Kafkas Univ Vet Fak Derg
22 (5): 765-770, 2016
DOI: 10.9775/kvfd.2016.15369

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Antimicrobial Activity Potential of *Enterococcus* spp. Isolated from some Traditional Turkish Cheeses

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Article Code: KVFD-2016-15369 Received: 15.02.2016 Accepted: 19.04.2016 Published Online: 20.04.2016

Abstract

Enterococci can produce enterocins which have antimicrobial activity against Gram-positive and also Gram-negative pathogenic, toxigenic and food-spoilage bacteria. The aim of this study was to determine the antimicrobial activity of *Enterococcus* spp. isolated from traditional Turkish cheeses such as Kashar, *Manyas, Sepet, Kelle, Mihalic, Tulum*. The isolates were tested against *Listeria monocytogenes, Listeria innocua, Listeria ivanovii, Staphylococcus aureus* and *Enterococcus faecalis* and also detected the presence of *ent*A and *ent*B genes of these isolates. Total 66 of enterococcal isolates were obtained from 34 of cheese samples and 25 of these isolates showed antimicrobial activity against tested reference bacteria by using agar spotting method. Also it was determined most of *Enterococcus* spp. carried enterocin encoding *ent*A and *ent*B genes. We concluded that these isolates or their enterocins may have a potential for food preservation, however they should be evaluated in terms of food safety.

Keywords: *Enterococcus, Antimicrobial activity, Enterocin, entA, entB, Traditional cheese*

Bazı Geleneksel Türk Peynirlerinden İzole Edilen *Enterococcus* spp.'nin Antimikrobiyal Aktivite Potansiyeli

Özet

Enterokoklar, patojenik, toksijenik ve gıdalarda bozulma yapan Gram-pozitif ve hatta Gram-negatif bakterilere karşı antimikrobiyal etkiye sahip enterosinler üretebilmektedir. Bu çalışmanın amacı, Kaşar, *Manyas*, *Sepet, Kelle, Mihaliç, Tulum* gibi geleneksel Türk peynirlerinden elde edilen *Enterococcus* spp. izolatlarının antimikrobiyal aktivitelerinin belirlenmesidir. İzolatların, *Listeria monocytogenes, Listeria innocua, Listeria ivanovii, Staphylococcus aureus* ve *Enterococcus faecalis*'e karşı aktiviteleri test edilmiş ve ayrıca izolatlarda *ent*A ve *ent*B gen varlığı araştırılmıştır. Çalışmada 34 peynir örneğinden toplam 66 adet enterokok izole edilmiş ve yapılan testte bunların 25 tanesinin test edilen bakterilere karşı antimikrobiyal aktivite gösterdiği belirlenmiştir. Ayrıca bu izolatların çoğunun enterosin kodlayan *ent*A ve *ent*B genlerini taşıdıkları görülmüştür. Bu izolatların ya da bu izolatlardan elde edilecek enterosinlerin gıda koruyucusu olarak kullanım potansiyeli olduğu ancak izolatların gıda güvenliği yönüyle değerlendirilmesinin gerektiği düşünülmektedir.

Anahtar sözcükler: *Enterococcus, Antimikrobiyal aktivite, Enterosin, entA, entB, Geleneksel peynir*

INTRODUCTION

In Turkey, there are more than 50 cheese varieties and the main produced cheeses are Turkish white cheese, Kashar, *Tulum*, *Lor* and *Cokelek*, etc. The cow's, ewe's and goat's milk are used in the production of these cheeses. Beside of these, there are some local cheeses such as *Abaza*, *Mihalic*, *Sepet* cheese, *Ezine* Goat's cheese, etc.[1-3].

Enterococci are important members of cheese microbial flora during ripening period [1,4] and have an important effect in developing taste and flavour of fermented cheeses, by their proteolytic and lipolytic activities [5-8]. Certain strains of *Enterococcus* spp. may be used as starter cultures, co-cultures or probiotics in the food industry [6-8]. Furthermore, it is known that some enterococci have pathogenic potential [9-13].

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Food safety, especially the control of food-borne pathogen bacteria such as *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus* have become an increasingly important concern in worldwide. The use of chemical preservatives in food industry has also increased and it is needed to create more natural food preservatives. So, naturally produced antimicrobial agents have a great interest in terms of food processing and also consumer concern [14,15]. Lactic acid bacteria and also enterococci may produce natural biopreservatives called bacteriocin. Bacteriocins show antagonistic effect especially against Gram-positive bacteria [6,16,17] and also Gram-negative pathogenic, toxigenic, and food-spoilage bacteria [17]. It is thought that bacteriocins have bactericidal mechanisms through pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA and inhibition of peptidoglycan synthesis [16]. It is suggested that bacteriocins can be more effective when used in combination with other antimicrobial hurdles such as organic acids, chelating agents or essential oils. This combine effect may provide to reduce the required bacteriocin levels for inhibition. It is expected to find novel bacteriocins with enhanced specificity and potency in the future perspective [15].

Enterococci may produce multiple bacteriocins. Enterocins A and B are most common bacteriocins that can produce by enterococcal isolates and may be found in the same isolates [18,19].

In this study, it was investigated the antimicrobial activity potential and enterocin encoding *ent*A and *ent*B genes of *Enterococcus* spp. isolated from some traditional Turkish cheeses against certain Gram positive bacterial strains such as *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivonovii*, *Staphylococcus aureus* and *Enterococcus faecalis*.

MATERIAL and METHODS

Isolation of Enterococcus spp. from the Cheese Samples

Thirty four samples including Kashar, *Manyas, Sepet, Kelle, Mihalic, Tulum, Orgu* cheeses and Turkish white cheeses were supplied from different cities in Turkey such as Manisa, Izmir, Balikesir, Trabzon and Tekirdag. Ten grams of each cheese sample were homogenized with 90 mL of a Maximum Recovery Diluent (MRD, Oxoid, United Kingdom). Decimal dilution series of samples were prepared in sterile MRD. Following inoculation on Kanamycin Aesculin Azide agar (Oxoid, United Kingdom) including kanamycin sulphate supplement, the samples were incubated at 37°C for 24-48 h, under aerobic conditions. The typical 1-3 black colonies surrounded by black zones were selected from each sample. Purification of colonies was done streaking onto Tryptic Soy agar (Merck, Germany). These isolates were detected at the genus level using Gram staining, catalase test and growth at 6.5% NaCl, 10°C, 45°C, and pH 9.6. Then, they were stored at -20°C in glycerol [20-22].

Determination of Antimicrobial Activity Potential of the Isolates

The antimicrobial activity potentials of 66 of *Enterococcus* spp. isolated from traditional Turkish cheeses were investigated by using agar spotting and well diffusion methods against some pathogens and spoilage bacteria such as *Listeria monocytogenes* ATCC 7644, *Listeria ivanovii* ATCC 19119, *Listeria innocua* ATCC 33090, *Staphylococcus aureus* ATCC 6538 and *Enterococcus faecalis* ATCC 29212.

Agar spotting test were performed by spotting 3 µL of an overnight *Enterococcus* spp. isolates onto the surface of Brain Heart Infusion agar (BHI, Oxoid, United Kingdom) plate and incubating at 37°C for 18-24 h. The plates were overlaid with 10 mL of BHI soft agar (0.7% agar) inoculated with 10 µL of the test culture (*Listeria monocytogenes* ATCC 7644, *Listeria ivanovii* ATCC 19119, *Listeria inocua* ATCC 33090, *Staphylococcus aureus* ATCC 6538 and *Enterococcus faecalis* ATCC 29212). After overnight incubation at 37°C for 18-24 h the plates were examined for clear inhibition zones at around spotted enterococcal isolates. The clear zone diameters were evaluated including the spotted culture [23].

Cell-free supernatants (CFS) were obtained from the enterococcal isolates which showed antimicrobial activity with agar spotting test. CFS which obtained by centrifugation at 12,000g at 4°C for 10 min were used in well diffusion methods for determination of antimicrobial activity potential of the isolates. CFS was then adjusted to pH 7.0 with 1 N NaOH. The wells were made using sterile hollow punches in freshly prepared lawns of the test culture (70 µL of an overnight culture grown in 10 mL BHI broth containing 0.7% agar). Thirty microliter of each neutralized CFS was placed into each well and plates were incubated aerobically for 18-22 h at 37°C. Antimicrobial activity was measured after incubation as a clear inhibition zone around the wells [24,25].

To test the proteinaceous nature of the inhibitors, the active cell-free supernatants, were subjected to various enzymes treatment trypsin and proteinase K, at 1 mg/mL at 37°C during 2 h. Then, the residual activity was detected against indicator strains as above. The absence of inhibition zones indicated protease sensitivity. The heat sensitivity was evaluated by exposing the supernatants to heat at 60°C, 100°C and 121°C for 15 min, then, the inhibitory activity was checked by well diffusion method [26].

Investigation of entA and entB Genes in the Isolates

Enterococcus spp. isolates (n = 25) which showed antimicrobial activity against tested pathogen bacteria were also evaluated in point of presence of *ent*A and *ent*B genes. The isolates were grown overnight at 37°C in BHI broth and the genomic DNAs of all isolates were extracted by phenol-chloroform procedure for the detection of enterocin encoding genes [22]. The bacteriocin (enterocin) encoding genes (*ent*A, *ent*B) were investigated by using

Polymerase Chain Reaction (PCR) with specific primers listed in *Table 1* [26]. The PCR protocole and also PCR components and concentrations were shown in *Table 2* and *Table 3*, respectively.

RESULTS

Total 66 of *Enterococcus* spp. were isolated from 34 of traditional Turkish cheeses samples including Kashar, *Manyas, Sepet, Kelle, Mihalic, Tulum, Orgu* cheeses and Turkish white cheeses.

The *Enterococcus* spp. were examined for their antimicrobial activity against some pathogen and food-spoilage bacteria and 25 of these isolates showed mono- or multi-antimicrobial activity against tested reference bacteria with a different zone diameter *(Table 4)*.

After evaluation of antimicrobial activity potential by using CFS in well diffusion method, pH adjustment, protease and heat sensitivity testing, it was observed the antimicrobial activity all 25 of *Enterococcus* spp. isolates *(Table 4)*. Twelve isolates had antimicrobial activity against *L. monocytogenes* ATCC 7644, seven, nineteen, sixteen and one isolates showed antimicrobial activity with different zone diameter against *Listeria innocua* ATCC 33090, *Listeria ivanovii* ATCC 19119, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 6538, respectively. Most of the isolates (n = 16) showed also multi-antimicrobial activity against tested pathogen and food-spoilage bacteria. It was observed that most of antimicrobial activity zone formed against *Listeria ivanovii* ATCC 19119 and *Enterococcus faecalis* ATCC 29212 test bacteria.

Table 1. *Specific primers of entA and entB genes*
Tablo 1. *entA ve entB genlerinin spesifik primerleri*

Enterocin Genes	Oligonucleotide Sequence	Product Size (bp)
*ent*A	f: AAATATTATGGAAATGGAGTGTAT	126
	r: GCACTTCCCTGGAATTGCTC	
*ent*B	f: GAAAATGATCACAGAATGCCTA	159
	r: GTTGCATTTAGAGTATACATTTG	

Table 2. *The PCR protocol for detecting enterocin structural genes*
Tablo 2. *Enterosin yapısal genlerinin belirlenmesinde kullanılan PZR protokolü*

Program	Temperature (°C)	Time	Cycle
First Denaturation	95	2 min	-
Denaturation	95	30 sec	
Anneling	56	30 sec	35
Extension	72	30 sec	
Final Extension	72	5 min	-

Table 3. *PCR components and concentrations for detecting enterocin structural genes*
Tablo 3. *Enterosin yapısal genlerinin belirlenmesinde kullanılan PZR bileşenleri ve derişimleri*

PCR Components	*ent*A (µL/tube)	*ent*B (µL/tube)	Final Concentration
Sterile bidistilled H_2O	15.7	16.2	-
10X buffer	2.5	2.5	1X
$MgCl_2$ (25 mM)	3.0	2.5	2.5 mM-3.0 mM
dNTP (2.5 mM)	0.3	0.3	0.3 mM/25 µL
Primer forward (10 pmol/µL)	1.0	1.0	10 mM/25 µL
Primer reverse (10 pmol/µL)	1.0	1.0	10 mM/25 µL
Taq polymerase (5U/µL)	0.5	0.5	2.5 U/25 µL
DNA (150 ng/µL)	1.0	1.0	150 ng/25 µL
Final volume	25.0	25.0	-

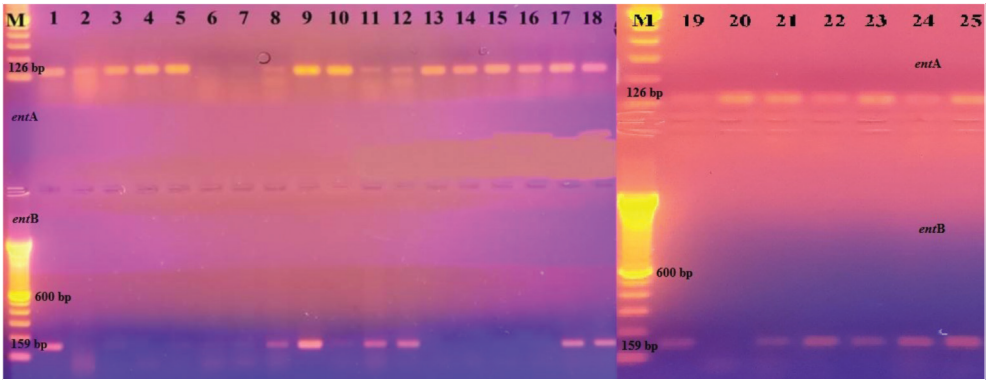

Fig 1. Enterocin structural genes in *Enterococcus* spp. isolated from traditional Turkish cheeses (1. LE 3-1, 2. LE 3-3, 3. LE 4-1, 4. LE 8-4, 5. LE 13-1, 6. LE 13-2, 7. LE 13-3, 8. LE 13-4, 9. LE 14-3, 10. LE 15-1, 11. LE 15-2, 12. LE 19-2, 13. LE 20-1, 14. LE 20-2, 15. LE 20-4, 16. LE 27-1, 17. LE 27-2, 18. LE 29-4, 19. LE 33-3, 20. LE 38-3, 21. LE 47-1, 22. LE 47-2, 23. LE 49-2, 24. LE 49-5, 25. LE 50-3)

Şekil 1. Geleneksel Türk peynirlerinden izole edilen *Enterococcus* spp. suşlarında enterosin yapısal genleri (1. LE 3-1, 2. LE 3-3, 3. LE 4-1, 4. LE 8-4, 5. LE 13-1, 6. LE 13-2, 7. LE 13-3, 8. LE 13-4, 9. LE 14-3, 10. LE 15-1, 11. LE 15-2, 12. LE 19-2, 13. LE 20-1, 14. LE 20-2, 15. LE 20-4, 16. LE 27-1, 17. LE 27-2, 18. LE 29-4, 19. LE 33-3, 20. LE 38-3, 21. LE 47-1, 22. LE 47-2, 23. LE 49-2, 24. LE 49-5, 25. LE 50-3)

Table 4. *Zone diameters of entA and/or entB positive Enterococcus spp. isolates against some test bacteria (mm)*
Tablo 4. *Test edilen bazı bakterilere karşı entA ve/veya entB pozitif Enterococcus spp. izolatlarının zon çapları (mm)*

Isolate No	Source	*L. ivanovii*	*L. innocua*	*L. monocytogenes*	*E. faecalis*	*S. aureus*	*entA*	*entB*
LE 3-1	Kashar cheese	15	-	7	15	12	+	+
LE 3-3	Kashar cheese	-	-	7	-	-	+	-
LE 4-1	Kashar cheese	9	-	-	7	-	+	+
LE 8-4	*Kashar cheese*	11	7	7	12	-	+	-
LE 13-1	*Manyas cheese*	11	-	-	13	-	+	+
LE 13-2	*Manyas cheese*	12	-	-	13	-	+	+
LE 13-3	*Manyas cheese*	11	-	10	10	-	+	+
LE 13-4	*Manyas cheese*	14	-	-	15	-	+	+
LE 14-3	*Kelle cheese*	17	15	20	15	-	+	+
LE 15-1	*Kelle cheese*	11	15	15	15	-	+	+
LE 15-2	*Kelle cheese*	-	-	15	-	-	+	+
LE 19-2	*Kelle cheese*	-	-	11	-	-	+	+
LE 20-1	*Sepet cheese*	10	-	10	10	-	+	-
LE 20-2	*Sepet cheese*	-	-	-	10	-	+	-
LE 20-4	*Sepet cheese*	7	-	-	10	-	+	-
LE 27-1	*Cerkes cheese*	10	-	-	-	-	+	-
LE 27-2	*Cerkes cheese*	12	17	-	-	-	+	+
LE 29-4	*Kelle cheese*	10	10	-	-	-	+	+
LE 33-3	*Mihalic cheese*	-	-	-	7	-	+	+
LE 38-3	*Orgu cheese*	12	12	15	15	-	+	-
LE 47-1	*Tulum cheese*	7	-	-	-	-	+	+
LE 47-2	*Tulum cheese*	15	-	-	-	-	+	+
LE 49-2	Kashar cheese	10	-	-	10	-	+	+
LE 49-5	Kashar cheese	-	-	20	-	-	+	+
LE 50-3	Turkish white cheese	8	10	13	12	-	+	+

It was showed that enterocin structural *ent*A and *ent*B genes in *Enterococcus* spp. isolated from traditional Turkish cheeses in *Fig. 1*. Eighteen of *Enterococcus* spp. isolates had both of *ent*A and *ent*B genes, while all of *Enterococcus* spp. isolates carried only *ent*A gene and eighteen of the isolates had only *ent*B gene *(Table 4)*. The LE 3-1 isolate from Kashar cheese, showed wide antimicrobial activity against *Listeria ivanovii* ATCC 19119, *L. monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 6538, respectively. The LE 14-3 isolate from *Kelle* cheese and LE 49-5 isolate from Kashar cheese showed most effective antibacterial activity against *L. monocytogenes* ATCC 7644 with 20 mm zone diameter *(Table 4)*.

DISCUSSION

Enterococcus species are found in human gastrointestinal tract, farm animals, and different foods such as meats, milk and cheeses [27]. In recent years, there has been enormous increase in the reports about enterococci used as starter cultures, co-cultures and probiotics [6-9]. The ability of enterococci to produce bacteriocins and to adapt to different environmental conditions are important characteristics for the food industry [6,8].

Several researches on determination of antimicrobial activity potential and enterocin encoding genes in *Enterococcus* species isolated from cheese samples were published [16,17,28-31]. Many of the findings are consistent with the results of our study. In a study performed by Renye et al.[29] 33 enterococcal isolates from Hispanic-style cheeses were screened for the production of bacteriocins and 5 *Enterococcus faecium* and 1 *Enterococcus durans* isolates which were inhibited the growth of *Listeria* spp. A PCR screen revealed that four *E. faecium* isolates contained nucleic acid sequences for multiple enterocins (*ent*A, *ent*P, *ent*L50AB and *ent*B). Similar to these results, enterococcal isolates contained multiple enterocin structural genes (*ent*A and entB) and showed antimicrobial activity against *Listeria* spp. (*L. monocytogenes*, *Listeria innocua* and *Listeria ivanovii) (Table 4)*. A study by Tuncer [30] also reported that three of *E. faecium* and one of *E. durans* isolates were found as bacteriocin producer strain obtained from Turkish Tulum cheese samples and most of these isolates showed antimicrobial activity against *Listeria innocua*. Favaro et

al.[16] determined bacteriocinogenic *E. faecium* isolates from Bulgarian homemade white brine cheese and it was reported that the isolates showed antilisterial activity and carried bacteriocin-encoding genes (*ent*A, *ent*B, *ent*P, *ent*L50B).

The *Enterococcus* spp. isolates which carried *ent*A and/or *ent*B genes did not show antimicrobial activity against all tested Gram positive bacteria such as *L. monocytogenes*, *S. aureus* and *E. faecalis* in this study. It was concluded that these enterococcal isolates may carry incomplete or nonfunctional bacteriocin genes or produce additional bacteriocins except *ent*A and *ent*B as mentioned by Abriouel et al.[32].

In conclusion, the single and/or multiple enterocin encoding genes (*ent*A and *ent*B) and also antimicrobial activity were detected in *Enterococcus* spp. isolates from traditional Turkish cheeses in this study. Although enterococcal isolates are widely used as starter and/or probiotic culture in food industry, this genus should be evaluated in terms of presence of virulence genes and acquired antibiotic resistance at the strain level. It is well known that enterococci, especially some of *E. faecium* and *E. faecalis* strains may have pathogenic potential, and cause infectious diseases in humans. However, the antimicrobial activity of *Enterococcus* isolates against *L. innocua*, *L. ivanovii, E. faecalis*, *L. monocytogenes* and *S. aureus* may create an opportunity for use them in food preservation.

Further studies are needed to purify and optimize the isolated enterocins. Safety evaluation of virulence genes and antibiotic resistance in these isolates is also necessary.

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