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Evaluation of Antimicrobial Activity of Probiotic *Lactobacillus* Strains against Growth and Urease Activity of *Proteus* spp.

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ABSTRACT

Background: Nowadays, the use of probiotic bacteria for the prevention and treatment of urinary tract infections is growing. *Lactobacillus*, as probiotic bacterial genus, is well known for its benefits for the human health.

***Methods*:** The effects of partially purified antimicrobial compounds (bacteriocins and biosurfactants) of *Lactobacillus* strains was assessed and their capacity to in vitro inhibit growth and urease production of various strains of *Proteus* spp, was studied. Inhibition of the urease production of *Proteus* spp. at sub-MIC levels was screened using spectrophotometry method.

***Results*:** Results revealed that semi-purified bacteriocins of *L. acidophilus* and *L. plantarum* showed a greater inhibitory activity on the bacterial urease, compared to biosurfactants of *L. rhamnosus*, *L. casei* and *L. fermentum* ($P < 0.05$).

***Conclusion*:** It can be concluded that bacteriocins may affect *Proteus* pathogenesis by inhibition of the bacterial urease activity and therefore eliminate the stone formation by these bacteria.

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Introduction

Proteus spp. can cause urinary tract infections in people using catheters for long term and those with urinary tract deficiencies (1). These bacteria have various virulence factors that increase their pathogenesis. One of these virulence factors, urease enzyme, plays a key role in the pathogenesis of the bacteria (2, 3). Urease enzymes (urea amidohydrolase, Ec: 3.5.1.5) have been found frequently in most of plants, molds, algae and invertebrates (4). Although these enzymes have various protein structures, all include the same mechanism of urine hydrolyze to CO_2 and NH_3. These enzymes contain nickel ions on the center of their active site and hence are classified as metal enzymes. Urine, a nitrogen compound excreted from humans and animals, is a substrate for these enzymes (5). *Proteus mirabilis* and *P. penneri* are the main causes of urinary and kidney stones followed by *P. vulgaris*. Urease increases pH during the urine formation. Increase in pH results in sedimentation of chemical compounds, including Ca^{2+} and Mg^{2+}, and subsequently formation of carbonate apatite stones ($Ca(PO_4).CO_3$) or struvite stones ($MgNH_4PO_4.6H_2O$). Stone formation and increased ammonium and pH are the most important features of *Proteus* spp. (6).

The effect of urease activity can be inhibited by various competitive or noncompetitive inhibitor compounds (7). Some of these compounds are produced by probiotic *Lactobacillus* strains. Numerous clinical and laboratory studies have proven that *Lactobacillus* spp. can protect consumers against urinary tract infections (8).

These beneficial bacteria show their antimicrobial activity by producing primary metabolites such as lactic, acetic, formic and benzoic acids, ethanol, carbon dioxide, hydrogen peroxide, diacetyl and acetone and secondary metabolites such as bacteriocins and biosurfactants (9). Bacteriocins are small proteins with bactericidal or bacteriostatic activity that inhibit the growth of a wide variety of Gram-positive and Gram-negative bacteria (10). These compounds are categorized in four major classes. In recent years, interests of studying microbiological, biochemical and molecular characteristic of bacteriocins have been raised due to their importance in medicine, agriculture and industries (11). Such properties has made probiotic bacteria suitable for the use as food additives and treatment of urinary tract infections (12, 13). Other useful compounds, biosurfactants, are surface active compounds known as emulsifiers and produced by probiotic microorganisms such as *Lactobacillus* spp. (14, 15). Several studies have shown that biosurfactants include antimicrobial activity (16, 17).

Due to the importance of bacteriocins and biosurfactants of *Lactobacillus* spp., the current study was carried out to evaluate the effects of these antimicrobial compounds on the urease activity of *Proteus* spp. Furthermore, the production conditions of these compounds were studied.

Materials and Methods

Bacterial strains and growth conditions

Five *Lactobacillus* strains and four standard strains of *Proteus* with and without urease activity were used. *Lactobacillus* strains were cultured in MRS broth for 24 h at 37˚C under anaerobic conditions. Test strains of *Proteus* spp. were cultured in Nutrient Broth (NB) and Muller Hinton Broth (MHB) media for 24 h at 37˚C under aerobic conditions.

Growth and acid production of Lactobacillus strains

Growth curves of *Lactobacillus* strains were analyzed due to effect on antimicrobial compound production. One hundred milliliters of MRS broth were micro-aerobically incubated at 37°C with 1% of overnight cultured *Lactobacillus* strains. Bacterial growth was assessed by measuring the optical density of cultures at 600 nm at various time intervals (18). To detect acid production, pH

changes were measured by pH meter at various time intervals (19).

Assessment of MIC, MBC and sub-MIC

Minimum inhibitory concentration (MIC) was assessed using broth microdilution method in 96-well plates. After addition of 100 µl of MRS broth to each well, 100 ml of *Lactobacillus* culture supernatant (centrifuged at 7000 g for 6 min at 4°C) were added to the first well and then serial dilutions were made. Then, 100 ml of 1.5×10^6 CFU/ml suspension of each test strain in Muller-Hinton broth were added to each well and incubated at 37°C. After 24 h, bacterial growth was detected by measuring the culture turbidity at 600 nm using a microplate reader. All experiments were carried out in three replicates. To assess the minimum bactericidal concentration (MBC), 10 ml of the well contents that did not show any turbidity were cultured on nutrient agar plates. MBCs were determined as the lowest concentration of the cell-free supernatant; at which, bacterial growth was not observed after two days of incubation (20).

Assessment of bacteriocin production

Lactobacillus acidophilus and *L. plantarum* were cultured in MRS broth for 24 h at 37°C under micro-aerobic conditions. Then, pH of culture supernatants was adjusted to 7 to remove the acid effect. To assess the sensitivity of neutralized cell-free bacteriocins, supernatants of *L. acidophilus* and *L. plantarum* were treated with pepsin (1 mg/ml), trypsin (1 mg/ml) and catalase (5 mg/ml) separately for 1 h at 37°C. Then, digested cell-free supernatants were screened for antibacterial activity using Microscale Optical Density Assay MODA (21).

Assessment of bacteriocin inhibitory activity

Antimicrobial activity of *Lactobacillus* culture supernatant and bacteriocin activities against test strains were carried out according to an original method by Lash et al. (2005). Briefly, 15 µl of the *Lactobacillus* culture broth were poured into a well of a 96-well plate and inoculated with 100 µl of an indicator strain by 10^7 CFU/ml. After 24 h of incubation at 37°C under aerobic conditions, the plate was read using a microplate reader at the wavelength of 600 nm. Differences between the control (media) and the experiment (15 ml *L. plantarum* and *L. acidophilus* supernatants) were reported as antibacterial activity (21).

Bacteriocin extraction

Ammonium sulfate precipitation was used to concentrate and precipitate bacteriocins from the producer strains. *Lactobacillus* strains were cultured in MRS broth at 37°C under micro-aerobic conditions until they reached the logarithmic phase. Cell-free supernatants were prepared from overnight cultures of L. acidophilus and *L. plantarum* by centrifugation at 10000 g for 10 min at 4°C. Supernatants were neutralized to pH 7 with 1N NaOH. Ammonium sulfate was added to crude bacteriocins to achieve 50% of saturation for *L. acidophilus* (22) and 90% of saturation for *L. plantarum* (21). Solutions were stirred overnight at 4°C. The precipitates were collected by centrifugation at 10000 g for 40 min and resuspended in 1 ml of sodium phosphate buffer (0.05 mM, pH 7). Suspensions were dialyzed against same buffer using 12,000 KDa dialysis tube for 48 h with four buffer changes (23). Activity of *L. acidophilus* and *L. plantarum* extracted bacteriocins was tested against *Proteus* strains using spot-on-the lawn method (24). Bacteriocin activity was calculated as the reciprocal of the highest two-fold serial dilution, showing a clear zone of growth inhibition of *Proteus* strains (25).

Assessment of biosurfactant production and antimicrobial activity

To assess the biosurfactant production, 15 ml of the overnight culture of *Lactobacillus* strains were added to 600 ml of MRS media and incubated at 37°C under micro-aerobic conditions until they reached the logarithmic phase. Cells were harvested by centrifugation (10000 g , 5 min at

10°C) and washed twice in demineralized water and then resuspended in 100 ml of PBS (10 mM of KH_2PO_4/K_2HPO_4 and 150 mM of NaCl, pH). Suspensions were incubated for two hours at room temperature with gentle stirring. Then, bacteria were removed by centrifugation and filtered through 0.22-mm filters (26, 27). Surface tension between water and hydrophobic surfaces was assessed using drop collapse test (28).

Assessment of bacterial urease activity

The bacterial urease activity was assessed using spectrophotometry. Fifty milliliters of enzyme-containing *Proteus* cultures in presence of various concentrations of antimicrobial compounds was added to cuvettes containing 3 ml of 3 mM sodium phosphate buffer (pH 6.8), 7 µg of phenol red per ml and 200 mM of urea. This was incubated for 24 h 37°C under aerobic conditions (29). Since the urease activity was affected by the pH and the amount of enzyme substrate (urea), effects of various environment conditions on the activity of urease were assessed.

Effect of pH on bacterial urease activity

The bacterial urease activity was studied in sodium phosphate buffer at various physiologic and pathologic pH values (5.5, 6, 6.5, 7 and 7.5) (28).

Michaelis-Menten plot drawing

The bacterial urease activity was assessed in sodium phosphate buffer with various concentrations of urea (29).

Statistical analysis

Data was analyzed using SPSS V.20 software, Ficher Exact Test and Student T Test. All tests were repeated three times and P values ≥ 0.05 were reported as significant.

Results

Growth and acid production of Lactobacillus strains

In the current study, *L. casei* (ATCC 39392) and *L. acidophilus* (ATCC 4356) reached the stationary phase at a longer time than other strains did. All the studied lactobacilli strains reached the stationary phase in less than 24 h (Diagrams 1 to 5). Supernatants of all *Lactobacillus* strains caused the reduction of culture media pH within 30 h; from which, *L. rhamnosus* (ATCC 7469) resulted in the lowest pH in the media (pH = 3.9).

Assessment of MIC, MBC and sub-MIC

Results of MIC, MBC and sub-MIC assessment are shown in Table 1.

Secondary metabolites from producing strains

Since the *Lactobacillus* strains were selected from standard strains, two strains of L. acidophilus ATCC 7002 and *L. plantarum* ATCC 8014 were used as the bacteriocin producer strains, while *L. fermentum* ATCC 9338 *L. casei* ATCC 39392 and *L. rhamnosus* ATCC 7469 were used as biosurfactant producer strains.

Effect of various conditions on antibacterial activity

After treatment of culture supernatants of bacteriocin-producing *Lactobacillus* strains by various enzymes and neutralization by acid, the antimicrobial activity of all strains was reduced significantly (Table 2).

Assessment of bacteriocin inhibitory activity

The highest bacteriocin dilution (1600 AU/ml) from *L. acidophilus* showed a clear inhibition zone on *P. vulgaris* (PTCC 1182) (Fig. 6).

Assessment of biosurfactant production and antimicrobial activity

Overall, *L. rhamnosus*, *L. fermentum* and *L. casei* produced biosurfactants. These biosurfactant containing compounds did not show any clear zone of growth inhibition for *Proteus* strains.

Assessment of bacterial urease activity

First, effects of pH and the urea concentration on urease activity were examined. Sodium phosphate buffer had the highest absorption rate at 560 nm and pH 7 after 24 h. This was the highest activity of urea hydrolysis (Fig. 7). When the urea concentration increased, the urease activity increased as well (Fig.8). Thus, 200 mM of urea at pH 7 were used in phosphate buffer in all experiments to assess the maximum urease activity. To assess the inhibitory effect of the extracted substances from *Lactobacillus* strains on the bacterial urease activity, rates of urea hydrolyses were tested in the presence or absence of various concentrations of antimicrobial compounds by monitoring the wavelength changes and phenol red indicator. It was revealed that the bacterial urease activity reduced in the presence of bacteriocins from L. acidophilus and *L. plantarum* at ½ MIC. When tested on P. mirabilis ATCC 7002 and *P. vulgaris* ATCC 7829, the most inhibition activity was seen for the L. acidophilus derived bacteriocin. The *L. plantarum* derived bacteriocin showed the highest anti urease activity on P. vulgaris PTCC 1182 at ½ MIC (Diagrams 9 to 11). Furthermore, bacteriocins from L. acidophilus and *L. plantarum* showed significant reduction on P. mirabilis ATCC 7002 urease activity (P = 0.016 and P = 0.042, respectively). It was shown that all semi purified lactobacilli bacteriocins and biosurfactants had significant reduction on the bacterial urease activity (P < 0.05), except for the *L. casei* derived biosurfactant on P. vulgaris ATCC 1182. The *L. acidophilus*, *L. plantarum* and *L. casei* derived bacteriocins and biosurfactants had significant reduction on the *P. vulgaris* ATCC 7829 urease activity (P = 0.032, P = 0.006 and P = 0.005, respectively).

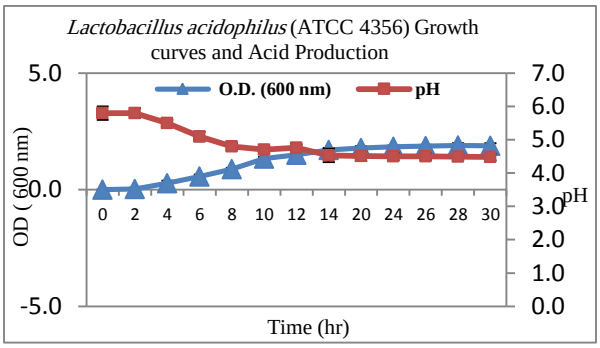

Figure 1. Growth curves and acid production statues of *L. acidophilus* (ATCC 4356) in MRS broth after 24 h of incubation at 37°C under micro-aerobic conditions.

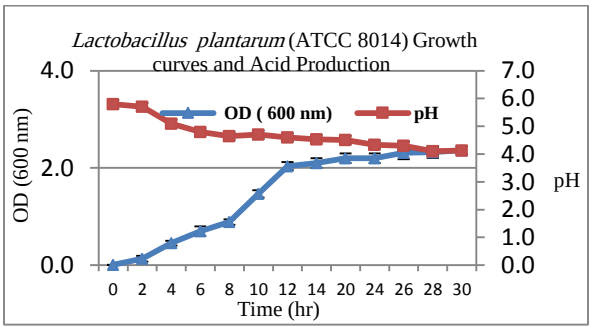

Figure 2. Growth curves and acid production statues of *L. plantarum* (ATCC 8014) in MRS broth after 24 h of incubation at 37°C under micro-aerobic conditions.

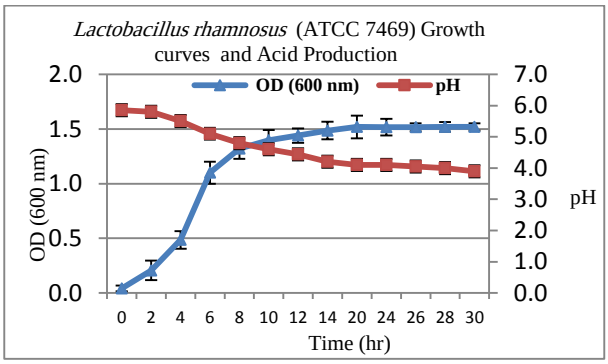

Figure 3. Growth curves and acid production statues of *L. rhamnosus* (ATCC 7469) in MRS broth after 24 h of incubation at 37°C under micro-aerobic conditions.

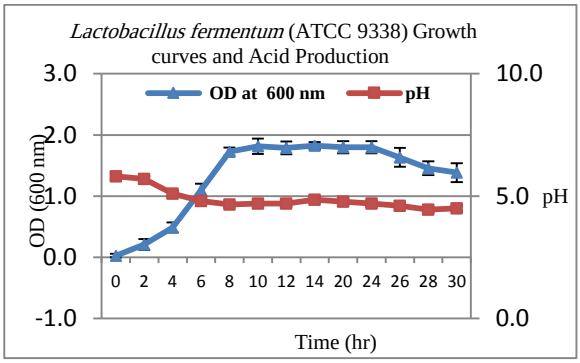

Figure 4. Growth curves and acid production statues of *L. fermentum* (ATCC 9338) in MRS broth after 24 h of incubation at 37°C under micro-aerobic conditions.

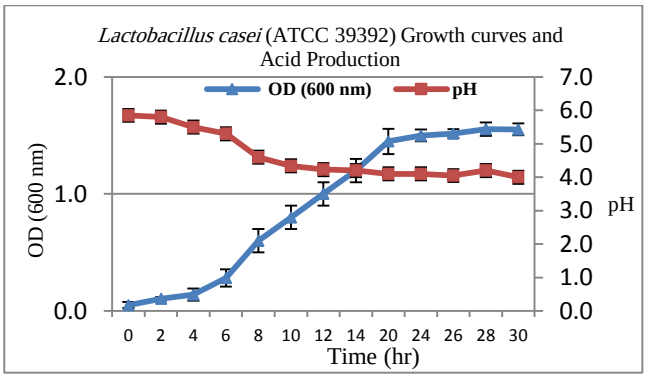

Figure 5. Growth curves and acid production statues of *L. casei* (ATCC 39392) in MRS broth after 24 h of incubation at 37°C under micro-aerobic conditions.

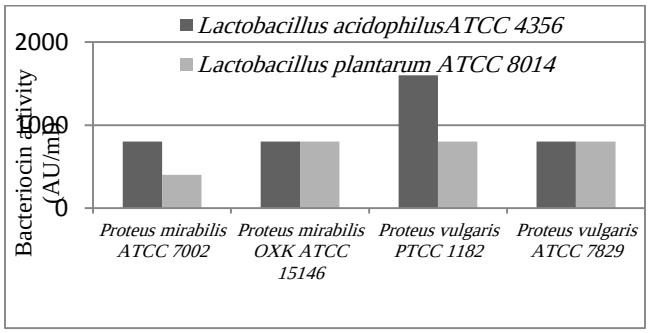

Figure 6. Bacteriocin activity of the lactobacilli pellet after ammonium sulfate precipitation.

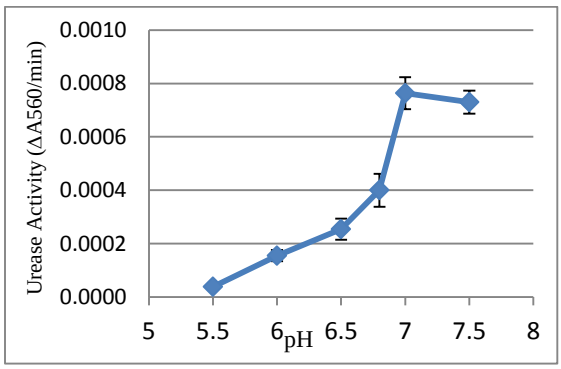

Figure 7. Study of effects of pH on urease activity of *P. mirabilis* ATCC 7002 after 24 h using spectrophotometry.

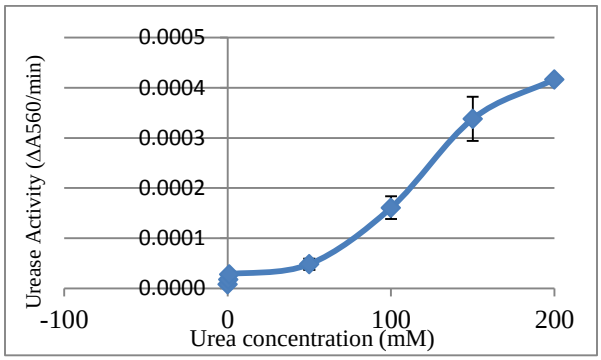

Figure 8. Study of effects of urea concentration on urease activity of *P. mirabilis* ATCC 7002 after 24 h using spectrophotometry.

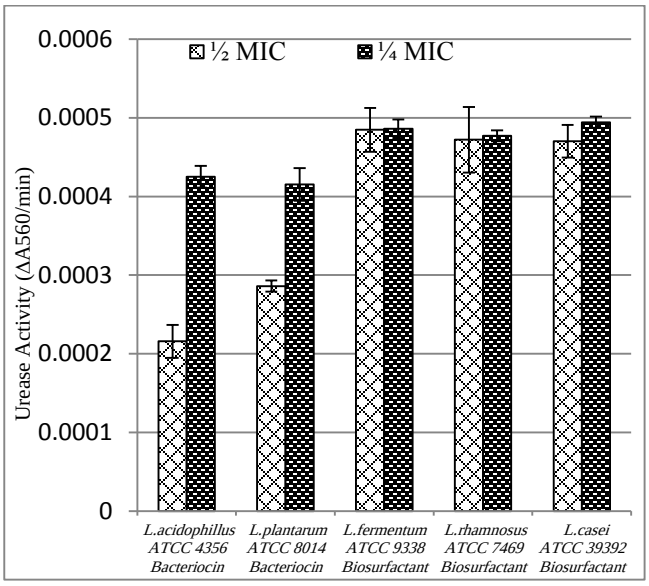

Figure 9. Study of effects of semi purified bacteriocin and biosurfactant on urease activity of *P. mirabilis* ATCC 7002 after 24 h of incubation at 37˚C using spectrophotometry.

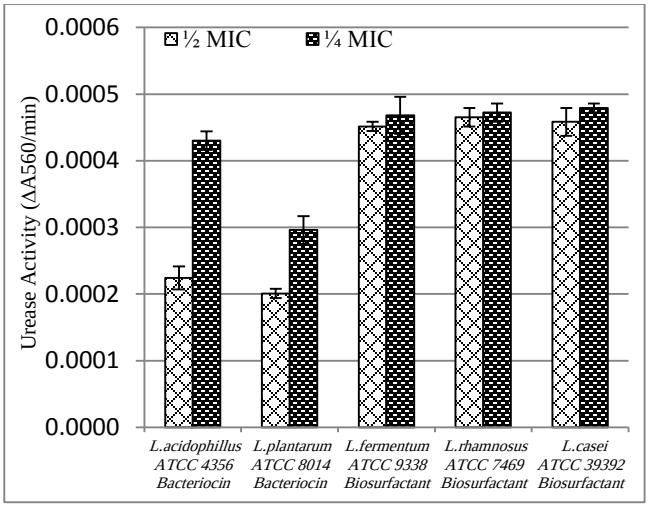

Figure 10. Study of effects of semi purified bacteriocin and biosurfactant on urease activity of *P. vulgaris* PTCC 1182 after 24 h of incubation at 37˚C using spectrophotometry.

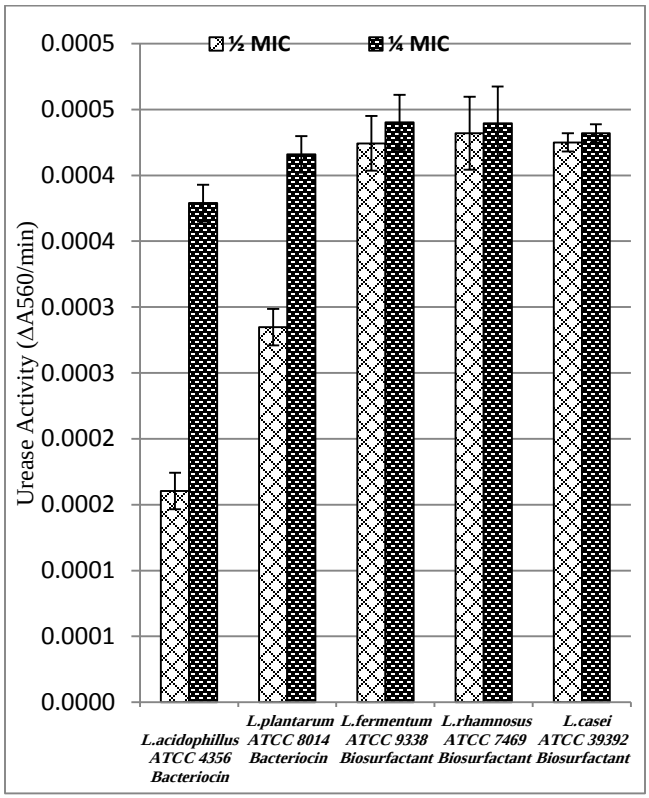

Figure 11. Study of effects of semi purified bacteriocin and biosurfactant on urease activity of *P. vulgaris* ATCC 7829 after 24 h of incubation at 37˚C using spectrophotometry.

Discussion

Proteus mirabilis and *P. vulgaris* are gram negative, motile and urease positive bacilli that include photolytic and hemolytic activities and are recognized among the most common urinary tract pathogens. These bacteria are known as the systematic and local infectious agents that can infect intestines (3). Urease inhibition is considered as the most important method in the treatment of stones formed due to the activity of urease enzymes. Urease enzymes are widespread in nature as well as urease inhibitors. Some urease inhibitors include plants extracts (31), 2-germa-γ-lactones (Gel) (32), urea analogs (33), boric and boronic acids (33), suramins (34) and heavy metals such as Hg, Zn and Cu (36). In recent years, many

scientists have studied use of probiotic and their metabolites for the treatment of urinary tract infections caused by *Proteus* spp. (25, 37). Mohankumar (2011) reported that *L. acidophilus* had inhibitory effects on the growth of *Proteus* spp.; most of these inhibitory effects were associated to the bacteriocin production (38). Secondary metabolites of bacteriocins and biosurfactants are produced in various phases of the bacterial growth cycle (39). It was also revealed that the highest amount of bacteriocins and biosurfactants is produced in the early stationary phase of bacterial growth (40). In the current study, the growth curves of lactobacilli strains showed that all investigated *Lactobacillus* strains reached to the stationary phase in less than 24 h. Therefore, extraction of bacteriocins and biosurfactants was carried out in this stage.

All *Lactobacillus* strains reduced the pH of media by producing organic acids after 24 h. Therefore, acid neutralization method was used. After neutralization of culture supernatants, the antimicrobial activity did not show any changes. Bacteriocin and bacteriocin-like substances are made directly in the form of polypeptide or prepolypeptide (41); therefore, the sensitivity of bacteriocins to various enzymes was assayed in this study. Results showed that pepsin and trypsin enzymes reduced the antimicrobial activity of the bacteriocin supernatant against the test strains, compared to untreated and neutralized supernatants (Table 3). Therefore, the antimicrobial effect of present bacteriocins was proven. This finding is similar to the previous finding by Lash et al. (2005) (21). Most antimicrobials (except organic acids) are sensitive to proteolytic enzymes. In the current study, bacteriocin concentration was carried out using ammonium sulfate precipitation and dialysis of the proteins. This study showed that all *Lactobacillus* strains produced biosurfactants in PBS (Diagram 1).

Since previous studies have shown that biosurfactant production occurs in the stationary phase (18); therefore, the first hours of *Lactobacillus* growth curves were studied in the current study. The major role of biosurfactants includes facilitating the absorption of solution materials in water by surface reduction in emulsification phase (42). Studies have demonstrated that biosurfactants can include antimicrobial activity (43). However, no antimicrobial effect was observed for the *Lactobacillus* derived biosurfactants in the current study, which is similar to a study by Tahmourespour et al. (44).

Results of the current study showed that *L. acidophillus* and *L. plantarum* semi purified bacteriocins could inhibit or reduce the urease activity in the three *Proteus* strains at sub-MIC levels. No considerable inhibition was seen for the lactobacilli derived biosurfactants. In this study, urease activity was studied at concentrations below MIC. Thus, inhibition of urease expression in *Proteus* spp. is not certainly linked to growth inhibition. It was demonstrated previously that the bacterial inhibitory activity was mainly linked to the quorum sensing mechanism (45, 46).

The Michaelis-Menten plot was studied and it was seen that the plot was a form of sigmoid. The sigmoid forming of the michaelis-menten plot is a result of the changed operation of the enzyme active site in response to the changes in environmental urea concentration. This is called positive homotropic behavior. This behavior is in the nature of enzyme molecule and shows the importance of the structure-operation relations. It is a regulatory response to the environmental condition changes; by increase in the urine concentration, the urease activity will increase spontaneously.

Table 1. Assessment of MIC, MBC and sub-MIC of the culture supernatant from various *Lactobacillus* strains against P. strains at 37°C.

Cell-free supernatant of *Lactobacillus* strains	***Proteus* strains**	**Concentration (% v/v)**			
		MIC	**½ MIC**	**¼ MIC**	**MBC**
L. acidophilus ATCC 4356	*P. mirabilis* ATCC 7002	25	12.50	6.25	25
	P. mirabilis OXK ATCC 15146	25	12.5	6.25	50
	P. vulgaris PTCC 1182	50	25	12.5	50
	P. vulgaris ATCC 7829	12.50	6.25	3.125	25
L. plantarum ATCC 8014	*P. mirabilis* ATCC 7002	25	12.5	6.25	25
	P. mirabilis OXK ATCC 15146	25	12.5	6.25	50
	P. vulgaris PTCC 1182	25	12.5	6.25	50
	P. vulgaris ATCC 7829	12.5	6.25	3.125	25
L. fermentum ATCC 9338	*P. mirabilis* ATCC 7002	50	25	12.5	50
	P. mirabilis OXK ATCC 15146	25	12.5	6.25	50
	P. vulgaris PTCC 1182	50	25	12.5	50
	P. vulgaris ATCC 7829	25	12.5	6.25	25
L. rhamnosus ATCC 7469	*P. mirabilis* ATCC 7002	50	25	12.5	50
	P. mirabilis OXK ATCC 15146	25	12.5	6.25	50
	P. vulgaris PTCC 1182	50	25	12.5	50
	P. vulgaris ATCC 7829	25	12.5	6.25	25
L. casei ATCC 39392	*P. mirabilis* ATCC 7002	25	12.5	6.25	50
	P. mirabilis OXK ATCC 15146	12.5	6.25	3.125	50
	P. vulgaris PTCC 1182	50	25	12.5	50
	P. vulgaris ATCC 7829	12.5	6.25	3.125	25

Table 2. Residual activity of the cell-free supernatant from bacteriocin producing *Lactobacillus* strains after various treatment conditions (pepsin, trypsin, catalase and neutralizing with NaOH).

Cell-free supernatant of *Lactobacillus* strains	**Pathogen**	**Supernatant treatment**				
		Untreated supernatant (pH = 4.8)	**Neutralized supernatant (pH = 7)**	**Pepsin**	**Trypsin**	**Catalase**
		Residual activity	**Residual activity**	**Residual activity**	**Residual activity**	**Residual activity**
L. acidophilus ATCC 4356	*P. mirabilis* ATCC 7002	100 %	72 %	36 %	52.6 %	45.8 %
	P. mirabilis OXK ATCC 15146	100 %	81 %	33%	58.5 %	49 %
	P. vulgaris PTCC 1182	100 %	70 %	27.3 %	54 %	34 %
	P. vulgaris ATCC 7829	100 %	73.2 %	28 %	51.2 %	50.6 %
L. plantarum ATCC 8014	*P. mirabilis* ATCC 7002	100 %	68 %	23.2 %	50.4 %	35 %
	P. mirabilis OXK ATCC 15146	100 %	86.6 %	44 $	70 %	60 %
	P. vulgaris PTCC 1182	100 %	62 %	33.3 %	35 %	52 %
	P. vulgaris ATCC 7829	100 %	72 %	58 %	48.8 %	47.5 %

Conclusion

Since urease enzyme plays a key role in the pathogenesis of *Proteus* spp., it can be concluded that compounds that deactivate or denature this enzyme can be effective in reduction of *Proteus* pathogenesis and therefore prevent stone formation by the bacteria. As demonstrated in the current study, *Lactobacillus* spp. are able to produce such compounds, including bacteriocins and biosurfactants. Use of LAB for the treatment of urinary tract infections is therefore suggested.

Conflict of interest

None decleared.

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