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Antilisterial Activity by *Enterococcus* Species Isolated from Traditional Cheeses

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ABSTRACT

In this study, it was aimed to detect the antimicrobial activity of 312 *Enterococcus* species against *Listeria monocytogenes*. Antimicrobial activity was detected by agar spot and well diffusion assay. A total of 201 enterococcal strains inhibited the growth of *L. monocytogenes* strains based on the agar spot test. Only 44 strains showed antimicrobial activity against *L. monocytogenes* strains using agar well diffusion assay. Of the 44 enterococcal strains screened, 6 *E. faecium* (2.99%) strains had a high antimicrobial effect against indicator *L. monocytogenes* strains. The antilisterial activity of 6 *E. faecium* strains had lost after treatment of proteinase K, trypsin and pepsin. The antimicrobial compounds of these strains could be a protein or peptides nature. *E. faecium* strains were more active against *L. monocytogenes* than *E. faecalis* strains.

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Geleneksel Peynirlerden İzole Edilen *Enterococcus* Türlerinin Antilisterial Aktivitesi

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ÖZ

Bu çalışmada, 312 adet *Enterococcus* suşunun *Listeria monocytogenes*'e karşı antimikrobiyel aktivitesinin saptanması amaçlanmıştır. Antimikrobiyel aktivite agar spot test ve kuyu difüzyon testleri ile tanımlanmıştır. Agar spot test sonucunda toplamda 201 enterokokkal suş, *L. monocytogenes* gelişimini inhibe etmiştir. Kuyu difüzyon testi sonucunda sadece 44 suş gıda kaynaklı patojene karşı antimikrobiyel aktivite göstermiştir. Taraması yapılan 44 suş içinden, 6 tanesi (%2,99) indikatör suşa karşı yüksek antimikrobiyel etki göstermiştir. Proteinaz K, tripsin ve pepsin uygulamaları sonucunda, 6 *E. faecium* suşunun antilisterial aktivitesi kaybolmuştur. Bu suşlardaki antimikrobiyel aktivitenin doğası protein veya peptit yapısındaki antimikrobiyel bileşiklerden kaynaklanabilir. *E. faecium* suşları, *E. faecalis* suşlarına oranla indikatör suşlara karşı daha fazla aktivite göstermiştir.

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Introduction

Foodborne pathogens cause serious diseases. Particular interest is focused on *Listeria monocytogenes* in the food industry, in this case, also (Bigot et al., 2011). Listeriosis outbreaks are associated with the consumption of contaminated food. *L. monocytogenes* is a Gram-positive, and non-spore forming bacterium. This pathogen can grow under anaerobic and aerobic conditions. Besides, it can survive even in extreme environmental conditions, such as high concentration of salts, wide temperature range, pH between 4 and 9.6, low water activities (Soni et al., 2014, Kurpas et al., 2018). There are many listericidal methods to eliminate of *L. monocytogenes* in food, including chemical and physical. As an alternative strategy, the use of bacteriocin to control *L. monocytogenes* as a food safety strategy is desirable (Arachchi et al., 2015). Nowadays, biocontrol strategies based on bacteriocin have received an increasing amount of interest because of their safety, practicability and economic feasibility (Lee et al., 2017). Many studies have investigated the effects of bacteriocin-producing enterococcal strains on *L. monocytogenes* in different food systems (Galvez et al., 2010, Trivedi et al., 2012, Huang et al., 2013).

Bacteriocins have ribosomally synthesized, small, cationic, and amphiphilic antimicrobial peptides or proteins. Bacteriocins which have produced by lactic acid bacteria (LAB) are very important due to their bactericidal activity against foodborne pathogenic and spoilage bacteria (Ohmomo et al., 2000, Hosseini et al., 2009). Their bactericidal activity on the sensitive cells is: i) depolarization of cell membrane, and ii) inhibition of cell wall synthesis (Dündar et al., 2015). *Enterococcus* species are known to be predominant LAB (Jurkovic et al., 2006). Although 64 *Enterococcus* spp. are now recognized, *E. faecium* and *E. faecalis* remain the most prominent species (Anonymous, 2020). The presence of *Enterococcus* spp. especially *E. faecalis* in food products is considered a sign of fecal contamination. However, some strains of *Enterococcus* species such as *E. faecium* SF68 have used as starter cultures, co-cultures, or probiotics (Toğay et al., 2014). In addition, more recently enterococcal strains have become accepted as part of the normal flora (Renye et al., 2009). They are frequently isolated from fermented dairy and meat products (Toğay et al., 2016). The ability of enterococci to produce bacteriocins had first noted by Kjems in 1955. Since then, bacteriocins which producing by enterococci (named as enterocins) have been described widely (Gaaloul et al., 2014). Enterocins belong to Class II bacteriocins,which were distinguished by their activity against *Listeria* spp. (Trivedi et al., 2012). To date, many enterococcal bacteriocins have been purified and characterized (Huang et al., 2013). One of them is also enterocin AS-48 produced by *E. feacalis* S-48. The cyclic peptide enterocin AS-48 is also commercial preparation (Galvez et al., 2010). This bacteriocin has broad bactericidal activity against the most Gram-positive bacteria including *L. monocytogenes* in meat, vegetable, and dairy products (Banos et al., 2016).

Thus, the objective of this study was to detect antimicrobial activities of *Enterococcus* spp. against *L. monocytogenes* strains.

Materials and Methods

Bacterial Strains and Growth Conditions

Three hundred and twelve different *Enterococcus* spp. strains, ninety-two different *Listeria monocytogenes* strains, three reference strains (*Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC BAA-2127 and *Listeria monocytogenes* ATCC 7644), and *Lactococcus lactis* LL27 (bacteriocin producer strain) were used, in this study. These strains have taken from the culture collection of Food Microbiology Laboratory, Department of Food Engineering, Ankara University, Ankara, Turkey. These 312 *Enterococcus* species were isolated from traditional cheese samples in Ankara (Şanlıbaba and Şentürk 2018) and 161 of these strains were identified as *E. faecium*, and 151 of *E. faecalis*. Also, 92 *L. monocytogenes* strains were also isolated from ready-to-eat foods in Ankara (Şanlıbaba et al. 2018). All of the strains used in this study were identified by 16S rDNA amplification and sequencing, previously. *Enterococcus* and *L. monocytogenes* strains were inoculated on Tryptic Soy Broth (TSB) (Sigma™, Germany). These strains incubated at 35°C for 24 h. In addition, *Lc. lactis* was propagated on M17 broth (Merck™, Germany) and incubated at 30°C for 24 h. All of the strains used in this study were stored at –20°C with 30% (v/v) glycerol (Merck™, Germany).

Antimicrobial Activity

Enterococcus spp. strains was screened for their antimicrobial activity spectrum against *L. monocytogenes* strains, using both agar spot tests and agar well diffusion assays

Agar Spot Test

For agar spot test, 92 *L. monocytogenes* and *L. monocytogenes* ATCC7644 strains were used to check sensitivity to the antimicrobial substance produced by *Enterococcus* spp. They were cultured in 10 mL TSB broth at 35°C for 18 h. One µL of overnight cultures were spotted onto the surface of Tryptic Soy Agar (TSA) plates containing 1.5% agar to allow the development of colonies. After 18 -24 h at 35°C, the plates were overlaid with 7 mL of the appropriate soft agar (0.7% agar) inoculated with cell suspension of the indicator strain at a final concentration of 10^5 CFU.mL^{-1}. After incubation for 24 h at 35°C, plates were checked for inhibition zones surrounding the colonies of the producer strains (Schillinger and Lüke, 1989).

Well Diffusion Assay

The antimicrobial activity of *Enterococcus* spp. was tested against *L. monocytogenes* strains according to the method described by Tagg and McGiven (1971). *Lc. lactis* LL27 was used as a reference strain for bacteriocin producer. The cell free culture supernatants of tested and reference strains were obtained by centrifuging (Hettich EBA 200) at 10.000 rpm at 4°C for 10 min. The supernatant was neutralized to pH 7 with 6.5 N NaOH and sterilized by filtering through a 0.45 µm pore-size cellulose acetate filter (Millipore™, France). TSA was poured into each sterile petri dish. The plates were overlaid with 7 mL

of the appropriate soft agar (0.7% agar) inoculated with 0.3 mL of an overnight culture of the listerial strains. After solidification, wells of 6 mm diameter were punched in the agar with a sterile steel borer. The cell free culture filtrates of the *Enterococcus* spp. strains were placed into each well. The plates were then incubated for 35°C and examined after 24 h for clear zones of inhibition. The antimicrobial activity was determined via measuring the diameter of the inhibition zone around the wells.

Examine for Inhibition Zones

The diameters (mm) of inhibition zones were scored as: NZ (no inhibition zone), + (a clear zone of 1-5 mm), ++ (a clear zone of 6-10 mm), and +++ (a clear zone of ≥11 mm) (Tezel, 2019).

Enzyme Treatments for Antimicrobial Activity

Cell free supernatants were treated with commercial digestive enzymes (Sigma^{TM}, Germany). Stock solutions (1 mg.mL^{-1}) of proteinase K and trypsin were prepared with 20 mM sodium phosphate buffer (pH 6.0). Pepsin was also prepared with distillated water. A 1:10 dilution of each enzyme was added directly to the cell free supernatant. It was incubated at 37°C for 2-3 h and then, heated at 90-95°C for 5 min. The untreated (control) and treated supernatants were tested in terms of antimicrobial activity using the well diffusion method described previously (Tagg and McGiven 1971, Renye et al., 2009).

Statistical Analysis

The experiments were performed in duplicate. All statistical analyses were done using the SPSS program (version 17; SPSS Inc., United States). The significant differences were determined using analyses of variance (ANOVA) at the probability level of $p<0.05$.

Results and Discussion

In the present study, it was screened the antimicrobial activity spectra of 312 *Enterococcus* spp. against different 92 *L. monocytogenes* strains using both agar spot and well diffusion methods. *Enterococcus* species identified as 161 *E. faecium* and 151 *E. faecalis*, and also indicator strains were characterized via 16S rDNA amplification and sequencing, previously. All enterococcal strains were tested primarily for their inhibitory actions against *L. monocytogenes* strains by agar spot test.

The agar spot assays were given in Table 1. Of these, 111 strains (35.58%) showed no inhibition zone against indicator strains. A total of 201 enterococcal strains inhibited the growth of *L. monocytogenes* strains. Sixty strains (19.23%) showed the lowest antimicrobial activity against indicator strains. However, 95 strains (30.45%) and 46 strains (14.74%) showed medium and high effect against indicator strains, respectively. When analyzed by species, 20 *E. faecium* strains (12.42%) had the lowest activity. In addition, 74 *E. faecium* strains (45.96%) and 26 of which (16.15%) showed medium and high antimicrobial activity against indicator strains, respectively. In contrast, 41 *E. faecium* strains (25.47%) did not show antimicrobial activity against indicator strains. Seventy strains (46.36%) from the 151 *E. faecalis* strains did not inhibit by any listerial strains. Also, it was found that forty strains (26.49%) of *E. faecalis* showed the lowest activity against indicator strains. However, 21 *E. faecalis* strains (13.90%) and 20 of which (13.25%) had medium and high inhibition zones against indicator strains, respectively. In this study, *E. faecium* strains (74.53%, 120/161) demonstrated more antimicrobial activity against indicator strains than *E. faecalis* strains (53.64%, 81/151) based on agar spot test.

Some strains of LAB may produce inhibitory substances such as organic acids (diacetly, reuterin, lactic acid, reutericyclin, hydrogen peroxide), antifungal compounds (propionate, phenyl-lactate, hydroxyphenyl-lactate), bacteriocins and bacteriocin like substance (Galvez et al., 2010, Akkoç et al., 2011, Turhan et al., 2018). A total of 201 *Enterococcus* strains showed inhibition zones against listerial strains based on the agar spot test were tested furthermore for detecting the antimicrobial agents responsible for the antimicrobial activity. For this purpose, the agar well diffusion assay was used in this study. Among these *Enterococcus* spp., 120 were *E. faecium* and 81 as *E. faecalis*.

Data in Table 2 indicate that antimicrobial activities of *Enterococcus* spp. against *L. monocytogenes* strains using agar well diffusion method ($p<0.05$). It was revealed by the agar well diffusion method that 157 strains (78.10%) were not active against *L. monocytogenes* strains, whereas only 44 strains showed inhibitory activities against indicator strains.Comerlato et al. (2016), in accordance with our results, reported that 5 out of 13 enterococci showed activity against *L. monocytogenes*. In accordance with our result, Toğay et al. (2016) were also reported that 25 out of 66 of enterococcal isolates showed antimicrobial activity against indicator strains. In contrast to our results, Schittler et al. (2019) observed that 307 out of 478 *E. faecium* isolates showed antagonistic activity against *L. monocytogenes*. In this study, while twenty-three enterococcal strains (11.44%) showed the lowest antimicrobial activity, fifteen strains (7.46%) and six strains (2.99%) of them showed medium effect and high effect against indicator strains, respectively. A total of 85 *E. faecium* strains (70.83%) and 72 *E. faecalis* strains (88.89%) did not inhibit any listerial strains. While 19 *E. faecium* (15.84%) and 4 *E. faecalis* (4.94%) strains showed lowest antimicrobial activities, 10 *E. faecium* (8.34%) and 5 *E. faecalis* (6.17%) strains had medium antimicrobial activities against *L. monocytogenes* strains. Besides, only 6 *E. faecium* strains (5.00%) showed high antimicrobial zones. However, *E. faecalis* strains did not show high antimicrobial activities. In accordance with agar spot test, *E. faecium* strains (29.17%) demonstrated more antimicrobial activity against indicator strains than *E. faecalis* strains (11.12%) based on agar well diffusion method. The results obtained remain in agreement with the previous study indicating that *E. faecium* strains showed high activities against spoilage or pathogenic bacteria, such as *L. monocytogenes* (Renye et al., 2009). However, in contrast to our study, Anandani and Khan (2014) reported that *E. faecalis* accounted for greater percentage (57.14%) of antibacterial activity than *E. faecium* (42.85%). Similarly, Turhan et al. (2018) found that 8 of *E. faecalis* and 6 of *E. faecium* strains were bacteriocinogenic.

Table 1. Antimicrobial Activities of *Enterococcus* spp. against *Listeria monocytogenes* Strains using Agar Spot Test*

Strains	Indicator *Listeria monocytogenes* Strains							
	NZ		+		++		+++	
	n	%	n	%	n	%	n	%
Enterococcus spp. (312 strains)	111	35.58	0	19.23	95	30.45	46	14.74
E. faecium (161 strains)	41	25.47	20	12.42	74	45.96	26	16.15
E. *faecalis* (151 strains)	70	46.36	40	26.49	21	13.90	20	13.25

NZ: No inhibition zone, +: 1 mm < zone> 5 mm (low effect), ++: 6 mm < zone > 10 mm (medium effect), +++: zone > 11 mm (high effect), * P<0.05

Table 2. Antimicrobial Activities of *Enterococcus* spp. against *Listeria monocytogenes* Strains using Well Diffusion Test

Strains	Indicator *Listeria monocytogenes* Strains							
	NZ		+		++		+++	
	n	%	n	%	n	%	n	%
Enterococcus spp. (201 strains)	157	78.10	23	11.44	15	7.46	6	2.99
E. faecium (120 strains)	85	70.83	19	15.84	10	8.34	6	5.00
E. faecalis (81 strains)	72	88.89	4	4.94	5	6.17	0	0

NZ: No inhibition zone, +: 1 mm < zone> 5 mm (low effect), ++: 6 mm < zone > 10 mm (medium effect), +++: zone > 11 mm (high effect)

The results obtained in this study indicate that 6 *E. faecium* strains may be of proteinaceous nature and belong to bacteriocins. In this study, it observed that the proteinase K, pepsin, and trypsin treatments had elimination effect on six antimicrobial active *E. faecium* strains. This may mean that the active substance is quite proteinaceous nature. The results of the present study are agreement with Renye et al. (2009) who found that 5 *E. faecium* strains produced active substance inhibited the growth of *Listeria* spp. El-Ghaish et al. (2011) identified that a total of 24 enterococcal isolates showed antagonistic properties against indicator strains.

In this study, interestingly, a total of 1.9% of enterococcal strains were found as potential bacteriocinogenic strains. Similarly, El-Ghaish et al. (2011) reported that 2 out of 503 enterococcal were produced bacteriocins. Favaro et al. (2014) showed that 4 out of 12 *E. faecium* strains were able to produce bacteriocin. Schittler et al. (2019) also observed that 28 out of 307 *E. faecium* strains showed bacteriocinogenic potential. In contrast to our study, Turhan et al. (2018) who found that 70% of enterococcal isolates produced bacteriocinogenic antimicrobial compounds.

Enterococcus species are responsible for the production of several types of bacteriocins (Omar et al. 2006, Ruiz et al. 2013, Terzic-Vidojevic et al. 2014). This fact may contribute to their colonization of habitats and their competitive edge over other bacteria. It must also be kept in mind that the production of bacteriocins may be affected by the composition of the culture medium, the optimum pH, aeration and growth temperature (Galvez et al., 2010). According to the result of the present study, it could be suggested that further analysis should be done with antimicrobial compounds of 6 *E. faecium* strains. It should be determined the effect of different pH, temperature, and enzyme applications. Also, bacteriocin production in different media should be controlled.

Conclusion

The 312 enterococcal strains were screened for antimicrobial activity since they may be useful to control the growth of *L. monocytogenes*. It was found that only 6 *E. faecium* strains produced active components which could be proteins or peptides. Bacteriocins might be useful as biological control agents, an alternative to chemical preservatives in the food industry. As a result, novel food processing technology is able to use in combination with bacteriocins as effective antilisterial steps in the food industry.

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