Original Article

The Effect of Bacteriocin Isolated From *Lactobacillus rhamnosus* on *Pseudomonas aeruginosa* Lipopolysaccharides

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Abstract

Background: Bacteriocins are heterogeneous inhibitory substances that could affect the bacteria belonging to the same genus. Both gram-positive and gram-negative bacteria produce bacteriocins. One of the best sources of producing bacteriocins is *Lactobacillus*. The aim of this study was to isolate and purify bacteriocin from *Lactobacillus rhamnosus* and assess its effects on *Pseudomonas aeruginosa* and synthesis of its lipopolysaccharide.

Methods: *L. rhamnosus* was prepared and cultured at MRS broth and incubated at 37ºC for 24 hours. Then, the medium was centrifuged for the isolation of bacteriocin and the supernatant was considered as bacteriocin. Antibacterial properties of different concentrations of bacteriocin (50, 100, 200, and 400 μg/mL) against *P. aeruginosa* were assayed by using agar diffusion and broth micro dilution methods. Also, the effect of bacteriocin against lipopolysaccharide synthesis in *P. aeruginosa* was analyzed by using one unit of minimum inhibitory concentration (MIC) for bacteriocin.

Results: The results showed that all bacteriocin concentrations had antibacterial activity against *P. aeruginosa*. The MIC value was 31.25 μg/mL and minimal bactericidal concentration (MBC) was 62.5 μg/mL. Also, the synthesis of lipopolysaccharide decreased during *P. aeruginosa* growth period, and it reached zero after 5 hours.

Conclusions: The results of this study showed the antibacterial effect of bacteriocin isolated from *L. rhamnosus* against *P. aeruginosa*. In addition, this bacteriocin prevented the lipopolysaccharide synthesis in *P. aeruginosa*.

Keywords: Bacteriocin, *Lactobacillus rhamnosus*, Lipopolysaccharide, *Pseudomonas aeruginosa*

Background

One of the most important culprits of health care associated infections and also respiratory infections in immunocompromised patients is *Pseudomonas aeruginosa*. This gram-negative pathogen can survive in different habitats such as soil and water (1,2), and it can cause a wide range of diseases such as septicemia, urinary tract infections, catheter-induced infections, meningitis, as well as soft tissue infections such as burn wounds and infections of eye and ear (3,4). *P. aeruginosa* can cause acute or chronic opportunistic infections (3). Chronic lung infection is seen in immunocompromised patients such as individuals with neoplasm and in cystic fibrosis disease. There are some important characteristics that help *P. aeruginosa* to cause morbidity and mortality such as biofilm formation, its intrinsic factors like motility coordination, and secreted substances (3). Biofilm formation has an important role in severe antibiotic resistance and it can be found on some medical devices. The annual rate of *P. aeruginosa* health care associated infections in the US, as declared by Centers for Disease Control and Prevention (CDC), is about 51 000. Unfortunately, about 13% of these infections are multi-drug resistant (MDR) with 6% mortality rate. The CDC has placed this pathogen in the

serious drug resistant threats group. According to the Food and Drug Administration (FDA) and CDC antimicrobial resistance bank report, only a few antibiotics are left for the treatment of MDR *P. aeruginosa*, such as polymyxin B and colistin. However, resistance to these last saviors has also been observed (5). To combat antimicrobial resistance, different strategies have been proposed such as developing new antibiotics, changing the antibiotic combination, and application of non-antibiotic alternative treatments. These alternative methods include bacteriophages, probiotics, nanoparticles, and phytotherapy. As developing new antibiotics is not anticipated to be easily achievable, the effort is mainly focused on alternative treatments.

One of these research scopes is probiotic organisms. The FAO/WHO have defined probiotics as live microorganisms which when administered in sufficient amount can cause health benefits for the host (6). Probiotics are now well known due to their health benefits for human, such as treatment of acute diarrheal disease, atopic dermatitis, inflammatory bowel disease, etc (7-9). *Lactobacillus rhamnosus* has been used in dairy products as a probiotic and studies have confirmed its effectiveness in treating some diseases; and many diverse strains of this bacterium are commercially available (10,11). It is a

member of lactic acid bacteria (LAB) group, which are known to produce lactic acid and are historically used in food industry as fermentative agents (12). LAB can also produce bacteriocins that are antimicrobial proteins or peptides, which are synthesized by ribosome. Principally, these proteinaceous compounds can play a role as an antagonist against genetically close related bacteria to their strain. The LAB and its metabolic products are generally regarded as safe.

Recently, some studies have approved the efficacy of these non-antibiotic treatments whether as an alternative or complementary method (13). Thus, such therapies may be considered as a potential novel strategy in management of P.aeruginosa infection.

As there is a hope that bacteriocins can be a good replacement for antibiotics, in this study we assessed the effect of bacteriocin isolated from *L. rhamnosus* on lipopolysaccharide of *P. aeruginosa*, as one of its virulence factors (14).

Methods
Bacteria and Growth Media
This study was conducted in Urmia Reference Microbiology Laboratory from March 2017 to September 2017. *L. rhamnosus* PTCC 1637 and *P. aeruginosa* PTCC 1558 were obtained from Persian Type Culture Collection (PTCC). *L. rhamnosus* PTCC 1637 was anaerobically incubated in MRS broth at 37°C for 24 hours and *P. aeruginosa* was grown at 37°C for 24 hours in Tryptic Soy Broth (TSB). Initially, the growth of both bacteria was confirmed with gram staining, catalase, oxidase, nitrite reduction, motility, indole production, H_2S production, and gelatin hydrolysis test (15-17). Then both bacteria were stored at -80°C in presence of 20% sterile glycine.

Extraction of Bacteriocin from Lactobacillus rhamnosus
The method proposed by Lakshminarayanan et al was used to extract bacteriocin. Briefly, a bacteriocin-producing microorganism, *L. rhamnosus* PTCC 1637 was anaerobically incubated in 20 mL MRS broth at 37 °C for 24 h. Bacterial cells were centrifuged to obtain cell-free supernatant that was used as bacteriocin. In parallel, the bacteriocin produced by this strain was purified by chromatography, following the procedure described by Lakshminarayanan et al (18). Then, bacteriocin was dried with lyophilizator and stored at -20°C in the form of powder.

Antibacterial Effects of Bacteriocin on Pseudomonas aeruginosa
Agar Disk Diffusion Method
Antibacterial property of different concentrations of bacteriocin (50, 100, 200, and 400 µg/mL) against *P. aeruginosa* extracted from *L. rhamnosus* were assayed by agar disk diffusion method (19). Briefly, the Mueller Hinton agar medium was poured onto the petri dishes and *P. aeruginosa* was cultured. To evaluate antibacterial properties, blank paper disks (made by Padtan Teb Co) were placed on the agar with a certain distance from each other and from the edge, then approximately 20 µL of different concentrations (50, 100, 200, and 400 µg/mL) of bacteriocin were added to the disks in a solution of dimethyl sulfoxide. Next, 30 µg/mL concentration of the cefixime antibiotic disk was used as the positive control, and the culture media containing bacteria were placed at 37°C for 24 hours. The antimicrobial activity was assessed by measuring the zone of inhibition for a pure culture of the organism and comparing the result of antibiotic inhibition by the Clinical and Laboratory Standards Institute (CLSI). These experiments were repeated three times to ensure each of the different concentrations of bacteriocin and antibiotics.

Determination of Minimum Inhibitory Concentration
The broth micro dilution method was used to determine the minimum inhibitory concentration (MIC) of bacteriocin. A single 96-well microdilution plate was used. At first, 100 µL of Mueller Hinton Broth (Merck, Germany) was poured in to designated wells. Then, 100 µL from 400 µg/mL concentration of bacteriocin was added in well 1. Then, serial two-fold dilutions using 100 µL pipette were done beginning at the second well and continuing through well 12. Finally, 100 µL of diluted suspension of *P. aeruginosa* (0.5 McFarland standard dilution) was added to all wells. After 24 hours of incubation at 37 °C, bacterial growth was evaluated. Turbidity was considered as bacterial growth. MIC was defined as the lowest concentration of the compound that had no macroscopically visible growth (20).

Determination of Minimal Bactericidal Concentration
To determine the minimal bactericidal concentration (MBC) values of bacteriocin, all well medium with no visible growth was removed and inoculated in TSB plates. MBC is defined as the lowest concentration at which 99% of the bacteria are killed (20).

Evaluation of the Effect of Bacteriocin on the Synthesis of Lipopolysaccharide
In this study, the effect of bacteriocin on the synthesis of lipopolysaccharide in *P. aeruginosa* was carried out by the method proposed by Goldman et al (21). In short, *P. aeruginosa* was cultured in 20 ml of TSB medium under aerobic conditions. Then, some MICs from bacteriocin were added to *P. aeruginosa* culture medium. Then, 3 µl from 0.5 mM N-acetyl-glucosamine solution was added to culture medium. After 12 hours, the culture medium was centrifuged and the bacterial precipitate was washed twice with sterile physiology serum. The bacterial specimen was sent to the Aria Chemical Company (Karaj, Iran) on ice to determine the amount of lipopolysaccharide through high-performance liquid chromatography.

Statistical Analysis

Prior to comparing the mean values (averages), the normality and uniformity of data were examined using Kolmogorov–Smirnov test. To analyze the data using SPSS software version 19, analysis of variance (ANOVA) and Tukey's tests were used. $P < 0.05$ was considered significant.

Results

The Results of Extraction of Bacteriocin from *Lactobacillus rhamnosus*

A total of 1 g of wet weight of bacteria and 230 µg of bacteriocin were extracted in powder form. Accordingly, the percentage of bacteriocin production by *L. rhamnosus* was 0.02%. The produced bacteriocin was white and water soluble.

The Results of Antibacterial Activity of Bacteriocin against *Pseudomonas aeruginosa*

The results showed that all produced bacteriocin concentrations could inhibit the growth of *P. aeruginosa*. In other words, by increasing the concentration of bacteriocin, the antibacterial properties of the bacteria increased. Accordingly, the largest diameter of the growth inhibitory region was recorded at a concentration of 400 µg/mL bacteriocin and a value equal to 25±1.15 mm was recorded (Figure 1). Also, the minimum diameter of the inhibition zone of bacteriocin *L. rhamnosus* for *P. aeruginosa* was 50 µg/mL, and a value equal to 9±0.3 mm was recorded. The amount of growth inhibition zone for other bacteriocin concentrations are presented in Table 1. The standard drug in this study was cefixime and the growth inhibition zone for this drug measured at a concentration of 30 g/mL was 13±0.9.

The results of MIC and MBC are presented in Table 2. Based on these findings, the MIC of growth for bacteriocin
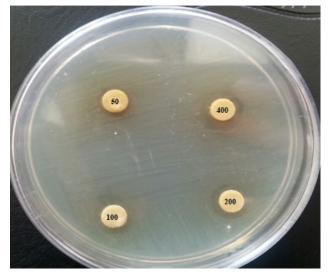

Figure 1. The Diameter of the Inhibition Zone of Bacteriocin *Lactobacillus rhamnosus* against *Pseudomonas aeruginosa*.

was 31.25 µg/mL and the MBC was 62.5 µg/mL. The MIC of growth and the MBC for cefixime were 25.2 and 15.5 µg/mL, respectively. The results showed that dilating liquid and distilled water had no negative effects on the growth of *P. aeruginosa*.

The Effects of Bacteriocin on the Synthesis of Lipopolysaccharide

The effects of *L. rhamnosus* bacteriocin on the synthesis of lipopolysaccharide in *P. aeruginosa* are shown in Figure 2. Based on this figure, it can be concluded that bacteriocin is able to disrupt the synthesis of lipopolysaccharides in bacteria. Also, the results showed that the synthesis of lipopolysaccharide decreased over time, and it reached zero after 5 hours. In this study, the bacteriocin level used was one MIC and no bacteriocin was added to the control group. As the Chart shows, over time and with increasing the concentration, lipopolysaccharide synthesis is reduced.

Discussion

Basically, probiotics are live microorganisms used to treat and prevent a number of infectious diseases. If possible, establishing a harmless beneficial organism in the device can prevent colonization of various microbial infections (22).

Inhibitory effects of probiotics are mainly attributed to manufactured products, such as antibiotic, bacteriocin, siderophore, lysozyme, protease, and pH alteration with

Table 1. Results of Antibacterial Activity of Bacteriocin *Lactobacillus rhamnosus* Against *Pseudomonas aeruginosa* by Agar Diffusion Method

No.	Bacteriocin Concentration (µg/mL)	Zone Diameter (mm)
1	50	9± 0.3 [d]
2	100	14±1.02 [c]
3	200	19±0.94 [b]
4	400	25±1.15 [a]
5	cefixime	13±0.9 [c]

Data were expressed as Mean ± SD.
Numbers with different letters in each column represent a significant statistical difference.

Table 2. Results of Determination of MIC and MBC Bacteriocin *Lactobacillus rhamnosus* Against *Pseudomonas aeruginosa*

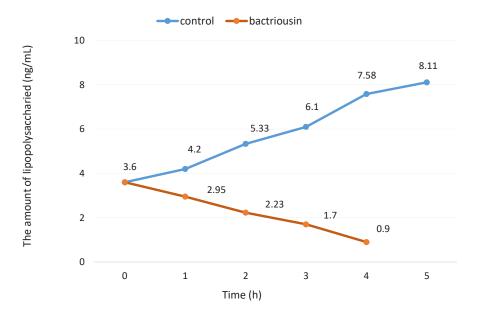| Material	Concentration (µg/mL)											
	2000	1000	500	250	125	62.5	31.25	15.5	7.25	3.5	1.75	0.85
Bacteriocin	-	-	-	-	-	MBC	MIC	+	+	+	+	+
Cefixime	-	-	-	-	-			MBC	MIC	+	+	+
Distilled water	+	+	+	+	+	+	+	+	+	+	+	+

Figure 2. The Synthesis Rate of Lipopolysaccharide Measured by High Performance Liquid Chromatography

the production of organic acids (23). The bacteriocin produced by LAB has bactericidal and or growth-inhibitory effects on sensitive bacteria (24).

Amin et al investigated the production of bacteriocin by two species of *L. plantarum* and *L. casei* against pathogenic bacteria as well as corrosive bacteria. Their results showed that produced bacteriocin inhibited the growth of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* (25). Also, Gharaei Fathabab et al demonstrated the antimicrobial activity of *L. plantarum* on *E. coli*, *Salmonella typhimurium*, *staphylococcus*, *Enterococcus faecalis* and *Citrobacter* (26).

Bacteriocins have inhibitory effects on pathogenic bacteria with different mechanisms, such as stopping DNA biosynthesis. In most cases, bacteriocins isolated from *lactobacillus* are low molecular weight proteins (2-10 kDa) that are resistant to heat, acid, and cold conditions. The lactic acid bacteria (LAB) plays a protective role against intestinal pathogens by producing short chain fatty acids and amino acids such as cysteine and glutamine. Therefore, it can be concluded that the inhibitory effect of *lactobacillus* is not solely due to an agent such as acid conditions of the supernatant or bacteriocin, and many factors are involved (27).

Many researchers have shown the inhibitory effect of various *Lactobacillus* against many gram-positive and gram-negative pathogens. In the present study, the antimicrobial effect of bacteriocin derived from *L. rhamnosus* was studied against *P. aeruginosa*; our results showed that bacteriocin has inhibitory effects on growth, which is consistent with the results of above-mentioned studies.

The first discoveries of bacteriocins were reported by Gratia et al in 1925.[28] They showed that some strains of *E. coli* do not allow the growth of similar strains by

production of compounds in the culture medium. This growth inhibitory substance was named colicin by Fredrico et al (28). Also, the properties of this material were studied and it was shown that this compound was diffusible in agar and cell membrane precipitates with using chloroform-acetone and is heat-resistant. Other studies showed the production of similar substances by strains belonging to the *Enterobacteriaceae* family, which includes *Enterobacter*, *Salmonella*, *Shigella*, *Proteus*, and *E. coli* (29).

Genetic research shows that colicin gene has a dominant hereditary characteristic that is not destroyed by transferring to other strain, and its action spectrum is only on the *Enterobacteriaceae* family. In addition, colicin producing strains are immune to bacteriocin products. Genes producing different types of colicins are on the plasmid.

Various studies have shown a wide range of antagonistic function of bacteriocins. Even bacteriocins sometimes include defective bacteriophages, which have less molecular weight than colicins. In this way, bacteriocins are divided into two main types: true bacteriocins and incomplete phage particles. Considering the effect of bacteriocins and their inhibitory power against strains close to manufacturer, researchers have been trying to create new compounds, including studies on protein engineering, production of vectors, regulatory expression of heterologous proteins, controlling the taste of fermented food, agriculture, and pharmaceutical application of bacteriocin (30).

Bacteriocin produced by LAB are valuable because their inhibitory and bactericidal activities are completely determined. For example, nisin is a bacteriocin produced by *L. lactis* which is massively manufactured, marketed, and used in the food industry. It is used as a food

preservative and also as an antagonist in over 50 countries (31). To date, bacteriocins have been purified and their genetic and biochemical properties have been studied. However, no study has surveyed the effect of bacteriocin on lipopolysaccharide.

The main limitation of this study was using only one strain. Hence, it is recommended that further studies include various strains, such as clinical ones.

Conclusions
The results of this study showed the antibacterial effect of bacteriocin isolated from *L. rhamnosus* against *P. aeruginosa*. In addition, this bacteriocin prevented the lipopolysaccharide synthesis in *P. aeruginosa*.

Conflict of Interests
None.

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