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Antibacterial Effect of Bacteriocinogenic Enterococci from Different Sources on *Listeria monocytogenes*

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ABSTRACT

In this study, antimicrobial activity of partially purified enterocins and crude bacteriocins from *Enterococcus* isolates with different sources was investigated against *Listeria monocytogenes* by disk diffusion assay. Totally 70% of enterococcal isolates (*Enterococcus faecalis* and *Enterococcus faecium* from food and clinical sources) were found as potential bacteriocinogenic strains. Both of food and clinical enterococcal isolates also exhibited antimicrobial properties against *L. monocytogenes*. Additionally, the present study detected that inhibitory activity was strain-specific. Both crude bacteriocins and partially purified enterocins from *E. faecium* isolates showed lower antimicrobial activity against *L. monocytogenes* than *E. faecalis* isolates. The inhibition diameters obtained with crude enterocins and partially purified enterocins were respectively ranging from 12.33 mm to 13.25 mm and from 8.66 mm to 9.25 mm. Crude bacteriocins retained antibacterial activity after heat treatment except 120 ºC and also remained functional at pH values between 3 and 11. As a result, it was considered that enterocins could be benefit in heated and acidic or basic food products as biopreservative.

Keywords: Bacteriocin; Enterocin; *Enterococcus* spp.; *Listeria monocytogenes*

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1. Introduction

Enterocin is novel bacteriocin produced by *Enterococcus* spp. and active against various pathogenic or food spoilage bacteria such as *Listeria* spp., *Clostridium* spp., *Staphylococcus* spp., *Bacillus* spp., *Campylobacter* spp. and *Escherichia coli* (Moreno et al 2003; Campos et al 2006; Javed et al 2010; Anandani & Khan 2014; Nami et al 2015). Bacteriocinogenic Enterococci strains, mostly *E. faecalis* and *E. faecium*, are isolated from different sources including vegetables, mostly fermented foods (cheese, sausages and other meat products), gastrointestinal system and various clinical specimens like urine, skin swab, pus and blood (De Vuyst et al 2003; Theppangna et al 2007; Ogaki et al 2016).

In recent years, there has been an increased tendency to use and study natural additives, such as natural antimicrobials and antioxidants. For this reason, bacteriocins have attracted more and more great attention (Savadago et al 2004; Yıldırım et al 2014; Ogaki et al 2016). The use of bacteriocins or bacteriocinogenic

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cultures seen as a useful biocontrol method in food preservation to decrease the growth of spoilage or pathogenic microorganisms (Nascimento et al 2010; Ogaki et al 2016). In general, many researchers focused on the effect of temperature, medium composition and pH in bacteriocin production, but there are insufficient information deal with effect of these factors on inhibitory activity of bacteriocins (Aymerich et al 2000; Meera & Devi 2012). Also, while most of the papers on enterocins have related to bacteriocinogenic enterococci from food sources, less attention has been given to isolates from clinical origins. The isolation of novel bacteriocins will be beneficial (Ogaki et al 2016). *L. monocytogenes* was most sensitive indicator to enterocins among pathogenic bacteria (Aymerich et al 2000; Nascimento et al 2010; Nami et al 2015). *Listeria monocytogenes* is one of the most important foodborne pathogens and resistant to adverse conditions including a wide range of temperatures and pH, high NaCl, sodium nitrite and various disinfectants. The prevention of *L. monocytogenes* growth in foods is highly difficult due to its resistance (Aymerich et al 2000). Investigators have come up with this problem from *L. monocytogenes* by using bacteriocins and described a large number of antilisterial bacteriocins (Ennahar & Deschamps 2000). Bacteriocins were mostly tested in meat and dairy product to inhibit *L. monocytogenes*. For example; enterocin from *E. faecium* DPC1146 had inhibitory effect on *L. monocytogenes* in milk. A drop in viable cell counts of *L. monocytogenes* in enterocin AS-48 added meat sausages was observed (Galvez et al 2008).

The aim of the present study was to isolate bacteriocinogenic enterococci from clinical and food sources and to evaluate the effect of temperature and pH on antibacterial activity of crude bacteriocins supernatant against *L. monocytogenes*.

2. Material and Methods

2.1. Samples and bacterial strains

In this study, enterococci strains identified before by VITEK-2 automated identification system in the University central laboratory were used. As seen in Table 1, a total of 20 enterococcal isolates were selected from clinical cases (5 of *E. faecalis* and 5 of *E. faecium*) and from foods (5 of *E. faecalis* and 5 of *E. faecium*). Enterococci strains isolated from food samples (white cheese, Tulum cheese, raw chicken meat, fermented sausages) were provided by Cukurova University, Food Engineering Department. Enterococcal isolates provided various clinical sources were collected from the Central Laboratory of Balcalı Hospital, Adana-Turkey during 2010-2011. All enterococcal isolates (food and clinical) were stored in Brain Hearth Infusion Broth (BHI-Fluka, Germany) including 10% sheep blood and 10% glycerol (v/v) at -20 °C. All enterococcal strains were subcultured twice prior to the experiments. Enterococci were grown in De Man, Rogosa and Sharpe broth (MRS broth; Merck, Darmstadt, Germany). *Listeria monocytogenes* ATCC7644 (Remel-USA) was used as indicator organism. *L. monocytogenes* was growth in BHI broth and stored at -20 °C in BHI broth supplemented with 20% (v/v) glycerol (Ogaki et al 2016).

2.2. Antibacterial spectrum of partially purified bacteriocins from enterococcal isolates

Enterocins were partially purified from food and clinically isolates of *E. faecium* and *E. faecalis* according to modificated method of Anandani & Khan (2014), Moreno et al (2003), Savadago et al (2004) and Yıldırım et al (2014). The enterococcal isolates were incubated for 48 h at 37 °C, in 250 mL MRS broth. After incubation, cells were removed by centrifugation (10000 g at 4 °C, 20 min), and pH of the cell free culture supernatant (CFS) was adjusted to pH 6.5 by the addition of 10 N NaOH to exclude antimicrobial effect of organic acid. Then, CFS was filter-sterilized (0.45 µm membrane-Millipore, Carrigtwohill, Ireland). The final concentration of sterile suspension was adjusted to 40% saturation of ammonium sulphate by slowly adding, and shaken overnight at 4 °C. The mixture was centrifuged (13000 g at 4 °C, 45 min) and after harvesting of the surface specimens and bottom pellets were performed resuspension in 10 mL sodium phosphate buffer (10 mM, pH 7). One volume of this suspension was mixed with 15 volumes of a methanol-chloroform (1:2, v/v) and then extraction of this mixture was performed at 4 °C for 1 h. The sample was centrifuged (15500 g, 4 °C, 30 min), the supernatant fraction decanted and the pellet air-dried. After resuspension of the pellet with 10 mL of ultrapure water (MilliQ; Millipore N.V., Brussels, Belgium) partially purified bacteriocin was obtained and was stored at -20 °C.

After purification, the antibacterial activity of enterocin was analyzed on Mueller Hinton Agar by disk diffusion assay against *L. monocytogenes* as a target (indicator) strain with a bit modification of previous reports (Yamato et al 2003; Savadago et al 2004; Campos et al 2006; Zheng et al 2015; Khalkhali & Mojgani 2017). Disk diffusion assay was used for detection of bacteriocin activity in enterococcal isolates. *L. monocytogenes* indicator strain at a 10^6 cfu mL^{-1} concentration was spread on Mueller-Hinton agar (Oxoid, England). Then, 100 µL portions of samples from enterococci were placed on paper disks (thick, 6 mm, Oxoid, England), which had previously been placed on the agar plates. The plates were incubated at 37 °C, for 24 h and translucent halos in the bacterial lawn surrounding the disks showed antibacterial activity. Diameters of inhibition zone around the disks were measured in millimeters.

Table 1- Inhibition zones from CB and partially purified enterocins obtained from each enterococcal isolate (as mm)

Code of samples	*Origin of samples*	*Species of isolate*	*Partially purified enterocins*	*CB*
F1	Urfa cheese	*E. faecalis*	8.00	12.00
F2	Fermented sausages	*E. faecalis*	10.00	12.00
F3	Raw chicken meat	*E. faecalis*	9.00	16.00
F4	Raw chicken meat	*E. faecalis*	0.00	0.00
F5	Fermented sausages	*E. faecalis*	10.00	12.00
F6	Antep cheese	*E. faecium*	10.00	14.00
F7	Erzincan Tulum cheese	*E. faecium*	0.00	0.00
F8	Hatay cow cheese	*E. faecium*	9.00	13.00
F9	Kasseri cheese	*E. faecium*	0.00	0.00
F10	Fermented sausages	*E. faecium*	8.00	10.00
C1	Clinical origin	*E. faecalis*	0.00	0.00
C2	Clinical origin	*E. faecalis*	11.00	12.00
C3	Clinical origin	*E. faecalis*	8.50	15.00
C4	Clinical origin	*E. faecalis*	8.00	12.00
C5	Clinical origin	*E. faecalis*	9.00	14.00
C6	Clinical origin	*E. faecium*	0.00	0.00
C7	Clinical origin	*E. faecium*	8.00	15.00
C8	Clinical origin	*E. faecium*	10.00	12.00
C9	Clinical origin	*E. faecium*	0.00	0.00
C10	Clinical origin	*E. faecium*	8.00	11.00

CB: crude bacteriocins (sterile cell free supernatant at pH 7)

2.3. Antibacterial spectrum of crude bacteriocins from bacteriocinogenic enterococcal isolates at different pH and temperature

The overnight bacteriocinogenic cultures were centrifuged at 10000 g for 30 min at 4 ºC. Cell free supernatants (CFS) were adjusted to pH 6.5 by the addition of 10 N NaOH and sterilized by filtration through a 0.45 µm membrane (Millipore, Carrigtwohill, Ireland). CFS was resuspended in 10 mM sodium phosphate buffer (pH 7) (Nascimento et al 2010; Nami et al 2015; Khalkhali & Mojgani 2017). This resuspended CFS (pH 7) was used as crude bacteriocins (CB) to detect antimicrobial spectrum of bacteriocinogenic cultures (Cintas et al 1998). The inhibitory spectrum of CB in different pH and temperature was studied by determining the antagonistic action of CB, against indicator organism (10^6 cfu mL^{-1}) by disk diffusion assay as mentioned above. The antibacterial activity was detected by measuring the clear zones around the disks containing CB. The clear inhibition zones were given in mm. Thermal stability of bacteriocinogenic enterococci was determined by incubation of CB at 60 °C, 70 °C, 80 °C, 90 °C, 110 °C, and 121 °C for 15 minutes. After incubation, bacteriocin samples were cooled to +4 °C (Campos et al 2006; Javed et al 2010). CB exposured to heat treatment was tested for antibacterial activity as described above. The pH stability of bacteriocinogenic enterococci was assayed at pH values 3, 5, 7, 9, and 11. pH level was adjusted with addition of 4 N HCl or 4 N NaOH to CB. For each test, 50 mL of CB was mixed with 2 mL of sodium phosphate buffer (10 mM) at each pH, and samples were incubated at room temperature (25 °C) for 2 h (Franz et al 1997; Javed et al 2010). The antibacterial activity in each sample was determined as described above.

3. Results and Discussion

Nowadays, there is a trend to detect novel enterocins from different origins due to their antimicrobial activity (Moreno et al 2003). Especially, it was focused on antilisterial activity of enterocins from *E. faecium* from various food such as meat products, fermented sausages, cheese (Ennahar & Deschamps 2000; Aymerich et al 2000; Marekova et al 2003; Vimont et al 2017). In our study, enterocins from different origins were compared in view of their antibacterial activity against *L. monocytogenes* strain. Both clinical and foodborne enterococci that are used in this paper may be candidate strains for practical use. However there is a needed more information for distinction among enterocins (Moreno et al 2003). Therefore, bacteriocinogenic enterococcal strains should be carefully and individually assessed for their safety and associated risk factors (Khalkhali & Mojgani 2017).

In this study, 20 strains of enterococci (10 *E. faecalis* and 10 *E. faecium*) from different sources were collected. Especially, *E. faecalis* and *E. faecium* were selected because pervious researchers reported that bacteriocinogenic strains are mostly belong to *E. faecium* and *E. faecalis* (De Vuyst et al 2003; Theppangna et al 2007; Özdemir et al 2011; Vimont et al 2017; Vijayakumar & Muriana 2017). As observed in Table 1, eight of *E. faecalis* strains and six of *E. faecium* (totally 70% of strains) were bacteriocinogenic and the rest of strains (totally 20% of strains) did not produce any bacteriocin.

3.1. Antibacterial activity of CB and partially purified bacteriocins from bacteriocinogenic enterococci

As seen in Table 2, both CB and partially purified bacteriocins had antibacterial effect on *L. monocytogenes*. Inhibition zones from CB varied from 12.33 mm to 13.25 mm whereas inhibition zones from enterocins were found between 8.66 mm and 9.25 mm. When enterococcal strains were compared, CB and partially purified enterocins from *E. faecalis* had highest inhibition effect. Inhibition zones by CB and enterocin from *E. faecalis* were measured respectively as 13.12 mm and 9.18 mm in diameter. *E. faecalis* accounted for greater percentage (57.14%) of antibacterial activity from the samples than *E. faecium* (42.85%) as reported in Anandani & Khan (2014). Similarly; De vuyst et al (2003) found that 58.7% of the *E. faecium* strains and 68.3% of the *E. faecalis* were bacteriocinogenic.

Table 2- The average of inhibition zones from CB and partially purified enterocins obtained from bacteriocinogenic enterococci (as mm)

Bacteriocinogenic enterococcal isolates	*CB*	*Partially purified enterocins*
Food isolates	12.71	9.14
Clinical isolates	13.00	8.92
E. faecalis	13.12	9.18
E. faecium	12.50	8.83
E. faecalis from food isolates	13.00	9.25
E. faecium from food isolates	12.33	9.00
E. faecalis from clinical isolates	13.25	9.12
E. faecium from clinical isolates	12.66	8.66

CB, crude bacteriocins

Antimicrobial effect of CB was found higher than partially purified enterocins. It was considered that presence of other inhibitory substances in CB caused additional antimicrobial activity (Zheng et al 2015). CB from clinical isolates led to higher inhibition than from food origin, while the opposite was observed for enterocin. As for isolate species, there are differences between antibacterial activity of isolate species (*E. faecalis* or *E. faecium*) and the effectiveness of the antibacterial activity of bacteriocinogenic enterococci is mostly relevant to the species. Klibi et al (2008) confirmed in our results that *E. faecalis* had higher antibacterial effect than *E. faecium* on *L. monocytogenes*. Generally, antimicrobial potential of enterococci was heterogeneous and strain-specific (Campos et al 2006; Nascimento et al 2010; Gómez et al 2012).

3.2. The effect of pH and temperature on antimicrobial activity of CB from bacteriocinogenic enterococci

Antibacterial activity of CB at different pH and temperature were presented in Table 3. CB exhibited a broader pH and temperature range of activity against *L. monocytogenes*. The activity of CB against *L. monocytogenes* was maintained in all pH range (3-11) and temperature grades except 120 °C, depending on enterococci strains. Antibacterial activity of CB reached maximum levels at pH 7 and 60 °C. CB from *E. faecalis* was found more resistant to pH and temperature deviations than *E. faecium*. Reduction in activity of CB from *E. faecalis* and *E. faecium* at 110 °C were found respectively at level of 26.30% and 20.97%. Especially, bacteriocins have lost their activity after the exposure to thermal stress at 120 °C and any inhibition zones did not observed on plates.

Antibacterial activity of the enterocins depends on the pH and temperature (Moreno et al 2003). Antibacterial effects of CB from our isolates were investigated at various pH and thermal conditions. Significant differences were recorded in the pH and thermal stability of the studied enterocins in accordance with Khalkhali & Mojgani (2017). As the temperature grades subjected to bacteriocins was increased, the inhibition zone was decreased in diameter. These results confirmed previous reports referring that the inhibitory action of the bacteriocinogenic enterococci reduced as temperature grade increased (Moreno et al 2003; Yamato et al 2003; Zhou et al 2014; Khalkhali & Mojgani 2017). These bacteriocinogenic enterococci may be applied as biopreservatives for various food products subjected to heat treatment such as pasteurization, cooking, sterilization (Campos et al 2006). Ghrairi et al (2008) detected that enterocin MMT21 exposured to heat treatment at 100 °C for 15 minutes did not exhibit any inhibition effect against *L. monocytogenes*. Bilgin (2008) reported that heat treatment at 90 °C for 30 minutes retains activity of enterocin HZ whereas as temperature increased to 110 °C and 121 °C for 15 minutes, a 50% and a 100% reduction in activity, respectively was observed. As seen our results, CB from *E. faecalis* strains were more resistant to increase in temperature grade than *E. faecium*. CB from clinical isolates was more sensitive to heat treatment than food isolates. Our results were confirmed by Uymaz (2009). Enterocins have mostly maintained inhibition effect both acidic and basic pH (Uymaz 2009). In general, they maintain their activity at diverse pH values between pH 4 and pH 8 (Ennnahar et al 1998). Ghrair et al (2008), enterocin produced by *E. faecium* MMT21 isolated from Tunisian Rigouta cheese had inhibitor effect on *L. monocytogenes* at pH between 2-10. Similarly, in our study, CB has continued its activity at pH ranging from 3 to 11. However, level of antibacterial activity of CB changed according to pH and highest inhibition effect was observed at pH 7. Antibacterial activity of bacteriocins gradually subsided as the pH values became more and more acidic or basic (Ennahar & Deschamps 2000; Javed et al 2010; Zhou et al 2014). As a matter of fact, the present study detected that there are a drop in activity of CB at high and low pH. Similar results were reported by various researchers: Bilgin (2008) stated that enterocin HZ by *E. faecium* obtained from local white cheese protect its activity in the range of pH 2-9, while half of its activity at pH 10, the majority of its activity at pH 11 and totally of inhibition activity at pH 12 was lost. Line et al (2008) reported that enterocin was active between pH 5.0 and 8.7 except pH 3.0 and above pH 9.5.

Table 3- The average of inhibition zones from crude bacteriocin (CB) obtained from bacteriocinogenic enterococci at different pH and temperature (as mm)

	Temperatures (°C)						*Acidity*				
Bacteriocinogenic enterococcal isolates	*60*	*70*	*80*	*90*	*110*	*120*	*pH 3*	*pH 5*	*pH 7*	*pH 9*	*pH 11*
Food isolates	12.54	12.25	11.54	11.17	10.04	0.00	9.88	10.46	12.71	9.25	9.13
Clinical isolates	11.96	11.42	10.92	9.96	9.50	0.00	9.50	9.75	13.00	11.42	10.38
E. faecalis	12.29	11.75	11.42	10.79	9.67	0.00	10.00	10.33	13.12	10.25	9.88
E. faecium	12.21	11.92	11.04	10.33	9.88	0.00	9.38	9.88	12.50	10.42	9.63
E. faecalis from food isolates	12.33	12.00	11.33	11.33	9.33	0.00	10.00	10.67	13.00	9.00	9.00
E. faecium from food isolates	12.75	12.50	11.75	11.00	10.75	0.00	9.75	10.25	12.33	9.50	9.25
E. faecalis from clinical isolates	12.25	11.50	11.50	10.25	10.00	0.00	10.00	10.00	13.25	11.50	10.75
E. faecium from clinical isolates	11.67	11.33	10.33	9.67	9.00	0.00	9.00	9.50	12.66	11.33	10.00

4. Conclusions

The present work detected that food and clinical enterococcal isolates had ability to form bacteriocinogenic affect against *L. monocytogenes*. This research clearly suggests the potential usefulness of the bacteriocins obtained from *E. faecalis* and *E. faecium* at broad pH and temperature range. Nonetheless, the inhibitory action of the bacteriocinogenic enterococci reduced as temperature grade increased. Enterocins has mostly maintained inhibition effect at both acidic and basic pH values. Their activity is the highest at neutral pH levels. Enterocins from food and clinical sources have potential to use in food industry as biopreservatives against *L. monocytogenes*. However, the relationship between bacteriocin production, hemolysis, antibiotic resistance and the presence of virulence factors should be individually evaluated to control the safety and risk factors of bacteriocins from food and clinical sources. As a result, enterocin producer enterococcal strains that are safe and their enterocins may be used for food preservation.

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