

Effect of Physical and Chemical Changes on the Antimicrobial Activity of Culture Supernatant Fluid of Lactic Acid Bacteria

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ABSTRACT

Background and Objective: Lactic acid bacteria are Gram-positive, catalase-negative, nonsporulating, either rod- or coccus-shaped bacteria that have beneficial effects on their hosts by producing antimicrobial substances such as lactic acid, hydrogen peroxide, bacteriocins and biosurfactants. Bacteriocins are antimicrobial peptides that are produced by bacteria and can inhibit the growth of other bacteria.

Methods: In this experimental study, bacteriocin production by Lactobacilli as known probiotic strains was evaluated in different physicochemical conditions. Antagonistic activity was evaluated using quantitative method of Microscale Optical Density Assay (MODA). After neutralization of acid and treatment with various enzymes, temperature, pH and NaCl conditions, the antimicrobial activity of culture supernatant fluid of *Lactobacillus acidophilus* and *L. plantarum* was investigated against pathogenic *Proteus*.

Results: The culture supernatant fluid of Lactobacilli was sensitive to proteolytic enzymes with relatively good stability to temperature. The antimicrobial activity was also present due to production of bacteriocin under different NaCl conditions (1 to 4% NaCl) and pH range of 5 to 8.

Conclusion: It seems that the antimicrobial liquid of *Lactobacillus* strains contains bacteriocin, which shows antimicrobial effects against pathogenic strains of *Proteus*. To investigate further this effect, some complementary studies should be performed.

Keywords: Bacteriocins, Lactobacillus, Proteus, Physicochemical.

INTRODUCTION

The term 'probiotic' is derived from the Greek language, meaning 'for life'. Lilly and Stillwell first used this term in 1965, as secretory factors of a microorganism that stimulate the growth of another microorganism versus the term antibiotic (1). In 1989, Roy Fuller provided a more comprehensive definition for probiotics; live microbial food supplements that cause useful and beneficial therapeutic and salutary effects in consumers by emplacement in intestine and regulating its microflora (2). Lactic acid bacteria and bifidobacteria are the most common microorganisms used in probiotics. Lactic acid bacteria are natural protectors of urinary tract with a long history in food and fermented products. A number of *Lactobacillus*, *Bifidobacterium* and other propionic bacteria strains are known as probiotics for their salutary effects (3). These bacteria are abundant in the nature and human digestive system and produce lactic acid through homolactic and heterolactic fermentation. Lactobacilli are specifically known as beneficial bacteria with the ability to break down proteins, carbohydrates and fats in food and also assist the body absorb essential nutrients such as minerals, amino acids and vitamins required by humans and animals.

These bacteria have strong antagonist activity against a large number of pathogenic and spoilage bacteria by producing organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins (4).

Bacteriocins are the peptides produced by some bacteria and lactic acid bacteria with antimicrobial activity that prevent the growth of other microbial strains (5). Most of them are deactivated by proteolytic enzymes, because of their peptidic nature. The weak range of activity and their peptidic characteristic distinguish them from antibiotics (6). Klaenhammer (1993) categorized the bacteriocins of lactic acid bacteria into four distinct classes based on their genetic profiles and biochemical properties. Class 1 includes the antibiotics that are small (<5kDa), surface-active peptides containing lanthionine, β -methylanthionine and dehydrated amino acids. Class 2 includes small (<10kDa), heat-resistant and non-lanthionine surface-active peptides. Class 3 is large (> 30kDa) and heat-resistant. Class 4 is the complex bacteriocins

containing proteins and other chemical moieties (lipid or carbohydrate) (7).

In recent years, the bacteriocins have shown anti-bacterial, anti-fungal and anti-viral activities that promoted their application in pharmaceutical and therapeutic compounds. Their application in natural ingredients, preservatives and salutary products, in controlling bacterial infections and in the treatment of urinary tract infection (UTI) has attracted the attention of many scientists (8-11).

Since different *Proteus* strains are the common causes of UTI (12) and bacteriocin-producing Lactobacilli may have a role against these bacteria (13), this study aimed to investigate the antimicrobial effect of bacteriocin in the culture supernatant fluid of Lactobacilli (as probiotic strains) under various physicochemical conditions.

MATERIAL AND METHODS

Bacterial strains used and culture conditions The standard probiotic strains of *L. acidophilus* (ATCC 4356) and *L. plantarum* (ATCC 8014) as bacteriocin-producing strains, and the pathogenic strains of *Proteus mirabilis* (ATCC 7002) and *P. mirabilis* OXK (ATCC 15146) as common causes of UTI were obtained from the collection center of the industrial infectious fungi and bacteria at the Iranian Research Organization for Science and Technology and the Pasteur Institute of Iran.

In order to produce an active bacterial culture, the purchased lyophilized powders of Lactobacilli were transferred to the tubes containing 5ml de Man, Rogosa and Sharpe (MRS) broth and then incubated at 37 °C with 5% CO₂ for 24 to 48 hours. They were then cultured on MRS agar plates for storage.

After reduction in brain heart infusion broth, the *Proteus* strains used to determine the antimicrobial properties were cultured in nutrient broth at 37 °C under aerobic conditions.

Preparing lactobacilli culture supernatant fluid

The studied *Lactobacillus* strains were grown in MRS broth and then incubated at 37 °C with 5% CO₂ in aerobic conditions for 24 hours. The pH of culture supernatant fluid of *Lactobacillus* strains were neutralized by sodium hydroxide (NaOH). The culture supernatant fluid containing bacteriocin was

centrifuged at 10000g for 10 minutes. The obtained fluid was then sterilized by filtration using 0.2-micron filters (14).

Catalase test and evaluating acid production

The catalase test was performed for all *Lactobacillus* samples obtained from their 48-hour liquid culture in the MRS.

One loop of fresh bacterial culture was transferred on the agar medium and one to two drops of hydrogen peroxide was added by a Pasteur pipette. The quick emergence and continuous production of gas bubbles indicate a positive reaction for the test (15).

In order to evaluate acid production, after incubation at 37 °C with 5% CO₂ in anaerobic conditions, the pH of the *Lactobacillus strains culture supernatant fluid* was measured using a pH meter (16).

Evaluation of bacteriocin production under various conditions

The effect of different enzymes on the antimicrobial activity of culture supernatant fluid of Lactobacilli

One mg/ml of pepsin and trypsin and 5mg/ml of catalase were separately added to the 24-hour culture supernatant fluid of bacteriocin-producing *Lactobacillus* strains. The pH of the fluid was neutralized with NaOH 1N. After one hour of incubation at 37 °C, the antimicrobial properties of the culture supernatant fluid treated with different enzymes was investigated against *Proteus* strains using Microscale Optical Density Assay (MODA) (17).

Relative extraction of Bacteriocin

One loop of bacteriocin-producing *Lactobacilli* was cultured in 250 ml Erlenmeyer flasks containing 50 ml sterile MRS broth. The flasks were incubated at 37 °C in Candle jar. When the optical density of sample reached 0.5 McFarland (1.5 x 10⁸ CFU/ml), 5% of it was inoculated into the flasks containing 100 ml MRS broth.

The antimicrobial agent was produced under suitable conditions with optimum temperature and pH. The pH was measured and recorded after 24 hour of incubation and changed to 6.5-7 range by adding sufficient amount of NaOH 1N.

After neutralizing in sterile conditions, the microbial suspension of culture medium was centrifuged at 6000g for 20 minutes at 4 °C to separate cell mass. Then, the sufficient amount of ammonium sulfate was weighed [50% saturation for *L. acidophilus* (18) and 90% saturation for *L. plantarum* (17)] and slowly

added to the flasks that were placed on ice. The samples were placed at 4 °C for one night to dissolve ammonium sulfate completely.

The contents of the flask including the resulted sediment and the sediment-containing solution were transferred to sterile tubes and the tubes were centrifuged at 10000g for 40 minutes at 4 °C.

The supernatant was removed and the resulted sediment was resuspended in one ml of 0.05M sodium phosphate buffer (pH 7). The result of dialysis (using dialysis bag with 12.000 KDa cut off) was used as semi-purified bacteriocin.

Evaluating the antimicrobial activity of Lactobacilli culture supernatant fluid

MODA was used to evaluate the antimicrobial activity of *Lactobacilli* culture supernatant fluid. In this method, a 96 microplate was used and 100 µl of *Proteus* strains tested (at a ratio of 1: 10000 in nutrient broth) was poured into each well. Next, 15 µl of semi-purified bacteriocin from cultured *Lactobacillus* strains was poured into the wells.

The optical density of the samples was read at 600nm (using ELISA reader) after 24-hour incubation at 37 °C. The difference between control wells (medium) and test wells (semi-purified bacteriocin from cultured *Lactobacilli*) was reported as the antimicrobial activity against pathogenic strains. Each test was performed in triplicate to reduce error.

In separate wells, the MRS broth was used as culture control and the wells without semi-purified bacteriocin were used as growth control (17).

Effect of different conditions on the antimicrobial activity of bacteriocin-producing lactic acid bacteria

Temperature: In order to evaluate the effect of temperature, the semi-purified bacteriocin was divided into 4 tubes. Tube 1, 2 and 3 were placed at 100 °C for 10, 30 and 60 minutes, respectively. Tube 4 was autoclaved at 121 °C for 15 minutes (17).

pH: For this purpose, pH of *Lactobacillus* culture medium (in the range of 4 to 10) was changed and after culture of various strains and 24-hour incubation in microaerophilic conditions with 5% CO₂, the antimicrobial activity of each sample was evaluated using MODA. Without changing the pH, the MRS medium (pH=5.7) was used as control (19). **Addition of NaCl:** the percentage of NaCl was changed in the culture medium (from 1% to 4%), and then the antimicrobial activity was

evaluated using MODA after 24 hours of growth in microaerophilic conditions with 5% CO₂ (20).

RESULTS

Antimicrobial activity of semi-purified bacteriocin

Table 1 shows the results of the antimicrobial activity of semi-purified bacteriocin using MODA. The results of reading absorbance at 600nm are after the addition of filtered (0.22 µm pore size) Lactobacilli culture supernatant fluid.

The antimicrobial activity of the culture supernatant fluid of *Lactobacillus* strains are shown as percentage of difference in the growth of bacterial cells compared to the control. A good inhibitory activity was observed against all tested *Proteus* strains.

The culture supernatant fluid of semi-purified bacteriocin-containing *Lactobacillus* strains showed 77.78% and 97.78% inhibitory activity

against all tested strains compared to the untreated control. The most significant inhibitory activity was observed against *P. mirabilis* OXK (ATCC 15146) with more than 97% inhibitory activity, in comparison with the untreated control.

L. acidophilus and *L. plantarum* strains also reduced the pH of culture medium to about 4.5 and 4, respectively. After treating the culture supernatant fluid of Lactobacilli with different enzymes as well as neutralization using NaOH 1N and one hour incubation, the percentage of difference between the optical density of the wells containing culture medium (control) and the wells containing Lactobacilli culture supernatant fluid (test) reduced significantly in all test cases. This means that the enzymes and neutralization of its acid affect the antimicrobial activity. Pepsin showed the greatest effect in each case. The results are shown in figure 1 and 2.

Table 1- Results of evaluating the effect of *L. acidophilus* and *L. plantarum* culture supernatant fluid on different *Proteus* strains by the MODA method and comparing the percentage of difference in the growth of bacterial cells

		Absorbance at 600 nm	Lactobacilli culture supernatant fluid
	<i>P. mirabilis</i> OXK(ATCC 15146)	<i>P. mirabilis</i> (ATCC 7002)	
0.683	0.507	Pathogenic bacteria (growth control)	<i>L. acidophilus</i> (ATCC 4356)
0.540	0.463	Culture medium (control)	
0.012	0.053	Culture supernatant fluid of <i>L. acidophilus</i> (test)	
97.78 %	88.55 %	Difference in the growth of bacterial cells (%)	
0.683	0.507	Pathogenic bacteria (growth control)	<i>L. plantarum</i> (ATCC 8014)
0.540	0.463	Culture medium (control)	
0.120	0.033	Culture supernatant fluid of <i>L. plantarum</i> (test)	
77.78 %	92.87 %	Difference in the growth of bacterial cells (%)	

DISCUSSION

Since *Proteus* treatment is of great importance in UTIs, the continual use of antibiotics can cause antibiotic resistance and treatment-resistant infections (21). In recent years, the use of probiotics such as various *Lactobacillus* strains has attracted a lot of attention for prevention and treatment of UTI as alternative treatments (22). Lactobacilli have strong antagonist activity against a large number of pathogenic bacteria by producing organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins (4). The role of lactic acid bacteria in the treatment of infections caused by *Proteus* has been observed (23). *L. plantarum* and *L. acidophilus* good inhibitory activities against UTI-causing strains are of great significance and importance. Other scientists (17, 18, 24, and 25) have considered such inhibitory activity against common pathogens that cause hospital infections and food spoilage. Lash et al. study (2005) on the bacteriocin extracted from *L. plantarum* (ATCC 8014) using MODA, showed more than 90% difference in the growth of bacterial cells against Gram-positive and Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Shigella flexneri*, *Salmonella Typhimurium*, *Staphylococcus aureus*, *Listeria innocua* and *S. epidermidis*. In agreement with our results, this indicates the presence of antimicrobial activity due to *Lactobacillus* bacteriocin (17). This inhibitory effect could also be due to high sensitivity of the MODA, its applicability and evaluating the antagonistic property of culture supernatant fluid containing bacteriocin and pathogen stains in the liquid medium. Various studies have demonstrated that *L. acidophilus* and *L. plantarum* can reduce or inhibit the growth of *P. mirabilis* as a common cause of UTI, through production of bacteriocins (26). Mohankumar study (2011) showed that *L. acidophilus* had an inhibitory effect on the growth of *Proteus* and found that this inhibitory effect was mainly due to production of bacteriocins by *L. acidophilus* (14). It is observed that the antimicrobial peptides or proteins produced by lactic acid bacteria often inhibit the growth of Gram-positive bacteria, while the low molecular weight compounds such as lactic acid have a higher inhibitory effect on the growth of Gram - negative

pathogens (27).

However, since the antimicrobial effect of potential lactic acid production by Lactobacilli in this study was eliminated by neutralizing the pH, the extracted antimicrobial compound performed well against *Proteus* (Gram-negative). De vuyst et al. also showed that the bacteriocin produced by *L. acidophilus* was more effective against Gram-negative bacteria in comparison with the gram-positive bacteria. Nevertheless, the antimicrobial compounds produced by *Lactobacillus* has shown different spectrum of effect on Gram-positive and Gram-negative bacteria in various studies (28). Since bacteriocins and the molecules similar to bacteriocins are directly made as polypeptides or pre-polypeptides (29). The sensitivity of bacteriocin existed in various enzymes (trypsin, pepsin and catalase) was measured, and the effect of H₂O₂ and produced acid was neutralized. The results showed a decreased inhibitory activity compared with untreated supernatant fluid (figure 1 and 2), emphasizes that the inhibitory compounds are bacteriocins with protein nature. Similar results were also observed in other studies (6, 17, 18, 29, 30). After confirmation of bacteriocin production, relative separation of these protein molecules was performed by ammonium sulfate deposition method and then dialysis of proteins. The effect of various physicochemical conditions on bacteriocin production and antimicrobial activity was measured. The results showed that the inhibitory activity is sensitive to heat, because its activity was eliminated at 100 °C for 10 minutes and 30 minutes in case of *L. acidophilus* and *L. plantarum*, respectively. Autoclaving at temperatures above 121 °C also eliminated the inhibitory property (figure 7 and 8). The inhibitory activity remained a little stable in the pH range, while the highest activity was observed in medium with pH 5-7 for both strains compared to the controls (figure 3 and 4).

In agreement with the results of other studies, the relative stability to heat and need for various culture medium conditions in terms of pH for production of the inhibitory compounds in the culture supernatant fluid of Lactobacilli confirms that these compounds are bacteriocins with protein nature (31-33).

CONCLUSION

The results of this study showed that *L. acidophilus* and *L. plantarum* have suitable inhibitory activity against Gram-negative bacteria (*Proteus*). These inhibitory compounds are capable to remain relatively stable when facing temperature and proteolytic enzymes. These antimicrobial liquids are able to produce bacteriocins in both strains in NaCl range of 1 to 4% and pH 5 to 7 and remain relatively resistant to temperature and pH conditions. These inhibitory compounds in the culture supernatant fluid of *L. acidophilus* and *L. plantarum* could be classified as bacteriocins, which have protein nature. Thus, they are considered suitable for use in food and pharmaceutical industries. Finally, it is suggested to conduct further molecular studies

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on the nature of these compounds and perform clinical trials.

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CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

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