

# Antimicrobial effect of probiotic *Lactobacillus spp.* on *Pseudomonas aeruginosa*

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**Objectives** Study the antimicrobial effect of probiotics produced from *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* on *Pseudomonas aeruginosa* isolated from burn and wound infection and their ability of protease production.

**Methods** Swab samples were collected from 70 patients admitted at Burns Center/Al-Yarmouk Teaching Hospital. Primary bacterial identification cultured on differential selective media and biochemical tests were done. The Vitek2 compact system (Biomérieux, France) was used to confirm the *P. aeruginosa* isolates by Gram negative identification card and the antimicrobial susceptibility Test Card to each isolate was performed. Protease production using skimmed milk agar 1% was performed. Crude bacteriocin produced from *L. acidophilus* (Holland & Barrett, USA) and *L. rhamnosus* (Health Gensis, USA) was extracted during log phase using MRS broth (24 h/ 37°C/ 5–10% CO<sub>2</sub>), then cool centrifugation was done (6000 rpm at 4°C for 10 min). Protein concentration of bacteriocin was estimated using Bradford assay using bovine serum albumin as standard.

**Results** Only 31 out of 48 isolates were identified as *P. aeruginosa*; 9 (45%) from wound and 22 (79%) from burn swabs. Antimicrobial susceptibility tests included 16 antibiotics; *P. aeruginosa* isolates showed multi-drug resistance for antibiotics. All *P. aeruginosa* isolates were having the ability for protease enzyme production. Antimicrobial effect of bacteriocin produced by *L. acidophilus* and *L. rhamnosus* on protease using plate diffusion method showed positive results. Protein concentration of bacteriocin produced by *L. rhamnosus* and *L. acidophilus* were 74 mg/mL, 44 mg/mL, respectively. The highest zone of antimicrobial effect by *L. rhamnosus* was 32 mm and by *L. acidophilus* was 25 mm using well diffusion method.

**Conclusions** *P. aeruginosa* showed a multi-drug resistance and had the ability to produce protease enzyme. Bacteriocin produced by *L. acidophilus* and *L. rhamnosus* showed an acceptable positive results to be used as potential alternative bio-remedy to overcome the multi-drug resistance dilemma.

**Keywords** antimicrobial effect, *Lactobacillus spp.*, bacteriocin, *Pseudomonas aeruginosa*, protease

## Introduction

*Pseudomonas aeruginosa* is a Gram-negative aerobic bacilli and the natural flora of the skin and intestinal tract that is also found in water and soil. It is an opportunistic pathogen and one of the main causes of nosocomial infections causes severe diseases like cystic fibrosis, urinary tract infections, acute purulent meningitis, otitis media, otitis externa, eye infections, wound and burn infections, septicaemia and infantile diarrhea.<sup>1</sup> The multiple resistant to the most commonly used antibiotics is quite common in *P. aeruginosa* due to possession of high number of virulence factors, which is attributable to a concerted action of multidrug efflux pumps with a chromosomally encoded antibiotic resistance genes and the low permeability of bacterial cellular envelopes as well as biofilm formation phenomenon.<sup>2</sup> Wound infections due to *P. aeruginosa* are especially difficult in burn patients, high percentage of wound infections will lead to sepsis with significant mortality rates.<sup>3</sup> Protease-deficient strains are generally less virulent than protease producers in burned mouse models.<sup>4</sup> Proteases are enzymes that can hydrolyze peptide bonds within peptides and proteins. *P. aeruginosa* secretes several proteases (protease IV, elastase B, elastase A, and alkaline protease), which play an important role during infection with *P. aeruginosa* and are a characteristic for invasiveness as determined in clinical strains.<sup>5</sup> These proteases are able to degrade a whole range of biological important host proteins such as fibrinogen, elastin, collagen and plasminogen as well as immunoglobulin G (IgG) and the complement components 3 and

C1q, which belong to the immune defense system.<sup>6</sup> So, there is a considerable interest in developing low cost large-scale alternative remedies to prevent or reduce the multi-drug resistance of *P. aeruginosa*. In this regard, probiotics may close the therapeutic gap. Probiotics are living microbial species, when administered in adequate amounts confer a health benefit to the host.<sup>7</sup> Probiotics have been proven to be useful in the treatment of several infections and gastrointestinal diseases such as acute diarrhea.<sup>8</sup> Multiple mechanisms have been proposed to justify the protective and therapeutic role of probiotics including lactose digestion, production of antimicrobial agents, pathogen exclusion and immunomodulation.<sup>9–11</sup> Commercially available probiotic preparations including lactic acid bacilli (*Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *L. plantarum*, *L. casei*, etc.) alone or in combination with *Streptococcus* and *Saccharomyces* species have shown the beneficial effects.<sup>12</sup> *Lactobacilli* are known to produce a variety of metabolic by-products in addition to biosurfactants. Some of which have antimicrobial activity including lactic acid, hydrogen peroxide, bacteriocins, and bacteriocin-like substances which has imperative biomedical advantages.<sup>13</sup> Bacteriocins are ribosomally synthesized low-molecular weight peptides or proteins with potential use in food preservation due to their bactericidal effects on food spoilage and pathogenic organisms.<sup>14</sup> Bacteriocins have unique applications in food processing and food safety because of their heat stability and sensitivity to proteolytic enzymes.<sup>15</sup>

The current study aimed to investigate the antimicrobial effect of the probiotic strains *L. rhamnosus* GG and *L. acidophilus* on *P. aeruginosa* isolated from wound and burn infection *in vitro*.

## Patients and Methods

### Bacterial Isolation, Identification and Antimicrobial Resistance

Swab samples were collected from 70 patients (both males and female with age range 1–64 years) admitted at Burns Center / Al-Yarmouk Teaching Hospital from the period November 2013 till February 2014. Primary bacterial identification cultured on differential and selective media then biochemical tests were done. The automated microbial identification using Vitek2 compact system (Biomérieux, France) was used to confirm the *P. aeruginosa* isolates by Gram Negative Identification Card (GN ID), which accommodates colorimetric reagent cards that are incubated and interpreted automatically and the Antimicrobial Susceptibility Test Card (AST) to each isolate was performed according to manufacturer's instructions.

### Protease Production

Protease production by *P. aeruginosa* isolates using skimmed milk agar 1% (Himedia, India) was performed to determine the proteolytic potency of the isolates using agar well diffusion assay.<sup>16</sup>

### Probiotic Preparation

Probiotic strains from commercially available capsule *L. acidophilus* (Holland & Barrett, USA) and *L. rhamnosus* GG (Health Gensis, USA) were isolated by suspending in each capsule in 10 ml of MRS broth (Himedia, India) then incubated anaerobically at 37°C for 48 hr.<sup>17</sup>

### Antimicrobial Assay

The antimicrobial spectrum from *Lactobacilli* spp. was determined using a loopful of each of the *Lactobacilli* isolates from the MRS agar slants was inoculated into tubes containing 10 mL of sterile MRS broth. These broth cultures were incubated anaerobically at 37°C for 48 hr. The following assays conducted as triplicate:

**Well diffusion method:** Sterile cotton swabs were dipped into the cultures of *P. aeruginosa* previously propagated in Brain Heart Infusion (BHI) broth (Difco, USA) for 24 hr at 37°C. The turbidity of the suspension was 0.5 McFarland and contained more than 10<sup>8</sup> bacteria. The inoculated with 100 µl of (1.5 × 10<sup>8</sup> cfu/ml) by swabbing over the entire surface of the nutrient agar (Himedia, India) plates was made. Wells (6mm diameter) were made on the cultured plates. Then 50 µl of *Lactobacilli* spp. bacterial suspension was inoculated into wells. After 24 hr of incubation at 37°C, each plate was examined for the zone of inhibition. Control for each zone was prepared using un-inoculated sterile MRS broth as negative control and acetic acid (33%) as positive control.<sup>18</sup>

**Agar disc method:** The above bacterial suspension of *Lactobacillus* spp. was incubated anaerobically on MRS agar at 37°C for 48 hr, agar discs with (6 mm) were made, then the agar discs were seeded on plates of nutrient agar cultured with 100 µl of (1.5 × 10<sup>8</sup> cfu/ml) of *P. aeruginosa*. The plates were

incubated for 37°C for 24 hr. The diameters of the inhibitory zones were measured including the diameters of the discs to the nearest whole number. Control was prepared using agar disc free of bacterial growth.<sup>19</sup>

### Extraction of Crude Bacteriocin

Crud bacteriocin produced from *L. acidophilus* and *L. rhamnosus* was extracted during log phase using MRS broth incubated anaerobically at 37°C for 24 hr. After incubation, the cultures were centrifuged (6000 rpm at 4°C for 10 min) to obtain culture-free supernatant which was filtered using 0.22 µm pore sterilized filter. The pH was adjusted to pH 7 with 1 M NaOH.<sup>20</sup>

### Determination of Protein Content

Protein content was determined using colorimetric at maximum absorption at 600 nm, using brilliant blue G-250 and Bovine Serum Albumin.<sup>21</sup>

### Antimicrobial Activity of Bacteriocin on Protease Production

The antimicrobial spectrum from bacteriocin was determined using a loopful of *P. aeruginosa* isolates from the BHI agar slants that was inoculated into tubes containing 5 mL of sterile BHI broth. These broth cultures were incubated at 37°C for 24 hr. The well diffusion assay conducted as triplicate. They inoculated with 10 µl of (1 × 10<sup>8</sup> cfu/ml) by swabbing over the entire surface of the skimmed milk agar plates. Wells (6 mm diameter) were made on the cultured plates. Then 10 µl of bacteriocin was inoculated into wells. After 24 hr of incubation at 37°C, each plate was examined for the zone of inhibition. Control for each zone was prepared using un-inoculated sterile BHI broth as negative control and phosphate saline (0.1 M/PH 7.0) as positive control.

### Statistical Analysis

The data are shown as the mean ± standard deviation (SD,  $n = 5$ ). The results obtained were analyzed using SPSS 18.0 program for Windows and by analysis of variance (ANOVA) with significance level set at  $P = 0.05$ .

## Results

From 70 swabs obtained from contaminated burn and wound infection, only 48 (69%) isolates were positive for primary bacterial isolation. Only 31 (65%) isolates out of 48 were identified as *P. aeruginosa* (9 wound infection and 22 burn infection) ( $P \leq 0.01$ ). Antimicrobial susceptibility tests included 16 antibiotics (Table 1).

Out of 31 *P. aeruginosa* isolates, 20 isolates showed multi-drug resistant for antibiotics (Table 2) ( $P \leq 0.01$ ).

All the 20 multi-drug resistant *P. aeruginosa* isolates were having the ability for protease production with potency (Table 3); ( $P \leq 0/05$ ). Figure 1 shows protease production for the isolate-11 and isolate-19 with duplicates.

Antimicrobial effect of *Lactobacilli* spp. on *P. aeruginosa* using agar disc and well diffusion method, (isolate-8, 11 and 19) were selected due to their high protease production and multi-drug resistance to many antibiotic. The inhibition zone was measured by millimeters as in Table 4. Statistically, the results were significant ( $P \leq 0.05$ ). The highest inhibition zone was by *L. rhamnosus* than *L. acidophilus* in all isolates in both methods.

The highest inhibition zone by *L. rhamnosus* was 20 mm on isolate-19, on isolate-11 was 25 mm, respectively; meanwhile,

Table 1. Antimicrobial susceptibility tests with resistant percentage of *Pseudomonas aeruginosa* isolates

No.	Antimicrobial	Resistant percentage (%)
1	Piperacillin	100
2	Ticarcillin	100
3	Ticarcillin clavoulanic acid	100
4	Cefazolin	100
5	Ceftriaxone	100
6	Tigecycline	100
7	Piperacillin Tazobactam	80
8	Amikacin	65
9	Gentamycin	80
10	Tobramycin	85
11	Imipenem	70
12	Meropenem	70
13	Cefepime	60
14	Ceftazidime	50
15	Ciprofloxacin	75
16	Levaofloxacin	75

Table 2. Multi antimicrobial resistant by *Pseudomonas aeruginosa* isolates

Number of antimicrobial	Resistant <i>P. aeruginosa</i> isolates	
	No.	%
4	2	10
5	2	10
8	2	10
9	1	5
12	1	5
13	5	25
14	7	35
Total	20	100
Chi square test ( $\chi^2$ )	—	9.017**

\*\*P ≤ 0.01.

Table 3. *Pseudomonas aeruginosa* protease production potency

No. of <i>P. aeruginosa</i> isolates	Protease production	No. of <i>P. aeruginosa</i> isolates	Protease production
Isolate-1	++	Isolate-11	++
Isolate-2	+	Isolate-12	+
Isolate-3	+	Isolate-13	++
Isolate-4	+	Isolate-14	+
Isolate-5	+++	Isolate-15	++
Isolate-6	+	Isolate-16	+
Isolate-7	++	Isolate-17	+++
Isolate-8	+++	Isolate-18	+
Isolate-9	++	Isolate-19	++
Isolate-10	+	Isolate-20	+

+ : Mild protease production; ++ : Moderate protease production; +++ : High protease production.

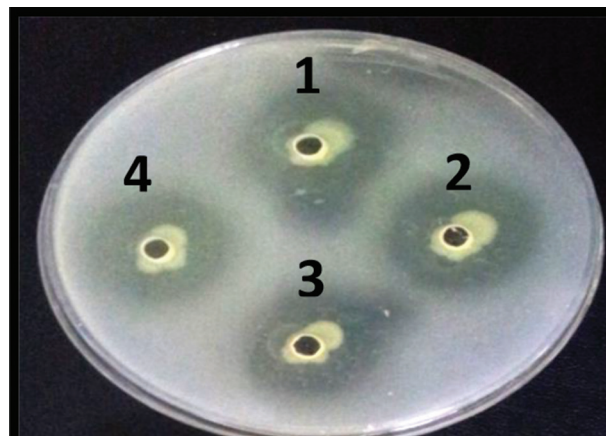


Fig. 1 *Pseudomonas aeruginosa* on skimmed milk agar 1%. (1 and 2) = duplicate of isolate-11; (3 and 4) = duplicate of isolate-19 using well diffusion method.

highest inhibition zone by *L. acidophilus* was 18 mm on isolate-19 using agar disc method as shown in Fig. 2.

The highest inhibition zone of bacterial suspension by *L. rhamnosus* was 27 mm on both isolate-11 and isolate-19; meanwhile, highest inhibition zone by *L. acidophilus* was 18 mm on isolate-19 using well diffusion method as shown in Fig. 3.

The highest inhibition zone of bacterial supernatant on isolate-19 by both *L. rhamnosus* and *L. acidophilus* was 32 mm, 25 mm, respectively, using well diffusion method as shown in Fig. 4.

Protein concentration of crud bacteriocin produced by *L. rhamnosus* GG and *L. acidophilus* were 74 mg/mL, 44 mg/mL, respectively. Fig. 5 shows the inhibitory effect of crud bacteriocin extracted from *L. rhamnosus* GG and *L. acidophilus* on protease production by *P. aeruginosa* (isolates-11 and 19) cultured on skimmed milk agar 1% after treated with crud bacteriocin comparing to untreated isolates.

## Discussions

*P. aeruginosa* is one of the important bacteria that can cause huge burdens for public health today due to its ability to adapt its genome and physiology during chronic infections. Major features making it a very successful opportunistic pathogen includes: virulence factors, biofilm formation, motility and quorum sensing.<sup>22</sup> According to Arqués et al. (2015) determining the antagonistic effect of probiotics on the growth of *P. aeruginosa* and the effectiveness of various bacteriocins of probiotics may be hindered by the proteolytic activity of microbial enzymes that are secreted only during active fermentation.<sup>23</sup> The present study basically focused on the bacteriocin, which is produced by a commercially available of *L. rhamnosus* GG and *L. acidophilus*. Bacteriocins, in general, share a narrow spectrum of antimicrobial activity; however, there are certain bacteriocins that exhibit a broad spectrum of antibacterial activity and are also capable of targeting viruses, protozoa and even fungi.<sup>24</sup>

The results revealed that, the probiotics of *L. rhamnosus* showed enhancement of inhibitory zone diameters in agar disc and well diffusion method (bacterial suspension and supernatant) rather than *L. acidophilus*. The narrow inhibitory zone using the agar disc method may be due to the limited number of the *Lactobacilli* cultured on MRS disc leading to its limited antimicrobial activity which come in accordance with

Table 4. Antimicrobial effect of *Lactobacilli* spp. in millimeters using agar disc method and well diffusion method

LSD value	Inhibition zones (MM)										Isolate
	CO <sup>-</sup>	Well diffusion method								Agar disc method	
		Supernatant				Suspension					
		CO <sup>+</sup>	<i>L. rhamnosus</i>	<i>L. acidophilus</i>	CO <sup>+</sup>	<i>L. rhamnosus</i>	<i>L. acidophilus</i>	<i>L. rhamnosus</i>	<i>L. acidophilus</i>		
7.02*	5	30	25	15	30	24	12	24	16	P8	
9.13*	5	33	30	18	31	27	16	25	16	P11	
7.53*	5	30	32	25	31	27	20	20	18	P19	
—	0.00 NS	6.02 NS	5.71*	6.38*	6.72 NS	5.22*	5.19*	5.83*	6.59*	LSD value	

\*P ≤ 0.05; NS: Non significant; CO<sup>+</sup>: Control positive (33% Acetic acid); CO<sup>-</sup>: Control negative (un-cultivated MRS broth).

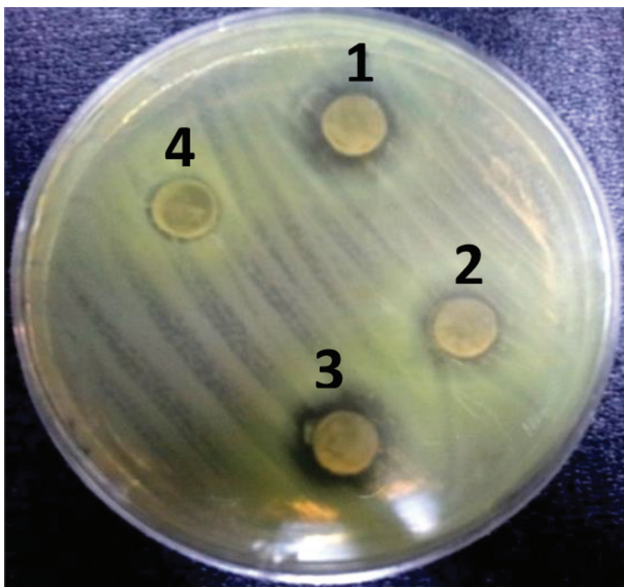


Fig. 2 Antimicrobial effect of *Lactobacilli* spp. on *P. aeruginosa* (isolate-11, 19) using agar disc method. (1) *L. acidophilus*; (2 and 3) duplicate of *L. rhamnosus* GG; (4) Negative control (un-cultivated MRS broth) using agar diffusion method.

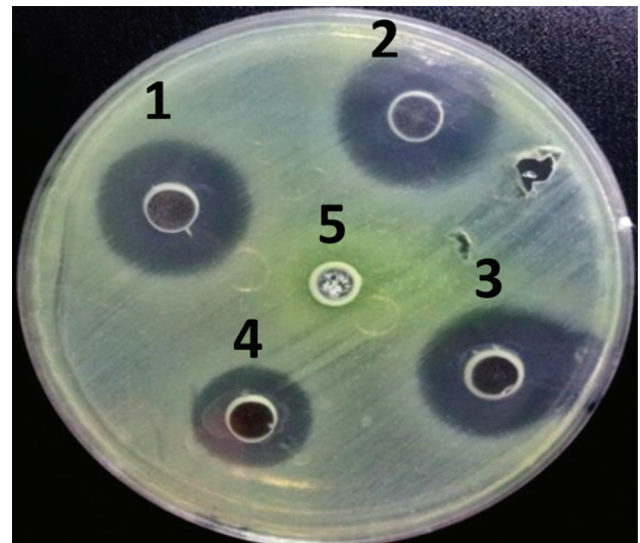


Fig. 4 Antimicrobial effect of bacterial supernatant (*Lactobacilli* spp.) on *P. aeruginosa* (isolate-19) using well diffusion method. (1 and 2) *L. rhamnosus* GG (duplicate); (3) *L. acidophilus*; (4); Positive control (33% Acetic acid); (5) Negative control (un-cultivated MRS broth).

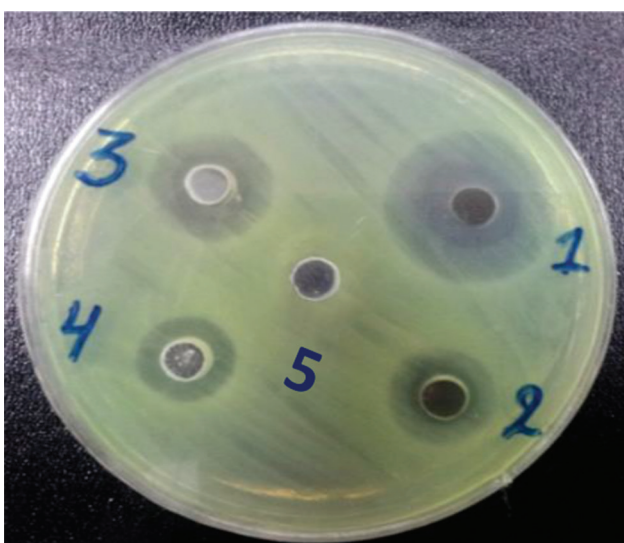


Fig. 3 Antimicrobial effect of bacterial suspension (*Lactobacilli* spp.) on *Ps. aeruginosa* (isolate-11, 19) using well diffusion method. (1) Positive control (33% Acetic acid); (2) *L. acidophilus*; (3 and 4) *L. rhamnosus* GG (duplicate); (5) Negative control (un-cultivated MRS broth).

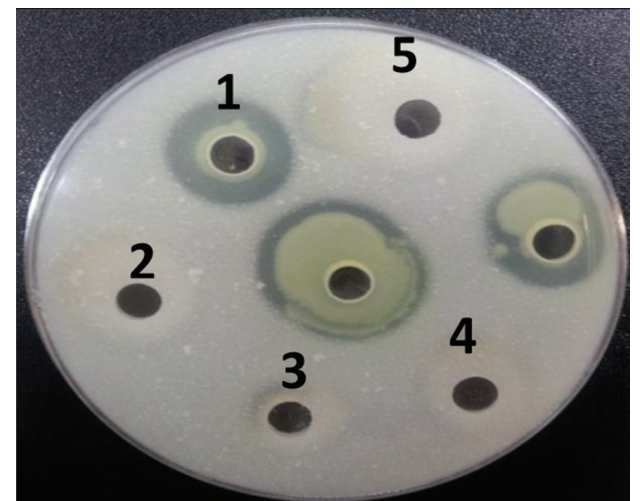


Fig. 5 Inhibitory effect of crude bacteriocin produced by *Lactobacilli* on protease production from *Ps. aeruginosa*. (1) Positive control (*P. aeruginosa*, isolate-19); (2) Negative control (un-cultivated Brain Heart broth); (3) *P. aeruginosa* isolate-11 treated with crude bacteriocin from *L. rhamnosus*; (4) *P. aeruginosa* isolate-19 treated with crude bacteriocin from *L. rhamnosus*; (5) *P. aeruginosa* isolate-19 treated with crude bacteriocin from *L. acidophilus*.

Paluszak et al. (2007).<sup>19</sup> The antimicrobial effect of bacterial suspension using well diffusion method showed more clearly inhibitory zone in diameters, the competitive exclusion between the pathogenic bacteria as well as to the presence of other secondary metabolites by *Lactobacilli spp.* such as the lactic acid, biosurfactant, and other fermentation product as well as bacteriocin may play a major role.<sup>25</sup>

The highest inhibitory zone was recorded using free cell supernatant which have remarkable potential for their antimicrobial activities which comes in compatible with a study by Daba and Saidi (2015) which study the inhibitory activity of bacteriocin producing lactic acid bacteria (LAB) that isolated from raw milk against *P. aeruginosa* and *Escherichia coli* using free cell supernatant and cell diffusion method.<sup>26</sup> Wala'a and Nibras (2013) found that bacteriocins from *L. acidophilus* exhibited activity against *Serratia marcescens* and that bacteriocin of *L. acidophilus* was stable at pH 4, 7 half of its activity was lost at pH 8 and whole activity was lost at other pH values.<sup>27</sup> Gho and Philip (2015) focus on isolate and purify the bacteriocin of *Weissella confusa* A3 from cow milk was shown to have inhibitory activity towards pathogenic bacteria as *Bacillus cereus*, *E. coli*, *P. aeruginosa* and *Micrococcus luteus*. The bacteriocin was shown to be heat stable and functioned well at low pH (2 to 6). Reduction of activity was shown after treatment with proteinase K, trypsin and peptidase that confirmed the proteinaceous nature of the compound.<sup>28</sup>

Ismaeel et al. (2013) investigated the biological applications of surlactin derived from *L. acidophilus* using different pathogenic strains and toward to *in vitro* (contact lenses) and *in vivo* (rabbits' eyes). Their results demonstrated the capability of surlactin to inhibit the adhesion of pathogens up to 60% without any antibacterial activity against *Staphylococcus aureus* using well diffusion method. The surlactin proved to be effective for treating the infection in rabbits' eyes with *P. aeruginosa* and that infection with *P. aeruginosa* (administrated to rabbits' eyes) can be prevented by using surlactin.<sup>29</sup> A study by Zho et al., (2015) revealed that bacteriocin from *L. acidophilus* XH1 inhibited *E. coli*, *S. aureus* and *B. anthracis*. It showed a wide range of antimicrobial activity at pH 1.0–5.0 while at 37–120°C, it was sensitive to trypsin, pepsin and papain, but insensitive to proteinase K and neutral protease.<sup>30</sup>

Many antibiotics, antimicrobial agents do possess protein as one of the major functioning fractions of the entire molecule.

The proteinaceous nature or peptides do contribute toward antimicrobial activity and have tremendous potential for treating and/or preventing the infectious diseases. Risk of microbial resistance can be reduced certainly with the help of such proteinaceous molecules. Protein rich with and without polysaccharide, phosphate fractions in cell-bound or cell-associated biosurfactant originated from *Lactobacillus spp.* have undoubtedly fulfilled this expectation proving to combat pathogens. Brzozowski et al. (2011) reported biosurfactant production by *L. fermenti* 126 and *L. rhamnosus* CCM 1825 having proteinaceous biosurfactant with an existence of polysaccharide and phosphates biosurfactant obtained *L. rhamnosus* CCM 1825 possessed more proteins and phosphates as compared with *L. fermenti* 126.<sup>31</sup>

Antimicrobial effect of bacteriocin production may contribute to probiotic functionality through three different mechanisms: firstly, as colonising peptides; secondly, bacteriocins function through direct inhibition of the growth of pathogens; and finally, bacteriocins may serve as signalling peptides/quorum-sensing molecules in the intestinal environment.<sup>32</sup> Bacteriocin can interfere with the bacterial cell wall enzyme production leading to inhibit their virulence factors such as potency of protease production.<sup>33</sup>

## Conclusions

In an effort to establish a new antimicrobial agent from lactic acid bacteria, novel strains capable of utilizing cheaper, renewable substrates, greater yields, and novel applications, which may act as bacteriostatic or bactericidal agents. Using probiotic bacteria toward therapeutic approaches can be highly significant in preventing and/or dealing with hospital-acquired infections may be an important undertaking.

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## Conflict of Interest

The authors declare no conflicts of interest. ■

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