bu amaca ulaşabilmek için virülans determinantlarının belirlenmesi gerekmektedir.

Anahtar Kelimeler: Gıda, *Enterococcus*, MALDI, sekanslama, antibiyotik direnci

INTRODUCTION

Enterococci are complex and essential members of lactic acid bacteria (LAB). Owing to their strong adaptability and resistance to extreme environmental conditions, they are ubiquitously found in nature, mostly in the gastrointestinal tracts of mammals as well as soil, water, and some of the food products, especially those of animal origin (1). It was assumed that the presence of enterococci in dairy products occurred due to inadequate hygiene practices as a consequence of solely direct fecal contamination (2). The Commission Regulation (EC) No 1441/2007 of 5 December 2007 'on microbiological criteria for foodstuffs' declares that enterococci in food is not always due to fecal contamination and therefore sets no limit for their presence in foods (3). They play significant roles during the fermentation of cheese products by enhancing the organoleptic characteristics generally by proteolysis, lipolysis, and citrate metabolism (4). They are also known to produce bacteriocins, so-called enterocins, which inhibit the growth of some food spoilage and pathogenic bacteria comprising *Clostridium* spp., *Bacillus* spp., *Listeria monocytogenes*, and *Staphylococcus aureus* (5). Because of the ability to be used as adjunct or co-culture, they have comprehensively been studied for possessing beneficial properties in different foods, especially in traditional raw milk cheeses (1).

Enterococci are not assumed as GRAS (Generally Recognized as Safe) unlike most of other LAB. The European Food Safety Authority did not recommend them in the QPS (Qualified Presumption of Safety) approaches (6). On the other hand, they are considered to be opportunist pathogens and are often associated with hospital-acquired infections. Because some of the species may be intrinsically resistant to some antibiotics and show the ability to acquire, accumulate, and transfer their plasmids encoding antimicrobial resistance genes or virulence traits to several food pathogens by horizontal gene transfer (7).

Enterococci can be found in raw milk as a consequence of direct or indirect fecal contamination, and due to their ability to resist extreme environmental conditions, they can survive through the cheese-making process and ripening (1). The researchers reported that enterococci populations in cheeses produced from raw milk might vary between 10^4 - 10^6 cfu/g and 10^5 - 10^7 cfu/g in curd and during ripening, respectively (8). Traditional 'sikma/ squeezed' cheeses are manufactured using raw milk (from raw sheep's or cows' milk or mixtures of these milks) coagulated with artisanal animal rennet without starter culture addition. Then the curd is pressed by using heavy stones before scalding at high temperatures. Scalded curd is molded by squeezing with the hand before ripening (9). Although squeezed cheese resembles 'traditional Sicilian cheeses', they differ in shaping and ripening processes. To date, *Enterococcus* spp. isolated from squeezed cheese samples have not been deeply investigated regarding their antibiotic and antimicrobial susceptibility.

For the identification of enterococci, traditional or molecular methods, in which 16S rRNA region of ribosomal DNA is amplified or species-specific primers are used without additional sequence knowledge, have been widely used so far (10). Since traditional methods take time as well as giving indecisive results, and molecular methods are expensive and require trained laboratory staff, alternative methods have been developed. Such as Matrix-Assisted Laser Desorption Ionization – Time of Flight - Mass Spectrometry (MALDI-TOF-MS) in which protein signatures of each microorganism is characterized and then compared to a reference spectrum with a succession log score ranging from 0.000 – 3.000 (11).

Based on the considerations mentioned above, this study aims to identify enterococci isolated from squeezed cheeses by MALDI-TOF-MS and 16S rRNA sequence analysis, to evaluate antibiotic resistance of the isolates to various clinically significant

antibiotics, and to determine their inhibition against different food pathogens.

MATERIAL and METHOD

Bacterial strains

The study has been conducted in Aksaray University and Abant Izzet Baysal University Food Microbiology laboratories in the summer of 2019. Eighty-four Enterococcus isolates were supplied by Aydin and Ardic (12) where detailed information of isolation sources were given. The isolates were identified on the species level by MALDI-TOF-MS (Bruker, Germany) as described before (12). Briefly, single colonies were smeared onto target polished steel plate (MSP 384; Bruker Daltonik GmbH, Karlsruhe, Germany), and overlaid with 2 µL α-cyano-4-hydroxycinnamic acid matrix solution in acetonitrile (Sigma-Aldrich):ddH2O:TFA (Sigma-Aldrich) (50:47.5:2.5, v/v). After crystallization, MALDI plate was inserted into Microflex TOF mass spectrometer (Bruker Daltonics, Germany) equipped with an N2 laser. The mass range used was 2.000-20.000 m/z. The MALDI-TOF mass spectra were analyzed with MALDI-bioTyper 3.0 software (Bruker Daltonics, Karlsruhe, Germany) with a laser intensity of 50 Hz. Results were recorded as log scores. Score values between 2.300-3.000 were evaluated to be true species identification, 2.000-2.300 were secure genus identification, but probable species identification, 2.000-3.000 were probable genus identification. A list of identified strains to have been used in the present study is in Table 1.

Molecular identification of the strains

The total DNA of the strains was extracted from overnight cultures in Tryptic Soy Broth (Merck, Germany) (TSB) following the protocol provided by Promega Wizard Genomic DNA Purification Kit (Promega, USA). The DNA concentration was evaluated spectrophotometrically by NanoDrop ND-2000 spectrophotometer (Thermo Scientific, USA). Finally, the DNA of the strains were stored at -20 °C until used as templates for amplification.

The DNA templates were used to amplify the 16S rRNA region. Amplification was performed as reported, using the forward primer (Amp-F) 5′-GAG AGT TTG ATY CTG GCT CAG-3’ and reverse primer (Amp-R) 5′- AAG GAG GTG ATC CAR CCG CA-3′ (Y is C or T; R is A or G). PCR mixture contained 1 µL of template DNA, 10 µL 5× PCR buffer, 0.4 µL dNTPs, 1 µL of 20 mM primers FP and RP, 0.25 µL 5 U Taq polymerase and sterile ddH2O up to 50 µL of total volume. The reaction was performed with the following program: 95 °C for 2 min, 20 cycles of 95 °C

Table 1. Bacterial strains previously identified by MALDI-TOF-MS

Enterococcus spp.	Number of isolates
Enterococcus faecalis	32
Enterococcus faecium	31
Enterococcus durans	13
Enterococcus gallinarum	4
Enterococcus casseliflavus	3
Enterococcus thailandicus	1
Total	**84**

for 30 s, 55 °C for 20 s, and 72 °C for 30 s with a final extension of 72 °C for 5 (13). The PCR products were purified using a GeneJET PCR purification kit (Thermo Scientific, USA) and analyzed on 1.2% agarose gel electrophoresis to check the amplification. Then the amplicons were sent to Soygen Biotechnology (Istanbul, Turkey) for sequencing. The sequence data were analyzed and performed via BLAST search in the GenBank database to identify the closest available reference sequences in the complete National Center for Biotechnology Information (NCBI) nucleotide collection (14). Accordingly, the accession numbers of the isoletes submitted to NCBI are MK962023 through MK962106. The 16S rRNA sequences of the strains were arranged using Molecular Evolutionary Genetics Analysis (MEGA) 7.0 software. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method with 1000 bootstrap replicates (15).

Antibiotic susceptibility testing

Antimicrobial susceptibility tests were performed by the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) (16). For this purpose, clinically significant antibiotics were spotted on Muller-Hinton Agar (Merck, Germany). These include; ampicillin (10 µg), penicillin (10 µg), teicoplanin (30 µg), vancomycin (30 µg), gentamycin (120 µg), streptomycin (10 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), tetracycline (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), norfloxacin (10 µg), oxacillin (1 µg) and rifampin (5 µg) (Bioanalyse, Turkey). Then the plates were incubated aerobically at 37 °C for 24 h. According to diameter of the inhibition zone, strains were categorized as susceptible, intermediate, or resistant by taking into account the criteria of the CLSI (16).

Growth inhibition of bacterial pathogens

An agar spot test was performed as described elsewhere (17) with minor modifications. Briefly, overnight cultures (10^6 cfu/mL) of each isolate to be tested were spotted onto the surface of Tryptic Soy Agar (TSA) (Merck, Germany) (containing 1.5%, w/v, agar) and incubated for 24 h at 37 °C. The pathogenic bacteria, which are *Listeria monocytogenes* ATCC 13932, *L. innocua* ATCC 33090, *Staphylococcus aureus* ATCC 43300 and *Escherichia coli* ATCC 25922 were inoculated into 8 mL of TSA (containing 0.7%, w/v, agar) at a final concentration of approximately $5x10^6$ cfu/mL and poured over the plate on which the potentially antimicrobial producer strain was grown. After incubation for 16-24 h at 37 °C, the diameter of the inhibition zone has been measured. Accordingly, 5-10 mm inhibition zone was evaluated as strong inhibition (++), whereas inhibition zone between 1-5 mm were assessed to be weak inhibition (+). No-diameter zone was evaluated to be no-inhibition as negative (-).

Statistical analysis

Analysis of variance (ANOVA, F test) to compare identification methods, and the correlation between antibacterial results was performed using SPSS 22.0.0 software (SPSS Inc., Chicago, USA) to analyze between groups. The level of significance of differences between treatments was determined at $p<0.05$.

RESULTS

Among 84 enterococcal strains: 33 (39.3%) were ascribed to *E. faecalis*, 29 (34.5%) to *E. faecium*, 14 (16.7%) to *E. durans*, 4 (4.8%) to *E. gallinarum*, 3 (3.5%) to *E. casseliflavus* and 1 (1.2%) to *E. thailandicus*. Comparing to MALDI-TOF-MS results, only three isolates have been characterized differently as a result of 16S rRNA sequencing, as indicated in Table 2. The difference between the two identification methods has not been found to be statistically significant ($p>0.05$).

Figure 1. represents the MEGA 7.0 alignments of the 16S rRNA genes of genetically close and distinct enterococcal strains indicating their phylogenetic relationship with the formation of different subgroups. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The analysis involved 84 nucleotide sequences. The cluster

alignments analysis indicates that *E. faecalis* differs from all other five species in a different cluster. *E. thailandicus* differs from *E. durans* with a number of nucleotides substitution, however, these two species were clustered within the same subgroup. Similar to the relationship between *E. faecium* and *E. durans*, *E. casseliflavus*, and *E. gallinarum* strains stay close to each other, as well.

Table 2. Comparative identification results of the strains which differ in two methods

Isolate	MALDI score	MALDI-TOF-MS	16S rRNA Sequencing
AU11	2.090	*E. faecium*	*E. casseliflavus*
AU24	2.165	*E. faecium*	*E. durans*
AU45	2.127	*E. casseliflavus*	*E. faecalis*

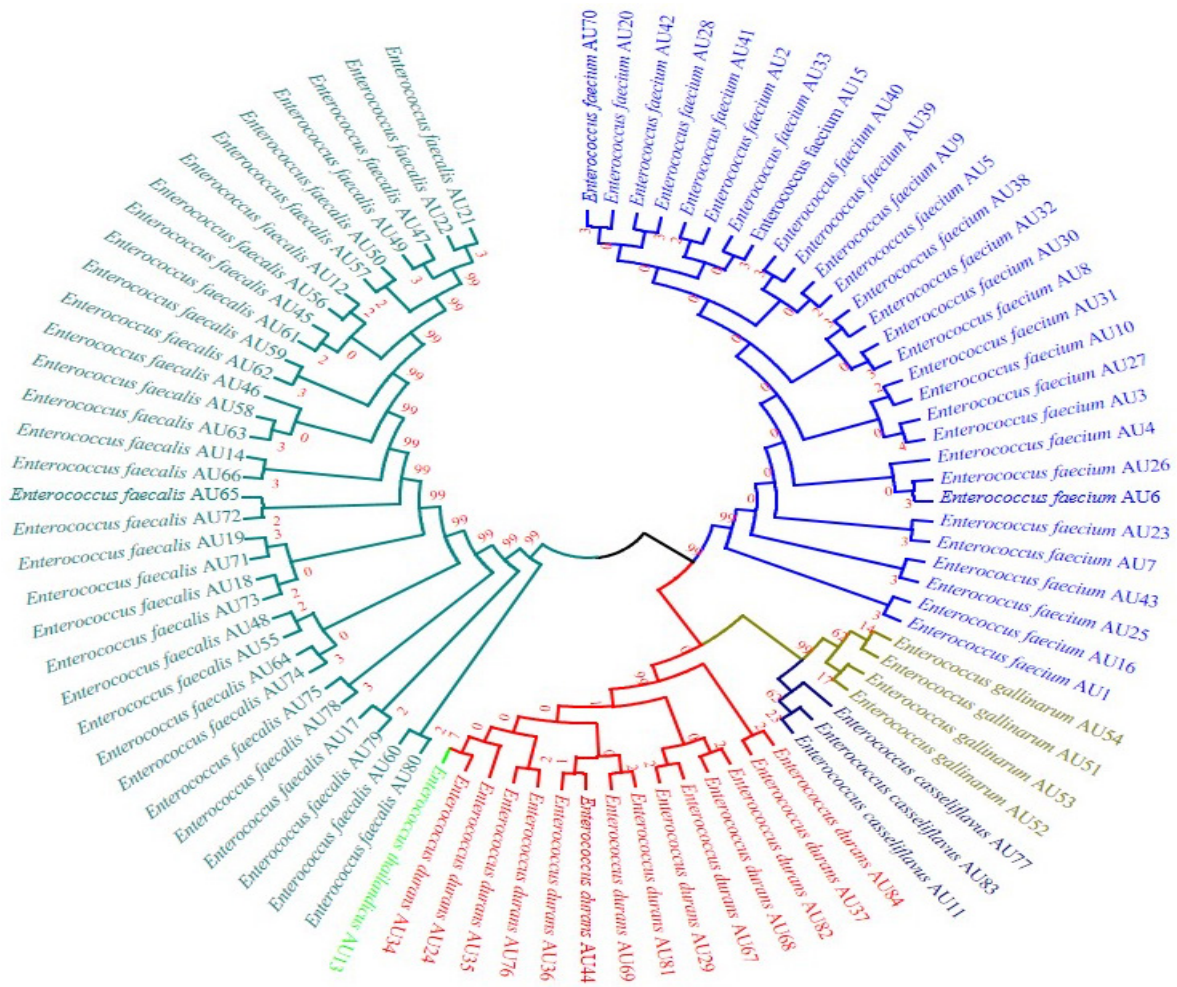

Figure 1. The evolutionary history was inferred using the Neighbor-Joining method

Numbers on the branches represent bootstrap values obtained from 1000 bootstrap replications

The results of antibiotic susceptibility testing, according to CLSI, are summarized as percentages of strains showing resistance (R), intermediate resistance (I) and no-resistance (S) in Table 3. Accordingly, analysis of the antimicrobial susceptibility of the 84 Enterococcus isolates revealed that all of the strains were found to be resistant to nalidixic acid (100.0%), which is followed by oxacillin (83.3%) and streptomycin (79.8%). On the other hand, all strains were assessed to be susceptible to norfloxacin. Along with this, *E. faecium* strains were more resistant to ß-lactam group antibiotics than other species. Among glycopeptides, a minimal amount of strains (n=3) showed intermediate-resistance to teicoplanin, while most of *E. faecalis* strains (93.9%) displayed either intermediate or strict resistance to vancomycin.

On the other hand, 62.1% of *E. faecium* strains were found to be susceptible to vancomycin. Among aminoglycosides, especially strains of *E. faecalis* (90.9%) and *E. faecium* (79.3%) exhibited more resistance to streptomycin rather than gentamycin. Furthermore, *E. faecalis* strains were assessed to be more resistant to chloramphenicol, tetracycline, and rifampin than others. Additionally, low frequencies of resistance (n= 6) have been detected to ciprofloxacin among all strains.

Table 3. Antimicrobial resistance profiles of enterococci against tested antibiotics (%)

Antibiotic	*E. faecalis* (n = 33)			*E. faecium* (n = 29)			*Enterococcus* spp.* (n = 22)			Total (n = 84)		
	S**	I**	R**	S	I	R	S	I	R	S	I	R
ß-lactams												
Ampicillin	87.9	-	**12.1**	58.6	-	**41.4**	77.3	-	**22.7**	73.8	-	**26.2**
Penicilin	100	-	**-**	44.9	24.1	**31.0**	100	-	**-**	80.9	8.4	**10.7**
Glycopeptides												
Teicoplanin	93.9	6.1	**-**	96.5	3.5	**-**	100	-	**-**	96.4	3.6	-
Vancomycin	6.1	63.6	**30.3**	62.1	17.2	**20.7**	81.8	9.1	**9.1**	44.1	34.5	**21.4**
Aminoglycosides												
Gentamycin	60.4	15.2	**24.4**	34.3	17.4	**48.3**	9.1	18.2	**72.7**	38.1	16.7	**45.2**
Streptomycin	-	9.1	**90.9**	6.9	13.8	**79.3**	13.7	22.7	**63.6**	5.9	14.3	**79.8**
Quinolones												
Ciprofloxacin	75.8	15.2	**9**	75.9	17.2	**6.9**	95.5	**-**	**4.5**	80.9	11.9	**7.2**
Amphenicols												
Chloramphenicol	15.1	15.1	**69.8**	48.3	10.3	**41.4**	45.5	4.5	**50.0**	35.7	9.5	**54.8**
Tetracyclines												
Tetracycline	6.1	39.4	**54.5**	62.1	13.8	**24.1**	50	45.5	**4.5**	36.9	28.6	**34.5**
Macrolides												
Erythromycin	24.3	24.2	**51.5**	17.3	31.0	**51.7**	27.3	13.6	**59.1**	22.6	23.8	**53.6**
Others												
Nalidixic acid	-	-	**100**	-	-	**100**	-	-	**100**	-	-	**100**
Norfloxacin	100	-	**-**	100	-	**-**	100	-	**-**	100	-	-
Oxacillin	-	15.2	**84.8**	-	31.1	**68.9**	-	-	**100**	-	16.7	**83.3**
Rifampin	18.2	15.2	**66.6**	44.8	31	**24.2**	59.1	18.2	**22.7**	38.1	21.4	**40.5**

*: *Enterococcus* spp. includes *E. durans*, *E. casseliflavus*, *E. gallinarum*, and *E. thailandicus* strains
**: S: susceptible; I: Intermediate-resistant; R: resistant
-: Not detected.

The summary of enterococci exhibiting resistance to at least three antimicrobial agents is given in Table 4. Accordingly, since enterococci are known to have resistance to nalidixic acid intrinsically, it was excluded while evaluating the multidrug resistance. Out of 84 isolates, 70 (83.3%) were found to be resistant at least three antibiotics, while 14 (16.7%) of the isolates did not carry multidrug resistance. Among species, *E. faecalis* was found to show more resistance phenotypes than other species.

The antibacterial results of enterococcal strains against selected food pathogens are summarized in Table 5. Most of the isolates showed low inhibition level against *E. coli* ATCC 25922 and *S. aureus* ATCC 43300. Along with this, 26 out of 33 strains of *E. faecalis* showed strong inhibition effects against *L. innocua* ATCC 33090, while three strains were found to have had a more weakly inhibition effect. It is followed by the strong effect of *E. faecalis* to *L. monocytogenes* ATCC 13932. Comparing to other Enterococcus species identified in this study, the inhibition effects of *E. faecalis* against *L. innocua* ATCC 33090, and *L. monocytogenes* ATCC 13932 were statistically found to be significant ($p<0.05$). Moreover, a strong correlation has been found on the antimicrobial effect of *E. faecalis* against these two food pathogens ($p = 0.941$).

Table 4. Resistance to multiple antibiotics detected among the strains

	E. faecalis (n = 33)	*E. faecium* (n = 29)	*Enterococcus* spp.* (n = 22)	Total (n = 84)
Resistance to 3 antibiotics	7	5	9	21
Resistance to 4 antibiotics	6	11	6	23
Resistance to 5 antibiotics	17	4	3	24
Resistance to 6 antibiotics	-	2	-	2

*: *Enterococcus* spp. includes *E. durans*, *E. casseliflavus*, *E. gallinarum*, and *E. thailandicus* strains
-: Not detected

DISCUSSION

Fast and reliable identification in food microbiology has become a real challenge. Since biochemical methods based on phenotypic characteristics take time and give indecisive results, these methods have been mostly replaced by PCR based molecular methods, particularly sequencing of the 16S rRNA region in bacteria (18). Sequencing requires an additional cost as well as being time-consuming. Alternative methods, such as MALDI-TOF-MS, are still of concern, which gives identification result on species level within a few minutes from a single colony grown on an agar plate (19). Therefore, the comparison of MALDI-TOF-MS and 16S rRNA sequencing, as well as antibiotic resistance and antimicrobial activity to various food pathogens, were aimed.

The results of this study revealed that 81 of 84 (96.4%) isolates were identified correctly on species level ($p>0.05$) and are in accordance with several studies, revealing that MALDI-TOF-MS could be a powerful tool for fast and reliable identification

Table 5. Antibacterial activity of Enterococcus strains against selected reference food pathogens strains

Pathogens	*E. faecalis* (n = 33)	*E. faecium* (n = 29)	*Enterococcus* spp.* (n = 22)
***L. monocytogenes* ATCC 13932**			
++	23 (69.6%)	8 (27.6%)	1 (4.6%)
+	5 (15.2%)	2 (6.9%)	3 (13.6%)
-	5 (15.2%)	19 (65.5%)	18 (81.8%)
***L. innocua* ATCC 33090**			
++	26 (78.8%)	9 (31.0%)	2 (9.1%)
+	3 (9.1%)	1 (3.4%)	2 (9.1%)
-	6 (18.1%)	19 (65.6%)	18 (81.8%)
***E. coli* ATCC 25922**			
++	1 (3.0%)	2 (6.9%)	4 (18.2%)
+	8 (24.2%)	12 (41.4%)	7 (31.8%)
-	24 (72.8%)	15 (51.7%)	11 (50.0%)
***S. aureus* ATCC 43300**			
++	2 (6.0%)	3 (10.3%)	5 (22.7%)
+	11 (33.3%)	8 (27.6%)	2 (9.1%)
-	20 (60.7%)	18 (62.1%)	15 (68.2%)

*: *Enterococcus* spp. includes *E. durans*, *E. casseliflavus*, *E. gallinarum*, and *E. thailandicus* strains
Results are expressed as a result of diameters of the inhibition zone
++ inhibition zone 5-10 mm, + inhibition zone 1-5 mm, −: no inhibition zone

(20). Apart from other studies, two of *E. faecium* strains and one *E. casseliflavus* strain have been characterized differently as a result of 16S rRNA sequencing. Strictly related species are likely a problem owing to similarities in ribosomal proteins that are the main proteins targeted by MALDI-TOF-MS (20). Besides, assessment of intensities of the common peaks obtained is sometimes not applicable for differentiation between phase variants, especially in the case of the high similarity of their profiles (21). Based on the MALDI scores obtained for Enterococcus isolates as seen in Table 2, which are below 2.300, are in accordance with the fact that scores between 2.000-3.000 should not be accepted as correct identification. According to results reported by Aydin and Ardic (12), 17 out of 84 isolates had MALDI scores between 2.000 - 2.300. 16s rRNA sequencing confirmed 14 of them, for which identification confidence is 82.35%. Since most of the researchers assume 2.000 and higher MALDI scores as correct identification on species level (2,44-45), care must be taken within this regard.

The 16S rRNA sequencing of the strains revealed the presence of *E. faecalis, E. faecium, E. durans, E. gallinarum, E. casseliflavus* and *E. thailandicus*. Among all, *E. faecalis* and *E. faecium* were reported to have been isolated from traditional cheeses as members of dominant microflora by researchers (22). The prevalence of *E. faecalis, E. faecium* and *E. durans* have been associated with geographical location and to lack of hygienic production conditions since they are the most abundant in the human gastrointestinal tract. At the same time, *E. casseliflavus* and *E. gallinarum* strains are ubiquitously found in environmental reservoirs (23). Fewer reports have been released regarding the identification of *E. gallinarum*, and *E. casseliflavus* (13) from cheese samples, which are known to be intrinsically vancomycin-resistant enterococci (VRE) and to cause bloodstream infections (24). To best of our knowledge, it is the first report revealing *E. thailandicus* as a result of 16S rRNA gene sequencing from traditional cheeses, which reveals that this species cannot only be found in meat and meat-based products. Therefore, the prevalence and the contribution of this species to cheese technology should be of interest as well as other non-dominant enterococci. Such as *E. hirae*, *E. gallinarum*, and *E. italicus*. This species was firstly isolated from sausage specimens in Thailand by Tanasupawat et al. (25). It is genetically similar to *E. hirae* by a ratio of 99.3-99.6% and assumed to be isolated from mostly bovine feces, which explains its prevalence in mostly meat and fermented meat products (25). Although genetically close species sometimes cause some problems when MALDI-TOF-MS is used due to similarities in ribosomal proteins, 16s rRNA sequencing indicated that MALDI-TOF-MS is capable of differentiating *E. thailandicus* from *E. durans*. These two species were clustered in the same subgroup in the phylogenetic tree constructed by Neighbor-Joining method with 1000 bootstrap (Fig 1).

The food chain is of great concern owing to being the main route for the introduction of antibiotic-resistant enterococci from animal and environment sources into the human gastrointestinal tract, where the genes might be transferred to pathogenic bacteria (7). In recent years, the concern about antibiotic resistance has increased dramatically. Raw milk cheeses harbor many sorts of bacteria, among which enterococci constitute a considerable portion (1). Among ß-lactams, glycopeptides, and aminoglycosides group antibiotics, only 5.9% of enterococci have been found to be susceptible to streptomycin. These results are in accordance with those reported by Karaalioğlu et al. (26). In combination with cell wall inhibiting antibiotics like penicillin, and ampicillin which were found to have inhibited the growth of the strains by rates of 80.9%, and 73.8%, are the antibiotics of choice for treating enterococcal infections, as well (27). Since streptomycin or gentamycin has been used in combination with penicillin in drinking water to treat some animals, there is a possibility that antibiotic-resistant enterococci may develop, and be transmitted by food (7). The results obtained in this study reveals that enterococci exhibited a lower resistance to glycopeptides than other groups. The reason for this is thought to arise from using ß-lactams and aminoglycosides more in common alone or combination.

VRE has spread globally and has become a significant cause of nosocomial infections as opportunistic pathogens. Treatment of VRE infections is hindered owing to resistance to multiple other agents (28). The emerge of enterococci resistance to vancomycin includes clinical overuse of antibiotics, cross-resistance, and use of avoparcin, which is a growth promoter in cattle, and which shares a chemical similarity with vancomycin causing the prevalence of vancomycin-resistant strains of bacteria (29). According to the results, the resistance between vancomycin (55.9%) and teicoplanin (3.6%) was found to be significant ($p<0.05$), which are two major glycopeptides. They constitute one of the few available therapies of severe infections that occurred by enterococci. Given the high number of cases, the

previously documented occurrence of transmission of VRE to people via the food chain, and the failure in the treatment, glycopeptides are classified as being of the highest priority (30). Since the cow milk is mostly used in the geographical regions in which squeezed cheeses are manufactured, the difference in the effect of these two antibiotics belonging to glycopeptides on the strains is noteworthy. When the results are investigated, *E. faecalis* strains displayed lower sensitivity than *E. faecium* did ($p<0.05$), which are in accordance with the results reported by Shridhar and Dhanashree (31).

Erythromycin, chloramphenicol, and tetracycline resistances, which are significant concerns for dairy enterococci, were 77.4%, 64.3%, 63.1%, respectively. Although the results reported by Sanlibaba and Senturk (22) show lower resistance profiles, the results obtained in this study are in accordance with those reported by Yogurtcu and Tuncer (32). The high prevalence of tetracycline and erythromycin resistance is in keeping with several studies revealing the ubiquitous presence of tetracycline and erythromycin resistance genes in the environment, and animal facilities, and due to the efficient transfer mechanisms of the resistance genes by conjugative plasmids and transposons (33,34). The reason for the difference between studies are thought to be often strain- and region-dependent.

The highest resistance was observed in nalidixic acid in all strains, which is an expected result since most of the enterococcal strains have been reported to have resistance to nalidixic acid intrinsically (35). Resistance to rifampin, which inhibits the transcription of mRNA, and evaluated to be an alternative drug to cure VRE infections, was found to be the highest among *E. faecalis* strains (81.8%). Our findings also indicate that all of the strains were either intermediate or strictly resistant to oxacillin, for which most enterococci have innate resistance (36).

Multidrug resistance refers to resistance at least three antimicrobial agents, which are found to be 83.33% (70 out of 84) in this study. Although Cariolato et al. (37) previously reported that multiple antibiotic resistance was uncommon among dairy enterococci, our results are not in accordance with what they stated. This frequency in multidrug resistance can be related to efficient mechanisms for the transfer of transposable genetic elements (33). On the other hand, *E. faecalis* strains showed more resistance phenotype than *E. faecium* did, which differs from the results revealed by Sanlibaba and Senturk (22) but resembles with those reported by Yuksel et al. (36). Therefore, thinking that resistance to a significant amount of antibiotics can be acquired from the strains sharing the same microflora or environment, the difference between the outcome of contrary studies can be attributed to working with strains obtained from different regions.

Antimicrobial metabolites synthesized by enterococci contain mainly hydrogen peroxide, and antibacterial peptides, namely bacteriocins/enterocins (13). Our results have revealed that *E. coli*, which is a Gram-negative bacterium, seems to be more resistant to the antimicrobial activity of enterococci to some extent than the Gram-positive bacterial strains which agree with the results reported by Veljovic et al. (38). As mentioned above, even though the results obtained from other studies are species- and strain-dependent, the anti-listerial effect of *E. faecalis* strains has been statistically found significant in this study ($p<0.05$), which resembles the results reported by some other researchers (39).

To sum up, the correlation between the results obtained from both MALDI-TOF-MS and 16s rRNA sequencing demonstrates that MALDI-TOF-MS is a fast, economical, robust, and reliable method for characterization of enterococci, thereby could be used as an alternative method on routine analysis. From the point of view of safety perspective, squeezed cheeses produced from raw milk without the addition of starter culture includes a great

amount of multiple antibiotic-resistant enterococci. On the other hand, the results of antibacterial activity revealed enterococci to be more effective against tested Gram-positive food pathogens. In this way, enterococci which do not carry multiple antibiotic resistance can be used in starter culture combinations in cheese manufacturing technology to inhibit the growth of Gram-positive food pathogen bacteria within the context of hurdle technologies, however, to this end, more studies on safety perspectives, such as determining virulent determinant, are required.

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