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ANTIBIOTIC RESISTANCE AND HEMOLYTIC ACTIVITY IN ENTEROCOCCI ISOLATED FROM TULUM CHEESE SOLD IN AKSARAY PROVINCE

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ABSTRACT
This study aimed to evaluate the hemolytic activity and antibiotic susceptibility of enterococci isolated from Tulum cheese samples sold in Aksaray province. The isolates were identified as *E. faecium* (*n* = 30), *E. faecalis* (*n* = 25), and *E. durans* (*n* = 7) as a result of 16S rRNA gene sequencing. Three strains (*E. faecalis* ATC12, *E. faecium* ATC49, and *E. faecium* ATC54) exhibited ß-hemolytic activity, whereas others were non-hemolytic. Enterococci were found to have an intermediary or high resistance to nalidixic acid (%100), oxacillin (92%), and streptomycin (72.6%), respectively. *E. faecalis* strains had more resistant phenotypes to various clinically significant antibiotics than *E. faecium* and *E. durans*. Multi-drug resistance was found in 41.93% of the isolates. According to the results, Tulum cheese produced in Aksaray could be a potential vehicle for the transmission of antibiotic resistance via the food chain.
Keywords: Tulum cheese, *Enterococcus*, hemolytic activity, antibiotic resistance

AKSARAY İLİNDE SATIŞA SUNULAN TULUM PEYNİRLERİNDEN İZOLE EDİLEN ENTEROKOKLARIN ANTİBİYOTİK DİRENÇLİLİKLERİ İLE HEMOLİTİK AKTİVİTELERİNİN BELİRLENMESİ

ÖZ
Bu çalışmada, Aksaray ilinde satışa sunulan tulum peyniri örneklerinden izole edilen enterokokların hemolitik aktivitesi ile antibiyotik duyarlılığının değerlendirilmesi amaçlanmıştır. İzolatlar, 16S rRNA gen sekanslaması sonucunda *E. faecium* (*n* = 30), *E. faecalis* (*n* = 25) ve *E. durans* (*n* = 7) olarak tanımlanmıştır. Üç izolat (*E. faecalis* ATC12, *E. faecium* ATC49 ve *E. faecium* ATC54) ß-hemolitik aktivite gösterirken, diğerlerinin hemolitik aktivite göstermediği saptanmıştır. İzolatların toplamda nalidiksik aside (%100), oksasiline (%92) ve steptomisine (%72.6) karşı orta veya yüksek derecede direnç gösterdiği saptanmıştır. *E. faecalis* suşlarının klinik olarak önem arz eden çeşitli antibiyotiklere *E. faecium* ve *E. durans* suşlarından daha dirençli fenotipleri olduğu görülmüştür. Çoklu antibiyotik direncinin %41.93 olduğu saptanmıştır. Sonuçlar değerlendirildiğinde Aksaray'da üretilen Tulum peynirinin gıda yolu ile antibiyotik direncinin yayılmasında tehlikeli bir araç olduğu kanısına varılmıştır.
Anahtar kelimeler: Tulum peyniri**,** *Enterococcus,* hemolitik aktivite, antibiyotik dirençlik

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INTRODUCTION

Enterococci are a group of lactic acid bacteria (LAB) ubiquitously found in the gastrointestinal tracts of mammals (Franz et al., 1999). The presence of enterococci in food occurs due to the lack of hygienic production conditions. They can be found in raw milk because of fecal contamination. Their high ability to resist adverse environmental conditions, such as low pH and high salt concentrations, enables them to survive through cheese-making and ripening processes (Giraffa, 2003). Although many reports have been published regarding their positive contributions to cheese making technology in point of view of aroma production (Martino et al., 2016) and inhibition of several foodborne pathogens (Maia et al., 2017), they are not recognized as safe, unlike other LAB. Thus, the use as a starter or probiotic is still under debate, and they arenot listed in the QPS (qualified presumption of safety) approaches declared by the European Food Safety Authority (EFSA, 2013). Multidrug-resistant and/or virulent enterococci (MRE;VE)give rise to several types of nosocomial infections, including urinary tract, surgical site, and bloodstream infections (Togay and Temiz, 2011; Kankaya et al., 2017). Cheese, specially produced from raw milk, is a serious vehicle for the transmission of MRE and VE that can persist in the human intestinal tract (Erginkaya et al., 2007; Jamet et al., 2012). Furthermore, enterococci have an efficient gene transfer mechanism through horizontal gene transfer and are able to transfer their plasmids encoding virulence genes in the human gastrointestinal tract with a broad range of bacteria, including pathogenic species, which makes treatments hindering (Jahan et al., 2015).Dissemination of MRE and VE is a serious problem worldwide, and the food chain is thought to be a significant vector for the transmission of these transposable genes. Numerous studies have reported the detection of VE and antibiotic-resistant enterococci for different kinds of cheeses (Jamet et al., 2012; Yogurtcu and Tuncer, 2013; Ispirli et al., 2017; Sanlibaba and Senturk, 2018; Ozdemir and Tuncer, 2020).

Tulum cheese is a very popular cheese consumed in Turkey, along with Turkish white cheese and Kashar cheese.The name 'Tulum' comes from 'goat's or sheep's skin bag' in Turkish, which is thought to have been used in replace of alternative packaging materials in the past and gives unique properties to cheese. Even though a small proportion of production takes place on an industrial scale, most of the production is made traditionally with no standard method (Tekinsen and Akar, 2017; Arslaner and Turkmen, 2020). It means that many species of bacteria, including the*Enterococcus* genus, may grow and dominate the microflora during ripening. Traditional production and consumption of tulum cheese are widespread throughout Turkey.This study was planned to be conducted in tulum cheese sold in different retails in Aksaray province of Turkey.

Antibiotic resistance of enterococci isolated from tulum cheese produced in different provinces of Turkey has been studied by several researchers (Yogurtcu and Tuncer, 2013; Ozdemir and Tuncer, 2020; Yerlikaya and Akbulut 2020). As a result of these studies the strains were found to be resistant to different kind of antibiotics, suggesting that resistance of a strains is mostly strain- and region-dependent. Tulum cheese is widely consumed in Aksaray province. To the best of our knowledge, there is no report regarding the antibiotic susceptibility and hemolytic activityof enterococci isolated fromtulum cheese produced in Aksaray. The present study aims to evaluate the antibiotic susceptibility and hemolytic activity of *Enterococcus* spp. isolated from Tulum cheese sold in different retail markets in Aksaray.

MATERIALS AND METHODS

Isolation of enterococci

A total of 22 traditionally produced Tulum cheese samples were collected from different local markets in Aksaray throughout the autumn of 2019. The samples were transferred to Aksaray University, Food Microbiology Laboratory, in ice containers to be analyzed within 24 h. Ten grams of each cheese was homogenized in 90 mL of ¼ Ringer solution and diluted serially. Serial dilutions were inoculated onto Kanamycin Esculin Azide Agar (KEA) (Merck, Germany) to be incubated at 37 ºC for 48 h. Typical *Enterococcus*

colonies with a black halo were purified using Tryptic Soy Broth (TSB) (Merck, Germany) and Tryptic Soy Agar (TSA) (Merck, Germany). The purified colonies were incubated in TSB at different temperatures (10 and 45 °C), at 6.5% NaCl concentration, and pH 9.6. Catalase activity and Gram staining were performed for each isolate. Catalase-negative and Gram-positive cocci were assessed to be *Enterococcus* at the genus level (Vos et al., 2011) and were stored in TSA containing 40% glycerol at 20 ºC.

Molecular identification

The total DNA of each isolate was extracted from the overnight cultures grown in TSB following the protocol provided by Promega Wizard Genomic DNA Purification Kit (Promega, USA). To amplify the 16S rRNA region of the ribosomal box, primers (Amp-F) 5'-GAG AGT TTG ATY CTG GCT CAG-3' and (Amp-R) 5'- AAG GAG GTG ATC CAR CCG CA-3' (Y is C or T; R is A or G) were used as forward and reverse, respectively (Baker et al., 2003). Polymerase Chain Reaction (PCR) mixture contained 1 µL of 50 ng template DNA, 10 µL 5× PCR buffer, 0.4 µL dNTPs, 1 µL of 20 mM each primer, 0.25 µL 5 U *Taq* polymerase and sterile ddH_2O up to 50 µL of total volume. The reaction was performed using a T100 thermal cycler (Bio-Rad, USA). It consisted of 95 ºC/2 min, 20 cycles of 95 ºC/30s, 55 ºC/20s, 72 ºC/30s with a final extension at 72 ºC for 5 min. PCR products were visualized on 1.2% agarose gel using 1x TAE buffer and purified with Wizard SV Gel and PCR Clean-Up System (Promega, USA) according to manufacturer instructions. The purified amplicons were sent to a commercial company for bidirectional sequencing (Macrogen, South Korea).

The sequence data were analyzed and performed via a BLASTn search in the GenBank database (http://blast.ncbi.nlm.nih.gov/) to match the closest available reference sequences. Accession numbers of the isolates submitted to the National Center for Biotechnology Information (NCBI) are MT393685 through MT393746. Phylogenetic and molecular evolutionary analyses of sixty-two isolates from this study and the corresponding *Enterococcus* isolates in the GenBank nucleotide database (*E. faecalis*: NR114782, NR115765, NR040789; *E. faecium*: NR042054, NR114742, NR113904; *E. durans*: NR113257, NR036922) were aligned with ClustalW (Thompson et al., 1994). Phylogenetic trees were constructed using the Maximum-Likelihood method with bootstrap analyses in Mega X software (Kumar et al., 2018) as previously described by Aydin et al. (2020) in detail.

Hemolytic activity

The hemolytic activity of the strains was determined by streaking the overnight cultures onto Columbia Agar (Merck, Germany) supplemented with 5% sheep blood. The plates were incubated aerobically at 37 ºC for 24-48 h. The plates showing clear zones around the colonies were evaluated to be ß-hemolytic, whereas no-zones around the colonies were interpreted as ϒ-hemolytic (Semedo et al., 2003).

Antibiotic susceptibility testing

The antibiotic susceptibility test was carried out by the disc diffusion method on Mueller-Hinton Agar (Merck, Germany). The discs containing the following antibiotics were obtained from a commercial institution (Bioanalyse, Turkey):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 Rifampicin (5 µg). Determination of susceptibility was evaluated according to the criteria offered by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2011; 2016).

RESULTS AND DISCUSSION

Isolation and identification of enterococci

In the present study, 62 presumptive enterococci, which are surrounded by a black halo on the KEA medium, were isolated and identified with traditional methods at the genus level. All of the isolates gave Gram-positive and catalase-negative reactions as well as grew well at 10 ºC, 45 ºC, 6.5% NaCl concentration, and pH 9.6. The molecular identification was accomplished by using two

primers targetting 1500 bp fragment of 16S rRNA, as previously described by Baker et al. (2003). The BLASTn results of sequence data of amplicons exhibited 99-100% of homology with different *Enterococcus* strains deposited in the GenBank. Accordingly, 62 *Enterococcus* isolates were identified as *E. faecium* (n = 30), *E. faecalis* (n = 25), and *E. durans* (n = 7). The phylogenetic analysis of 16S rRNA by the Maximum-Likelihood method revealing polymorphism between species is demonstrated in Figure 1. The percentage of replicate trees where the associated taxa clustered together in the bootstrap test is indicated adjacent to the branches. The phylogenetic tree divided 70 isolates (those from this study and GenBank as reference strains) into two main clusters. *E. faecalis* strains differed from *E. durans* and *E. faecium* strains, sharing more similar homology.

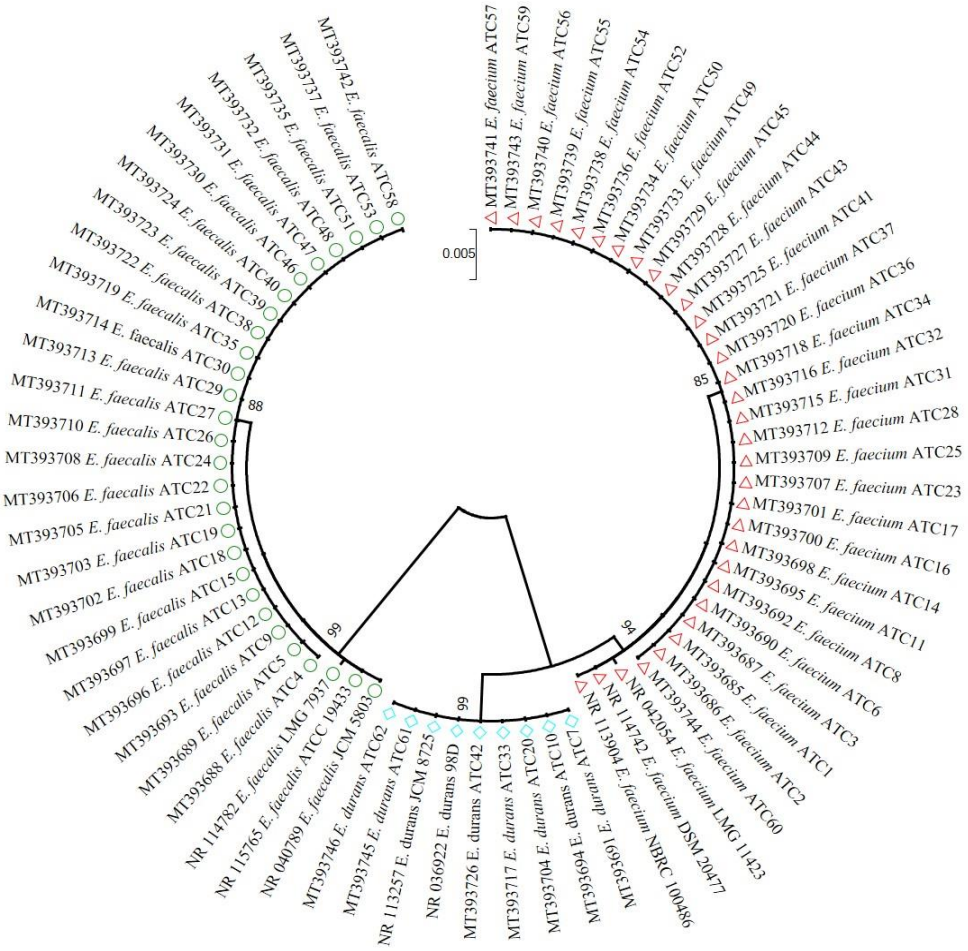

Figure 1 Maximum likelihood tree was constructed using 16S rRNA sequences of enterococci from this study and corresponding isolates from GenBank.

Enterococcus genus is reported to dominate the microflora of artisanally made cheeses during the ripening period (Kirmaci et al., 2016; Domingos-Lopes et al., 2017). They may have both technological importance and pose a health risk. Similar results, where *E. faecalis*, *E. faecium*, and *E. durans* were most frequently isolated, have been reported by several researchers in different kind of artisanal Turkish cheeses (Bulut et al., 2005; Dagdemir and Ozdemir, 2008; Ispirli et al., 2017; Aydin and Ardic, 2019).They are the most abundant *Enterococcus* species in the human

gastrointestinal tract (Giraffa, 2003). Our results are also in accordance with those reported by Oner et al. (2004) and Yogurtcu and Tuncer (2013) in Tulum cheese samples collected from Isparta and Ankara provinces of Turkey.

Hemolytic activity

E. faecalis ATC12, *E. faecium* ATC49, and *E. faecium* ATC54 strains showed ß-hemolytic activity, whereas others were ϒ-hemolytic, which indicates the absence of hemolysis on the agar medium supplemented with sheep blood. Haemolysin is a toxin encoded in either plasmid or the chromosomal DNA, and it is a significant virulence factor that affects the severity of the disease (Pérez-Pulido et al., 2006). Similarly, Psoni et al. (2006) and Trivedi et al. (2011) reported ß-hemolytic*Enterococcus* isolates from different cheese samples. Conversely, Yogurtcu and Tuncer (2013) did not find any ß-hemolytic strains among 47 *Enterococcus* strains from Turkish Tulum cheese. Non-hemolytic enterococci are advantageous in tulum cheese produced in Aksaray province;however, the strains showing negative ß-hemolysis phenotypically may harbor thehemolycins/cytolysin encoding genes. These genes may be silent, and their expression can change according to the environmental conditions, as reported by Semedo et al. (2003) and Pérez-Pulido et al. (2006).

Antibiotic suspectibility patterns

Sixty-two molecularly identified enterococci have been screened for their susceptibility to 14 different antibiotics using the disc diffusion method. The results obtained,according to CLSI, are indicated in Table 1. All strains were sensitive to norfloxacin (100%), which was followed by penicillin (93.3%), ampicillin (92%), teicoplanin (85.3%), chloramphenicol (79.7%), and ciprofloxacin (77.3%). Numerous studies have been carried out regarding the antibiotic resistance of enterococci from several kinds of traditional cheeses both phenotypically and in molecular ways (Citak et al., 2004; Yogurtcu and Tuncer, 2013; Furlaneto-Maia et al., 2014; Bulajić et al., 2015; Yuksel et al., 2015; Ispirli et al., 2017; Russo et al., 2018).Yogurtcu and Tuncer (2013) isolated 47 enterococci from tulum cheese and found them fully sensitive to ampicillin, chloramphenicol, gentamycin, penicillin, sulphamethoxazole, and vancomycin. They reported intermediary and high resistance to erythromycin (72.3%), streptomycin (55.3%), and tetracycline (34%).

The food chain is a great vector that transmits the antibiotic resistance from animal and environmental sources to the human gastrointestinal tract by means of bacteria having an efficient gene transfer mechanism, such as the *Enterococcus* genus. Enterococci are regarded as intrinsically resistant to ß-lactam group antibiotics, such as penicillin and ampicillin, due to their prolonged use for the treatment of enterococcal infections (Garrido et al., 2014). However, the strains were mostly susceptible to ampicillin (92%) and penicillin (93.3%). Similar results were obtainedfor different traditional cheese samples, as reported by Yogurtcu and Tuncer (2013) and Bulajić et al. (2015). On the other hand, Pesavento et al. (2014) and Sanlibaba and Senturk (2018) reported relatively higher ampicillin resistance than penicillin.

Our isolates were mostly sensitive to vancomycin (70.7%); however, 80% of *E. faecalis* strains were resistant to vancomycin at an intermediate or strong level.In the past, most of the resistance in enterococci to vancomycin was caused by the use of avoparcin as a growth promoter. The use of avoparcin is forbidden in Turkey. Therefore, vancomycin-resistant enterococci occur mostly due to the overuse of antibiotics and cross-resistance. (Miller et al., 2016). Yerlikaya and Akbulut (2020) reported that all of the *Enterococcus* strains were fully sensitive to vancomycin in İzmir Tulum cheese. Similar results were also reported by Ozdemir and Tuncer (2020). On the other hand, Citak et al. (2004) reported very high resistance (96.7 %)for*E. faecalis* strains. The isolates were found to be resistant to gentamycin at a higher ratio than streptomycin, which arethe two aminoglycosides. Similar results were reported by Yogurtcu and Tuncer (2013) in Tulum cheese produced in Isparta province.

Table 1. Antimicrobial resistance patterns of enterococci against tested antibiotics (%).

Antibiotic	*E. faecalis* (*n* = 25)			*E. faecium* (*n* = 30)			*E. durans* (*n* = 7)			Total (*n* = 62)		
	S*	I*	R*	S	I	R	S	I	R	S	I	R
Ampicillin	80	-	20	100	-	-	100	-	-	92	-	8
Penicillin	100	-	-	86.6	6.7	6.7	85.7	-	14.3	93.3	2.7	4
Teicoplanin	84	16	-	76.8	6.7	16.5	100	-	-	85.3	8	6.7
Vancomycin	20	24	56	90	3.3	6.7	100	-	-	70.7	9.3	20
Gentamycin	36	56	8	50.5	36.3	13.2	42.9	57.1	-	53.3	38.7	8
Streptomycin	40	28	32	23.1	53.8	23.1	14.3	28.6	57.1	27.4	40.3	32.3
Ciprofloxacin	80	12	8	63.7	23.1	13.2	57	14.3	28.7	77.3	12	10.7
Chloramphenicol	68	8	24	73.5	16.5	10	100	-	-	79.7	9.3	12
Tetracycline	44	24	32	76.8	16.5	6.7	85.7	14.3	-	70.6	18.7	10.7
Erythromycin	52	20	28	67	19.8	13.2	71.3	14.3	14.3	61.2	19.4	19.4
Nalidixic acid	-	-	100	-	-	100	-	-	100	-	-	100
Norfloxacin	100	-	-	100	-	-	100	-	-	100	-	-
Oxacillin	4	4	92	16.5	3.3	80.2	-	28.7	71.3	8	5.3	86.7
Rifampicin	24	12	64	59.4	13.2	27.4	57	14.3	28.7	54.6	10.7	34.7

*: S: Susceptible; I: Intermediate-resistant; R: Resistant

Even though low levels of resistance were observed for tetracycline and erythromycin among all enterococci, *E. faecalis* strains showed higher resistance comparing to *E. faecium* and *E. durans* strains. Cariolato et al. (2008) reported *Enterococcus* spp. from different dairy materials were found to be resistant to tetracycline (30.8%) and streptomycin (25.6%) at low levels. The prevalence of erythromycin and tetracycline resistance is often thought to arise from the efficient gene transfer mechanism by means of plasmids and transposons since the resistance genes of these two antibiotics are ubiquitous in the environment and animal facilities (Patterson et al., 2007). Besides, tetracycline is used as a growth promoter in livestock (Wegener, 2003), which may explain the relatively high level of resistance in *E. faecalis* strains.

All of the strains were highly resistant to nalidixic acid. Most of the enterococci have intrinsic resistance to nalidixic acid. Similar results were reported by Yuksel et al. (2015) and Sanlibaba and Senturk (2018). Conversely, much lower resistance to nalidixic acid (62%) was reported by Furlaneto-Maia et al. (2014). Rifampicin resistance waswidespread among *E. faecalis* strains. Seventy-four percent of *E. faecalis* strains were intermediately and highly resistant to rifampicin, which inhibits the transcription of mRNA of the cell (Miller et al., 2014). Similar results were obtained by Sanlibaba and Senturk (2018), whereas higher resistance patterns were reported for *E. faecium* and *E. faecalis* from different traditional cheeses (Yuksel et al., 2015). In total, *E. faecalis* strains were found to be more resistant to ampicillin, vancomycin, gentamycin, chloramphenicol, tetracycline, erythromycin, and rifampicin comparing to *E. faecium*, and *E. durans* strains.

Table 2. Resistance to multiple antibiotics detected among the strains.

	E. faecalis (*n* = 25)	*E. faecium* (*n* = 30)	*E. durans* (*n* = 7)	Total (*n* = 62)
Resistance to 3 antibiotics	2	4	3	9
Resistance to 4 antibiotics	4	6	1	11
Resistance to 5 antibiotics	0	4	0	4
Resistance to 6 antibiotics	0	2	0	2

Multi-drug resistance (MDR) can be defined as resistance to at least three antibiotics (Sanlibaba and Senturk, 2018).The summary of MDR is given in Table 2. According to our results, 41.93% (n = 26) of the strains were resistant to at least three antibiotics. *E. faecium* strains had more MDR patterns than *E. faecalis* and *E. durans* strains which is contrary to results reported by Gomes et al. (2008) and Pesavento et al. (2014). *E. faecium*ATC 49 and *E. faecium*ATC54 were resistant to six antibiotics, including streptomycin and gentamycin, which are clinically significant drugs to cure infections caused by MRE. These aminoglycosides are not affected by intrinsic enzymes produced by enterococci and have the ability to work with ß-lactam group antibiotics in synergism (Miller et al., 2014). According to results obtained by Yogurtcu and Tuncer (2013), only twoof the strains were resistant to tetracycline and a high level of streptomycin. Similar to those results, Cariolato et al. (2008) also stated that MDR is uncommon among enterococci from dairy origin. On the other hand, Sanlibaba and Senturk (2018) reported that MDR was 70.9% among enterococci isolated from traditional cheeses in Turkey. Relatively lower incidence (24.59%) in enterococci isolated from traditional and industrial cheeses was also reported by Bulajić et al. (2015). The difference is thought to be mostly strain- and region-dependent, taking into account that resistance to a significant amount of antibiotics can be obtained from the environment due to the efficient gene transfer mechanism of enterococci.

CONCLUSION

The present study mainly focused on the hemolytic activity and antibiotic susceptibility of enterococci isolated from Tulum cheese samples produced in Aksaray. Sequencing of the16S rRNA gene revealed that *E. faecium* and *E. faecalis* constituted the dominant *Enterococcus* microbiota of tulum cheese samples. Most of the strains did not show hemolytic activity, which is a significant virulence factor. *E. faecalis* strains had more resistance phenotypes to clinically essential antimicrobial agents than *E. faecium* and *E. durans*. High percentage (41.93%) of enterococci were MRD. Our results demonstrated that Tulum cheese widely consumed in Aksaray province could be a potential source for the transmission of antibiotic resistance. However, the phenotypic determination of antibiotic resistance or hemolytic activity may not be sufficient to explain the virulence determinants. Silent genes may be present within the genome or plasmids. More extensive genetic studies are required to determine potential virulence genes.

CONFLICT OF INTEREST

There is no possible conflict of interest among the authors.

AUTHOR CONTRIBUTION

Sule AYHAN and Mustafa ARDIÇ were responsible for collecting the cheese samples as well as evaluating hemolytic activity and antibiotic susceptibility. Halil İbrahim KAHVE and Furkan AYDIN performed the molecular analyses and wrote the article.

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