



## Antimicrobial and Anti-Swarming Effects of Bacteriocins and Biosurfactants from Probiotic Bacterial Strains against *Proteus* spp.

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### ABSTRACT

**Background:** *Proteus* spp. belongs to the family of *Enterobacteriaceae*. These bacteria are Gram-negative and motile microorganisms and known as the third most common causes of urinary tract infections. The aim of the current study was to investigate the effects of some secondary metabolites from probiotic strains of *Lactobacillus* spp. on swarming and growth of *Proteus mirabilis* and *P. vulgaris*.

**Methods:** After determination of optimal conditions for the growth and production of antimicrobials, bacteriocins and biosurfactants were partially purified from *Lactobacillus* culture supernatants. Then, effects of the purified compounds on growth and swarming migration of *Proteus* spp. were examined in the presence of various concentrations of semi-purified compounds.

**Results:** Results showed that the partially purified bacteriocins inhibited *Proteus* spp. swarming distance and had a significant reduction on the bacterial growth curves. Biosurfactants in a solvent form did not have any considerable effects on factors produced by *Proteus* spp.

**Conclusion:** According to the results, the secondary metabolites, especially bacteriocins or bacteriocin-like substances derived from *Lactobacillus* strains, can inhibit or reduce growth and swarming migration of *Proteus* spp. which are considered as the bacteria major virulence factors.

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## Introduction

The genus *Proteus* belongs to the family *Enterobacteriaceae* and includes five species; *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. myxofaciens* and *P. hauseri* with three unnamed genotypes (1). These bacteria are widespread in natural inhabitants such as contaminated water, soil and sewage and play an important role in decomposition of organic materials with animal origins. Unlike their saprophyte characterization in natural environments such as human microflora and animal gastrointestinal tract, *Proteus* spp. also is known as opportunistic pathogens that involve in various infections. They are reported as the third most common causes of urinary tract infections (2). *Proteus* spp. include a variety of virulence factors such as toxins (hemolysin and endotoxin), enzymes (urease, proteases and amino acid deaminases), fimbriae and flagella (3, 4). Compared to other urinary tract pathogens, *Proteus* spp. have a multicellular migration behavior called swarming. This occurs through a set of bacterial differentiation of short vegetative rods (2–4  $\mu\text{m}$  in length) into nonseptated, multinucleated bacilli with 20 to 80  $\mu\text{m}$  in length, which is accompanied by 50 to 500-fold increase in number of flagella. These differentiated cells have been introduced as the virulent form of *Proteus* spp. and most of the major virulence factors express during swarming differentiation. It has been suggested that the bacterial invasion to urinary tract (bladder and kidneys) and other virulence factor expression such as exo-enzymes, proteases and ureases are directly linked to the swarming phenomenon (5). Due to the importance of *Proteus* spp. for the public health, researchers have been looking for solutions to overcome the problems caused by these pathogens. One of these solutions includes the use of lactic acid bacteria (LAB).

For centuries, the normal microbial flora has been known to have the potential inhibitory effects on urinary tract infections (6). Some species of this microbial flora (microflora) belong to LAB and are

generally named probiotics. According to Fuller's definition, probiotics are live microbial food supplements that induce beneficial effects in their hosts via regulation and replacement of the intestinal microflora (7). LAB and Bifidobacteria are the most common microorganisms used as probiotics. One of LAB, *Lactobacillus* spp., protect their hosts against urinary tract infections by various mechanisms: 1) *Lactobacillus* spp. (living and non-living forms and cell wall components) attach to the urinary tract epithelial cells and inhibit the attachment of pathogens(8,9); and 2) *Lactobacillus* spp. can prevent urinary tract infections by producing inhibitory compounds. These bacteria produce various antimicrobial compounds such as lactic acid, hydrogen peroxide, bacteriocins and biosurfactants (2, 10).

Bacteriocins are peptides produced by some bacteria such as LAB and include antimicrobial specifications. Bacteriocins include inhibitory effects against a wide range of Gram-positive and Gram-negative bacteria. 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). Other compounds produced by LAB with antimicrobial features are biosurfactants. These compounds are able to reduce surface tension due to formation of hydrogen bonds and hydrophobic-hydrophilic interactions (12).

Use of *Lactobacillus* spp. in reduction or inhibition of swarming as a potential virulence factor of *Proteus* spp. has been less considered. The aim of the present study was to investigate the effect of various concentrations of bacteriocins and biosurfactants from *Lactobacillus* probiotic strains on swarming and growth of *Proteus* spp.

## Materials and methods

### *Bacterial strains*

All bacterial strains were provided by Iranian Research Organization for Science and

Technology (IROST), including five strains of *L. acidophilus*, *L. plantarum*, *L. fermentum*, *L. casei* and *L. rhamnosus* as antimicrobial producing microorganisms and four strains of *Proteus* with wild type and mutant swarming genotypes (Table 1).

#### *Growth conditions and media*

The appropriate media for the culture of lactic acid producing bacteria included MRS broth and MRS agar (described first by De Man and Rogosa) incubated for 24 to 48 h at 37 °C in an atmosphere containing 5% CO<sub>2</sub>. The uropathogen *P. mirabilis* and *P. vulgaris* were cultured in nutrient broth and Muller-Hinton broth for 24 h at 37 °C under aerobic conditions. All media were purchased from Merck Company (Germany).

#### *Optimal growth conditions of Lactobacillus strains*

To assess optimal growth condition of *Lactobacillus* strains, a variety of temperature and pH ranges tested as physicochemical conditions. To assess the effect of various temperatures on *Lactobacillus* growth, 50 ml of MRS broth were cultured with 1 % overnight culture of *Lactobacillus* strains and then incubated at temperatures of 15, 25, 30, 37, 40 and 45°C under micro-aerobic conditions. To assess the effect of various pH values on *Lactobacillus* growth, MRS broth adjust to pH values of 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, 10 and 11, then 1% overnight culture of *Lactobacillus* strains were cultured. After 24 h incubation under micro-aerobic conditions, growth status of *Lactobacillus* strains assessed using spectrophotometer (11).

Assessment of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and sub-MIC.

MIC was assessed using serial broth microdilution method in 96-well plates (JET BIOFIL, Canada). One hundred microliters of MRS broth were added to each well and filtered cell-free supernatant of *Lactobacillus* strains with 100% v/v were added to the first well. Then, serial

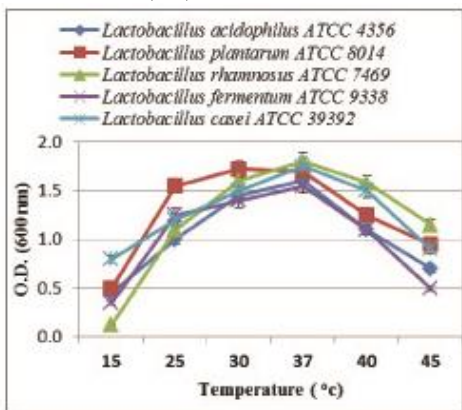
dilutions were prepared for each supernatant and 100 µl of the test bacterial suspension with approximately  $1.5 \times 10^6$  CFU/ml cell density in Muller-Hinton broth (assessed via optical density at 600 nm) were added to each well. Plates were then incubated for up to 24 h at 37 °C under anaerobic conditions. MIC was reported as the lowest concentration of the bacterial cell-free supernatants; at which, bacterial growth did not occur. The bacterial growth was detected based on turbidity (OD = 600 nm) using microplate reader. All experiments were carried out in three replicates. Positive and negative controls included overnight bacterial culture in broth and sterile broth, respectively. Ten microliters of the lowest concentrations of broth with no *Proteus* growth in presence of *Lactobacillus* supernatant were cultured on nutrient agar media. MBC was reported as the lowest concentration of the bacterial cell-free supernatants; at which, no bacterial growth was seen after two days of incubation (13).

#### *Extraction of bacteriocins*

Ammonium sulfate precipitation method was used to concentrate and precipitate bacteriocins from bacterial strains. A 0.5 Mcfarland ( $1.5 \times 10^8$  CFU/ml) suspension was prepared from bacterial strains. Then, 5% of this suspension were inoculated into 100 ml of MRS and incubated for 24h at 37 °C and optimal pH in a micro-aerophilic atmosphere. Culture broth media were adjusted to pH 6.5 by adding sufficient amounts of 1N NaOH. The bacterial Cell-free supernatant was collected by centrifugation at 6,000 g for 20 min at 4°C. Ammonium sulfate was added to crude bacteriocins to achieve 50% saturation for *L. acidophilus* (14) and 90% saturation for *L. plantarum* (15). Solutions were stirred overnight at 4 °C. Precipitates were collected by centrifugation at 10,000 g for 40 min and resuspended in 1 ml of sodium phosphate buffer (0.05 mM, pH 7) and then dialyzed against the same buffer using 12,000 KDa dialysis tubing for 48 h with 4 buffer changes.

Assessment of bacteriocin activity

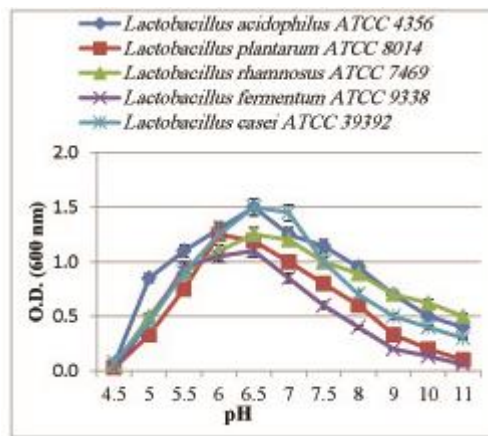
Bacteriocin activity of *L. acidophilus* and *L. plantarum* semi purified bacteriocins were tested against *P. mirabilis* and *P. vulgaris* using spot-on-lawn method (16). The bacteriocin activity was quantified by spotting 5 µl aliquots of the extracted bacteriocins onto the surface of BHI soft agar (0.7%) plate which was inoculated with 1% of indicator strains. The plates were incubated at 37 °C for 24 h. Bacteriocin activity (AU) was defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of *Proteus* strains (17).



**Figure 1.** Growth status of *Lactobacillus* spp. in MRS broth after 24 h of incubation at various temperatures and pH 5.7 with micro-aerobic conditions.

Extraction of biosurfactants

*Lactobacillus* strains were grown overnight in 600 ml MRS broth under micro-aerophilic conditions. Bacterial cells were harvested by centrifugation (10,000 g for 5 min at 10 °C) and washed twice in demineralized water and then resuspended in 100 ml of PBS (10 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> and 150 mM NaCl, pH 7). These were incubated at room temperature for 2 h with gentle stirring. Then, bacteria were removed by centrifugation and the supernatant was filtered using 0.22-mm filters (Millipore, USA). Ten



**Figure 2.** Growth status of *Lactobacillus* spp. in MRS broth after 24 h of incubation at various temperatures and various pH with micro-aerobic conditions.

milliliters of the supernatant were used immediately in adhesion assay and the rest was used to investigate biosurfactant inhibitory effects on *Proteus* growth and swarming (18).

Measurement of surface tension

To test the ability of extracted biosurfactants to decrease the surface tension between water and hydrophobic surfaces, drop collapse test was carried out as follows: 25 µl of the biosurfactant containing solvent were pipetted as a droplet on a hydrophobic surface such as parafilm. The flattening and spreading of the droplet on coating surface were followed over a period of time. Droplet was allowed to dry and the diameter of the dried droplet was reported, compared to distilled water as control. Then, droplets were stained with methylene blue (19).

Growth inhibition of selected pathogens

*P. mirabilis* and *P. vulgaris* growth curves were plotted in presence and absence of various concentrations of antimicrobials (MIC and sub-MIC) using spectrophotometry. One percent of the overnight culture supernatant (pH = 7) of *L. acidophilus* and *L. plantarum* and biosurfactant containing solvents from *L. fermentum*, *L. rhamnosus*

and *L. casei* was added to 50 ml of  $10^9$  CFU/ml growing cells from *P. mirabilis* and *P. vulgaris* in MHB. Bacterial growth was assessed by measuring optical density at 600 nm during various time intervals up to 18 h at 37 °C under aerobic conditions. Sterile broth without antimicrobials was used as growth control (20).

#### Assessment of *Proteus* swarming inhibition

Standard CLSI agar dilution method was used to test extracted antimicrobials for anti-swarming activity against *Proteus* spp. To calculate optimal agar concentration for the bacterial swarming migration, Muller-Hinton media with various agar contents (1–3%) were tested. Extracted antimicrobials were added to optimal Muller-Hinton agar at sub-MIC concentrations ( $\frac{1}{2}$  MIC and  $\frac{1}{4}$  MIC). Plates were inoculated with 5  $\mu$ l of each *Proteus* strain ( $10^7$  CFU ml<sup>-1</sup>) and incubated at 37 °C under aerobic conditions. The bacterial swarming migration was monitored after 24 h. Muller-Hinton agar without antimicrobials was used as growth control. Furthermore, MRS broth was used to investigate its possible effects on bacterial growth or swarming. All experiments were carried out in triplicate (21).

## Result

#### Optimal growth conditions of *Lactobacillus* strains

Assessment of *Lactobacillus* growth conditions in various temperatures showed that the highest optical density of the bacteria has been occurred at 30 and 37 °C for all strains, which means that the bacterial duplication time is shorter at these temperatures (Figure 1). The most growth rates of *Lactobacillus* strains were seen at pH 6, 6.5 and 7. The optical density and duplication time have been decreased by increasing the pH (Figure 2).

#### Assessment of MIC, MBC and sub-MIC

Results of MIC, MBC and sub-MIC assays are shown in Table 2.

#### Assessment of bacteriocin activity

The highest bacteriocin activity (1600 AU/ml) was seen for that produced by *L. acidophilus* ATCC 4356 and tested against *P. vulgaris* PTCC 1182. The lowest bacteriocin activity (400 AU/ml) was seen for that produced by *L. plantarum* ATCC 8014 and tested against *P. mirabilis* ATCC 7002 (Table 3).

**Table 1.** Bacterial strains used in this study.

Bacteria	ATCC	PTCC	Characteristics	Reference
<i>L. acidophilus</i>	4356	1643	Bacteriocin producing	16
<i>L. plantarum</i>	8014	1058	Bacteriocin producing	23
<i>L. casei</i>	39392	1608	Biosurfactant producing	47
<i>L. rhamnosus</i>	7469	1637	Biosurfactant producing	47
<i>L. fermentum</i>	9338	1638	Biosurfactant producing	47
<i>P. mirabilis</i>	7002	-	Positive swarming	atcc.org
<i>P. mirabilis</i> OXK	15146	1076	Negative swarming	IROST
<i>P. vulgaris</i>	-	1182	Positive swarming	IROST
<i>P. vulgaris</i>	7829	1312	Negative swarming	IROST

**Table 2.** Assessment of MIC, MBC and sub-MIC of the culture supernatant of *Lactobacillus* spp. against *Proteus* spp. at 37 °C.

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

**Table 3.** Bacteriocin activity of *L. acidophilus* and *L. plantarum* against *Proteus* spp.

Bacteriocin activity (AU/ml)	<i>P. mirabilis</i> (ATCC 7002)	<i>P. mirabilis</i> OXK (ATCC 15146)	<i>P. vulgaris</i> (PTCC 1182)	<i>P. vulgaris</i> (ATCC 7829)
<i>L. acidophilus</i> (ATCC 4356)	800	800	1600	800
<i>L. plantarum</i> (ATCC 8014)	400	800	800	800

**Figure 3.** Reduction of surface tension using biosurfactants produced by *L. rhamnosus* (a), *L. fermentum* (b) *L. casei* (c), compared to distilled water (d), as control using drop collapse assay.

#### Measurement of surface tension

Results showed that *L. fermentum*, *L. rhamnosus* and *L. casei* reduced surface tension on parafilm due to biosurfactant production, compared to distilled water (Figure 3).

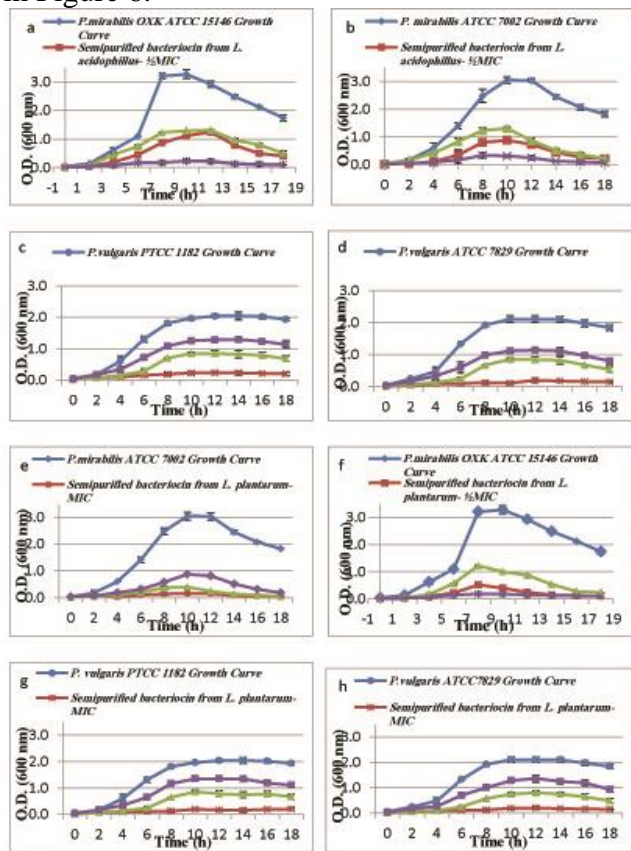
#### Growth inhibition of selected pathogens

Results showed that both bacteriocin producing *Lactobacillus* strains were able to reduce the growth of *Proteus* spp. (Figure 4a–h). The most growth reduction has been seen for *L. acidophilus* and *L. plantarum* bacteriocins against *P. mirabilis* ATCC 7002 and *P. mirabilis* OXK ATCC 15146, respectively.

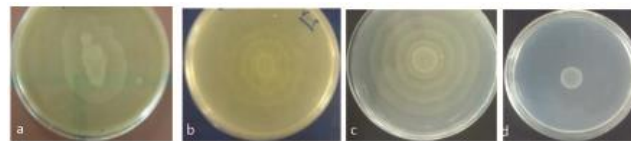
#### Assessment of *Proteus* swarming inhibition

Effects of the extracted antimicrobial compounds from *Lactobacillus* strains on swarming migration of *Proteus* spp. was tested on agar with various concentrations. Results showed that *Proteus* spp. could swarm on Muller-Hinton media containing 1 to 3% agar; from which, 1.5% agar contained media was reported as the most appropriate media for the bacterial swarming. Thus, this media was used for further experiments. Although the bacterial growth was not inhibited in the presence of extracted bacteriocins and biosurfactants at sub-MIC levels, semi purified bacteriocins from *L. acidophilus* and *L. plantarum* decreased the bacterial swarming migration in all swarming-positive *Proteus* strains. *L. acidophilus* ATCC 4356 showed the most anti-swarming activity in both swarming positive strains. However, results showed no significant reduction in the bacterial swarming migration in the presence of biosurfactants (Figure 5). A significant relationship was seen between *L. acidophilus* derived bacteriocins and *P. mirabilis* ATCC 7002 and *P. vulgaris* PTCC 1182 (P value = 0.046 and P value = 0.019, respectively). Effects

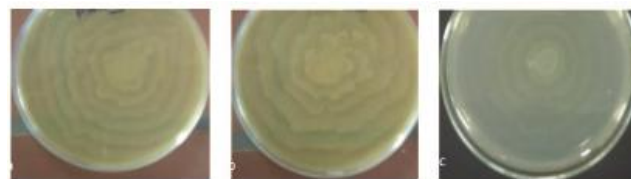
of extracted biosurfactants at sub-MIC are shown in Figure 6.



**Figure 4.** Growth curves of *P. mirabilis* ATCC 7002 (a), *P. mirabilis* OXK ATCC 15146 (b), *P. vulgaris* PTCC 1182 (c), and *P. vulgaris* ATCC 7829(d) in the presence of *L. plantarum* ATCC 8014 semi-purified bacteriocin at MIC, 1/2 MIC and 1/4 MIC in Muller-Hinton broth for up to 18 h of incubation at 37 °C using spectrophotometry. The most reduction in growth curve has been seen for *Proteus mirabilis* ATCC 7002 and *P. mirabilis* OXK ATCC 15146 in considering to *L. acidophilus* and *L. plantarum* produced bacteriocins, respectively.



**Figure 5.** Swarming migration of *Proteus* spp. in the presence of *L. acidophilus* (a) and *L. plantarum* (b) after 24 h of incubation at 37 °C (d). *P. mirabilis* OXK (ATCC 15146) negative control.



**Figure 6.** Effect of biosurfactants from *L. fermentum* (a) and *L. rhamnosus* (b) at 1/2 MIC on swarming migration of *P. mirabilis* ATCC 7002 in 1.5 % Muller-Hinton agar after 24 h of incubation at 37 °C compared to positive control (c).

### Discussion

Various virulence factors have been identified in *Proteus* spp. Swarming phenomenon is one of these virulence factors, which is important because of its prominent role in pathogenesis of the bacteria. Expression of virulence factors such as urease, protease and hemolysine has a higher rate in swarmer forms of *Proteus* spp. Indeed, this swarming phenomenon interferes with microbiology research and diagnosis procedures; hence, the inhibition of swarming migration in *Proteus* spp. is important (21). The idea that *Lactobacillus* spp. could displace harmful microorganisms such as pathogens on mucosal surfaces was first suggested nearly a century ago (22). Therefore, the inhibitory effect of various concentrations of *Lactobacillus* derived bacteriocins and biosurfactants on growth and swarming of *P. mirabilis* and *P. vulgaris* was investigated in the current study.

Study of optimum growth conditions of *Lactobacillus* strains showed that the maximum



bacterial density was achieved after 24 h incubation at 30 and 37 °C on MRS media with pH 5.5 to 7. To extract lactobacilli secondary metabolites (bacteriocins and biosurfactants), ammonium sulfate precipitation has been used because these compounds are non-dialysis, heat resistant, catalase resistance and susceptible to proteolytic enzymes (23). Various studies have shown that bacteriocins from *L. acidophilus* and *L. plantarum* can inhibit or reduce the growth of *Proteus* spp. as common urinary tract infection (UTI) agents. Mohankumar showed that *L. acidophilus* had inhibitory effect on growth of *Proteus* spp. and reported that the inhibitory effect was associated with bacteriocins (24). In the current study, suppressing effect of semi-purified bacteriocins on *P. mirabilis* and *P. vulgaris* growth curves was assessed. The most significant reduction on growth of *Proteus* spp. was linked to *P. mirabilis* (ATCC 7002) and *P. mirabilis* OXK (ATCC 15146) at sub-MIC levels. However, biosurfactants did not show any considerable reduction in *Proteus* growth curves. This might occur due to the impurity of biosurfactants.

In this study, *L. acidophilus* (ATCC 4356) and *L. plantarum* (ATCC 8014) bacteriocins had a significant reduction on swarming migration of *P. mirabilis* (ATCC 7002) and *P. vulgaris* (PTCC 1182). Addition of various compounds to culture media can inhibit the swarming of different bacteria. For example, plant extracts (21, 23, 25), urea (26), resveratrol (27), monoterpenes (28) and fatty acids (29) have been shown to be effective on bacterial swarming; however, these compounds can be toxic for a number of bacteria. In the current study, bacterial swarming was studied alternatively using antimicrobial concentrations below MIC; by which, the growth of bacteria is not completely inhibited.

This study demonstrated that biosurfactants producing strains of *Lactobacillus* (*L. fermentum*, *L. rhamnosus* and *L. casei*) reduced active-surface compounds in PBS solution. The main physiological role of biosurfactants includes facilitating the absorption of solvents in water by

reducing the surface tension and mediating the maximum possible attachment of microbial cells to the organic compounds (30).

In the current study, the ability of extracted biosurfactants from *Lactobacillus* strains on *P. mirabilis* and *P. vulgaris* swarming migration was examined as well as extracted bacteriocins. It was seen that extracted bacteriocins showed a stronger inhibitory effect against swarming of *Proteus* spp., compared to that of extracted biosurfactants. Many studies have been carried out on inhibitory mechanisms of bacterial swarming, including reviews of Stankowska (30) and Armbruster (5). They suggested that quorum sensing could regulate the virulence gene expression and hence swarming in many bacteria. Although quorum sensing system in *Proteus* spp. has not clearly been demonstrated, there is much evidence that this system can play a role in differentiation of swarming and other virulence factors in *Proteus* spp. First, swarming differentiation in *Proteus* spp. is a multi-cell differentiation activity and regulated by population density (21). Second, it is known that swarming differentiation of other virulence factors such as urease, hemolysin and proteases are coordinately regulated by swarming in *Proteus* spp. Although swarming differentiation is a population density dependent process, expression of other virulence factors is possibly regulated by population density mechanisms (31). Third, it has been demonstrated that RsmA protein as a regulator protein of quorum sensing system can inhibit 3-oxohexanoyl homoserine lactone, a signal molecule of this system. When the coding gene transferred to the genome of *P. mirabilis*, the bacterial swarming was inhibited (30). Evidence indicates that a quorum sensing system exists in *Proteus* spp., which plays a role in swarming migration of the bacteria. Therefore, *Lactobacillus* derived metabolites possibly inhibit swarming by interfering with this mechanism.

## Conclusion

The results from the current study showed that lactic acid bacteria such as *L. acidophilus* and *L. plantarum* could have therapeutic and prophylactic effects on one of the most common causative agents of urinary tract infections, *Proteus* spp. Since antibiotic resistance is increasing in *Proteus* spp. and due to importance of these pathogens for the public health, further research on use of bacteriocins from *Lactobacillus* spp. for the prevention or treatment of *Proteus* infections is necessary.

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## Conflict of interest

None declared conflicts of interest.

## Financial disclosure

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