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The inhibitory effect of bacteriocin produced by *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus plantarum* ATCC 8014 on planktonic cells and biofilms of *Serratia marcescens*

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Background/aim: The spread of antibiotic-resistant pathogens has resulted in the need for new treatments. The aim of the present study is to investigate the effect of bacteriocin from *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus plantarum* ATCC 8014 on planktonic and biofilm forms of *Serratia marcescens* strains.

Materials and methods: The direct antagonism of the *L. plantarum* and *L. acidophilus* cell-free supernatant on *S. marcescens* cultures was determined using an optical density assay. The bacteriocin was partial purified by ammonium sulfate precipitation. Its molecular weight was analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The effect of bacteriocins on the biofilm of *S. marcescens* strains was then determined with 2,3,5-triphenyl tetrazolium chloride.

Results: The purified bacteriocin from *L. plantarum* ATCC 8014 and partially purified bacteriocin from *L. acidophilus* ATCC 4356 displayed noticeable inhibitory activity against planktonic and biofilm forms of *S. marcescens* strains. SDS-PAGE analysis revealed that the apparent molecular weight of bacteriocin from *L. planetarium* was 63 kDa, and that of bacteriocin from *L. acidophilus* was 68 or 48 kDa.

Conclusion: The bacteriocins could be effective compounds to control surface-attached pathogenic bacteria and can be used as therapeutic agents after acceptable in vivo experimentation.

Key words: Bacteriocin, biofilm, probiotic, antibiotic alternatives, *Serratia marcescens*

1. Introduction

Biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix that adheres to biotic and abiotic surfaces (1). The biofilm structure increases the survivability of bacteria by 1000 times when they are exposed to high concentrations of antimicrobial agents, such as antibiotics, when compared with the planktonic form (2). The limitation of the penetration of antimicrobial compounds into the biofilm structure is one of the most important reasons for the high resistance of bacteria in biofilm (3,4). This characteristic is medically important because it results in the contamination of hospital equipment and medical implants (5,6). According to a report by the National Institutes of Health, nearly 70% of microbial infections are associated with a bacterial biofilm (5). Therefore, the killing and removal of biofilm cells are the main targets of infection control strategies (7).

Serratia marcescens, a gram-negative opportunistic human pathogen classified as a member of the Enterobacteriaceae, has been recognized as the causative organism for urinary tract infections, wound infections, and nosocomial infections, including pneumonia, septicemia, and meningitis (8). One of the main problems associated with *S. marcescens* infection is the increase in its resistance against a great number of antibiotics (9). Many bacteria gain the ability to tolerate antibiotics by forming biofilms, and treatment of biofilm-related bacterial infections is far more difficult (10). Therefore, it is essential to find a new therapeutic approach for the treatment of *S. marcescens* biofilm-related infections without using antibiotics. Bacteriocins, which are proteinaceous compounds with bacteriostatic or bactericidal activity against other bacteria, are appropriate substitutes for traditional antibiotics (11). Among them, bacteriocins produced by lactic acid bacteria have attracted increasing attention, because they are active in a nanomolar range

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and have no toxicity (12). The present study examined the antimicrobial effect of bacteriocin-like substance (BLS) derived from *Lactobacillus* strains on the planktonic and biofilm forms of *S. marcescens*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Lactobacillus plantarum (ATCC 8014), *Lactobacillus acidophilus* (ATCC 4356), *Serratia marcescens* (ATCC 13880), and *Serratia marcescens* (ATCC 19180) were purchased from Iranian Research Organization for Science and Technology. The *Lactobacillus* bacteria were grown in Man-Rogosa-Sharpe broth (MRSB; Merck, Darmstadt, Germany) and incubated at 37 °C in an anaerobic jar for 24 h and maintained on MRS agar plates (MRSA; Merck). *S. marcescens* strains were grown in nutrient broth (NB; Merck) and incubated at 37 °C for 24 h.

2.2. Cell-free culture supernatant preparation

L. acidophilus ATCC 4356 and *L. plantarum* ATCC 8014 were incubated in MRS broth at 37 °C for 18 h and 24 h, respectively. Cell-free supernatant (CFS) was obtained from each bacterial culture broth by centrifugation at 7000 × *g* for 10 min at 4 °C. The supernatant was then sterilized by filtration through a 0.2-µm syringe filter (Millipore, Bedford, MA, USA) (13,14).

2.3. Screening antibacterial properties

The inhibitory effect of supernatant on *S. marcescens* strains was examined by microscale optical density assay (MODA). To each well of a 96-well plate, 100 µL of diluted (1:10,000 in NB) test culture was added. This was done to 5 wells for each test culture; to the first well, nothing was added (no supernatant or media); to the second, 15 µL of the CFS was added; the remaining CFS was adjusted to pH 6.0 with 10 N NaOH in order to rule out possible inhibition effects due to organic acids, and then 15 µL of the pH-adjusted CFS was filtered and added to the third well. The neutralized CFS was then treated with 5 mg mL^{-1} catalase (Sigma) at 25 °C for 1 h to eliminate the possible inhibitory effect of H_2O_2 and filtered, then loaded into the fourth well. To confirm the production of a proteinaceous compound, CFS with antimicrobial effect after acid neutralization and H_2O_2 elimination was treated with 1 mg mL^{-1} of proteolytic enzymes including pepsin and trypsin (Sigma) at 37 °C for 2 h and added to the fifth well. For all wells MRS broth was applied in similar conditions to the CFS as a control. Each series was run in duplicate on the same plate. The plate was then incubated at 37 °C for 24 h. After incubation, the absorbance was read by a microplate reader at 600 nm. The difference in absorbance between the control and samples was used to report antibacterial activity (15).

2.4. Partial purification of bacteriocin

An amount of 100 mL of CFS obtained from *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 using the MODA method to show that the antimicrobial effect is related to the protein compound was salted out respectively in 70% and 90% saturated ammonium sulfate and then the precipitate was collected by centrifugation at 11,000 × *g* for 45 min at 4 °C. The resultant pellets from *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 were dissolved in 2 mL of Tris-HCl buffer (10 mM, pH 7.4) and then the suspension of *L. plantarum* ATCC 8014 was dialyzed against Tris-Mg_2SO_4 buffer (5–10 mM, pH 7.4) using dialysis tubing (12,000 kDa; Sigma) for 48 h (16).

2.5. Determination of protein concentration

Protein concentration of the bacteriocin in the supernatant was determined by the Bradford method using bovine serum albumin as the standard protein (17).

2.6. Molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The extracted proteins from *L. plantarum* ATCC 8014 and *L. acidophilus* ATCC 4356 were analyzed using SDS-PAGE. A 14.4-µg sample of *L. acidophilus* ATCC 4356 and a 19.2-µg sample of *L. plantarum* ATCC 8014 (sample buffer 5X) were loaded. The electrophoresis was run at 120 V/cm for 4 h. The molecular weight of the protein(s) was determined by comparison with the protein molecular size marker (prestained protein ladder, 11–180 kDa, CinaClon, Tehran, Iran). After electrophoresis, the proteins were visualized using the Coomassie Blue R250 staining method (18).

2.7. Determination of minimum inhibitory concentration (MIC)

The *S. marcescens* strains were precultured in Mueller Hinton broth (MHB; Merck) medium at 37 °C overnight, inoculated into fresh MHB medium, and incubated until an optical density (OD) of 0.05 at 595 nm was attained. Next, 20 µL of 2-fold dilutions (MIC range: 0.12–0.007 mg/mL for *L. acidophilus* ATCC 4356 and 0.16–0.01 mg/mL for *L. plantarum* ATCC 8014) of the desired peptides was added to wells of a 96-well flat-bottomed polystyrene plate (JET BIOFIL, Guangzhou, China) containing 80 µL of bacterial culture. The microtiter plate was then incubated at 37 °C for 18 h with shaking. Finally, absorbance at 595 nm was read using a microplate reader. The MICs were defined as the lowest concentration of peptide that completely inhibited the growth (19).

2.8. Inhibitory effect of bacteriocin against planktonic state

The *S. marcescens* strain, precultured in MHB medium at 37 °C overnight, was inoculated into fresh MHB medium and incubated until an OD of 0.136 at 595 nm was obtained (equal to 0.5 McFarland). Then 100 µL of the peptide

solution associated with *L. plantarum* with a concentration of 0.8 mg/mL and *L. acidophilus* peptide solution with a concentration of 0.6 mg/mL was added to the wells of 96-well plates containing 100 µL of bacterial suspensions. In a control well, 100 µL of sterile phosphate buffer saline (PBS) was added to the bacterial suspension and incubated at 37 °C with shaking. Ultimately, absorbance was read at 595 nm using a microplate reader (20,21).

2.9. Inhibitory effect of bacteriocin against biofilm

The *S. marcescens* strain was grown in Minimal Broth Davis (MBD; Sigma-Aldrich, St. Louis, MO, USA) medium supplemented with 0.2% (w/v) glucose and 0.5% (w/v) casamino acid at 37 °C overnight, and then it was diluted 1:100 with fresh MBD medium and 200 µL of the bacterial suspension was added to a 96-well flat-bottomed polystyrene plate and incubated at 37 °C for 24 h. The wells containing sterile MBD and bacterial suspension without treatment were used respectively as the blank and control. After biofilm formation, the medium was aspirated, and then the plate was washed 3 times with 200 µL of PBS to remove unbound cells and 100 µL of the peptide solution was added to each well and kept at room temperature for 1 h. The solution of peptides was refreshed during the 1-h treatment due to the high density of cells in the biofilms and the elimination of the effect of low volumes (100 µL) of the peptides. The same conditions were maintained for control wells and PBS was used for refreshing instead of peptide solution as the negative control. After removal of the wells' contents, biofilms were washed with PBS 3 times and the walls were stained with 2,3,5-triphenyl tetrazolium chloride (TTC) 2% w/v (Sigma) for 2 h. TTC staining was used to measure the active metabolism and respiration of bacterial cells that survived the treatment with peptide solution. The wells were then rinsed and filled with 33% glacial acetic acid (v/v). After 15 min of incubation, the plates were vigorously shaken and the absorbance was read at 450 nm (22–25).

2.10. Scanning electron microscope (SEM) observation

Glass slides of 2 cm in diameter were immersed in 12-well flat-bottomed polystyrene plates containing 5 mL of MBD supplemented with 0.2% (w/v) glucose and 0.5% (w/v) casamino acid for 24 h. A total of 100 µL of a 10^8 CFU mL^{-1} overnight culture was then added and the plates were incubated for 24 h in a shaking incubator. After incubation, the glass slides were removed and rinsed with sterile PBS to remove nonattached cells and resuspended in peptide solution from *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014. Over the course of 1 h the peptide solution was replaced 3 times, and then the glass slides were washed 3 times with PBS and fixed with 2.5% (v/v) glutaraldehyde for 2 h at 4 °C, then washed twice with PBS and dehydrated for 10 min using a gradient ethanol series of 30%, 50%, 70%, 90%, and 100% (v/v). The samples were then dried prior to coating with gold and studied using a SEM (VEGA, TESCAN, Brno, Czech Republic) (26).

2.11. Statistical analysis

The experiments were conducted in triplicate. The statistical analysis was conducted using SPSS 20. Significance levels were set at $P < 0.05$. After assumptions of normality and variances of homogeneity were checked, one-way analysis of variance (ANOVA) and independent samples t-tests were performed.

3. Results

3.1. The inhibitory effect of *L. plantarum* and *L. acidophilus* supernatant measured by MODA

The inhibitory effect of CFS of *Lactobacillus plantarum* and *L. acidophilus* on *S. marcescens* could be attributed to the presence of BLS. The data presented in Figure 1 show the inhibitory activity of the *L. plantarum* and *L. acidophilus* supernatants measured by MODA. Comparison of the nontreated supernatant of both the strains with that of the control (MRS medium) revealed an inhibitory effect of the supernatant from both the strains on *S. marcescens* ATCC 13880 and *S. marcescens* ATCC 19180 ($P < 0.05$ and $P < 0.05$, respectively) (Figures 1a and 1b). Subsequently, the supernatant of both the strains of *Lactobacillus* was neutralized with 10 N NaOH to remove the organic acids ($P < 0.05$ and $P < 0.05$, respectively), treated with catalase to eliminate the possible effect of H_2O_2 ($P < 0.05$ and $P < 0.05$, respectively), and compared with the control (Figures 1a and 1b). The results indicated that organic acids and H_2O_2 had no effect on the antimicrobial activity of the supernatant. However, digestion with proteolytic enzymes (pepsin and trypsin) resulted in the loss of antimicrobial activity of the supernatant of both the strains of *Lactobacillus* on *S. marcescens*, indicating the protein nature of the produced antimicrobial substance ($P > 0.05$) (Figures 1a and 1b).

3.2. Molecular weight of protein produced by *L. plantarum* and *L. acidophilus* supernatant

The dialyzed supernatants of *L. plantarum* and *L. acidophilus* were analyzed using SDS-PAGE, and the results are shown in Figure 2. The *L. acidophilus* gel showed 2 bands of 48 and 68 kDa (Figure 2a), whereas the *L. plantarum* gel showed a specific band of about 63 kDa (Figure 2b).

3.3. Determination of MICs

The MICs of proteins from the *L. acidophilus* and *L. plantarum* supernatants against *S. marcescens* strains were determined using a microliquid dilution method and the results are shown in the Table. The highest MIC was associated with proteins from *L. acidophilus* against *S. marcescens* ATCC 13880 (>0.12 mg/mL).

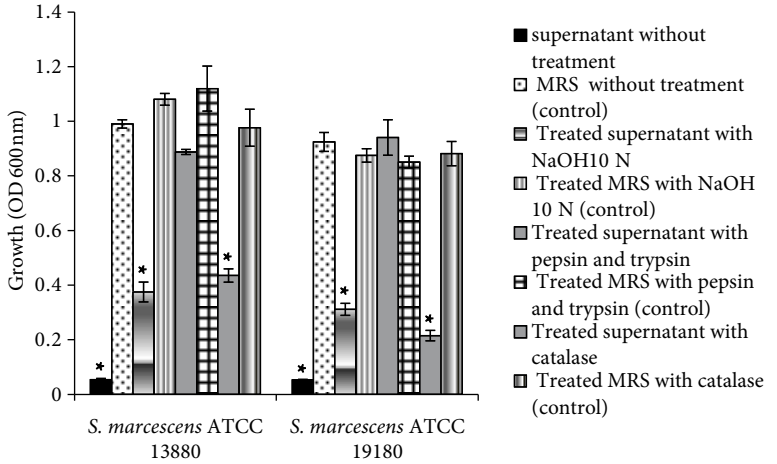

Figure 1a. MODA of cell-free supernatant from *L. acidophilus* ATCC 4356 on *S. marcescens* strains' growth. Data represent the mean and standard deviation (±SD) of 3 different experiments performed in triplicate. *P < 0.05 (Student's t-test).

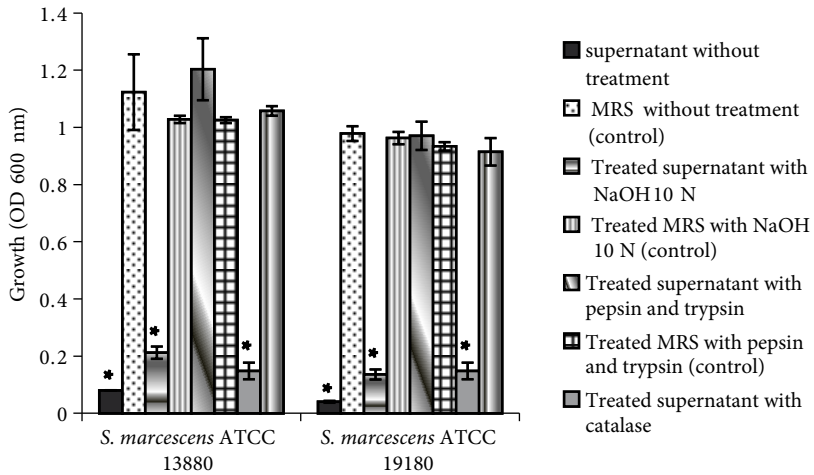

Figure 1b. MODA of cell-free supernatant from *L. plantarum* ATCC 8014 on *S. marcescens* strains growth. Data represent the mean and standard deviation (±SD) of 3 different experiments performed in triplicate. *P < 0.05 (Student's t-test).

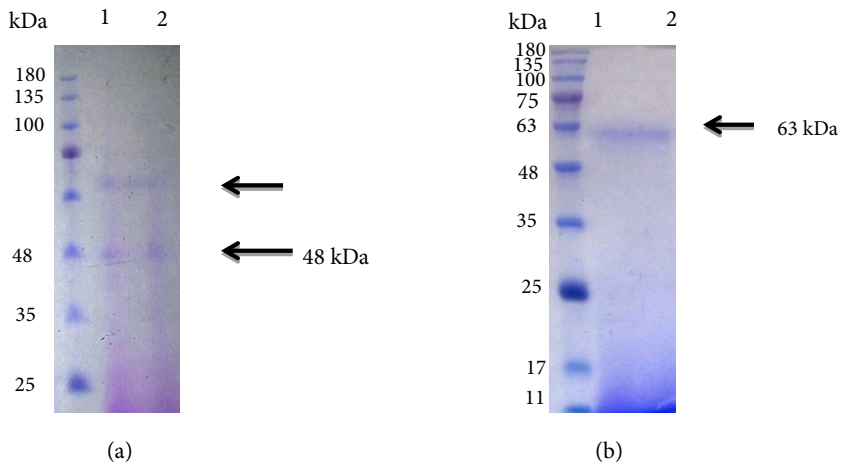

Figure 2. SDS-PAGE of BLS from *L. acidophilus* ATCC 4356 (a) and *L. plantarum* ATCC 8014 (b). a: Lane 1: Protein molecular size marker (prestained protein ladder); Lane 2: Protein bands stained with Coomassie Blue R250 (70% ammonium sulfate-saturated). b: Lane 1: Protein molecular size marker; Lane 2: Protein bands stained with Coomassie Blue R250 (90% ammonium sulfate-saturated).

Table. Minimum inhibitory concentrations (MICs) of antimicrobial peptides (mg/mL).

Bacterial strain	MIC of antimicrobial peptide (v/v)	
	Bacteriocin produced by *L. acidophilus* ATCC 4356 (0.12–0.007 mg/mL)	Bacteriocin produced by *L. plantarum* ATCC 8014 (0.1–-0.01 mg/mL)
S. marcescens ATCC 13880	>0.12	0.08
S. marcescens ATCC 19180	0.12	0.08

3.4. Inhibitory effect of bacteriocins against planktonic cells of *S. marcescens* strains

Assessment of the antimicrobial activity of bacteriocin produced by the 2 *Lactobacillus* strains against *S. marcescens* strains suggested that the BLS obtained from both the strains could inhibit the planktonic cells of *S. marcescens* (Figure 3). In particular, when compared with the control mode (PBS), the BLS derived from *L. acidophilus* ATCC 4356 showed a statistically significant inhibitory effect on the planktonic cells of *S. marcescens* ATCC 13880 and *S. marcescens* ATCC 19180 ($P < 0.05$ and $P < 0.05$, respectively). Similarly, the protein compounds obtained from *L. plantarum* ATCC 8014 were also capable of inhibiting the planktonic cells of *S. marcescens* ATCC 13880 and *S. marcescens* ATCC 19180 ($P < 0.05$ and $P < 0.05$, respectively).

3.5. Inhibitory effect of bacteriocins against *S. marcescens* strains' biofilm and analysis by SEM

After treating the 24-h *S. marcescens* biofilms with BLS produced by *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014, the biofilms were stained with TTC dye, which stains only living cells. The results indicated that 1 h of treatment with 3 replacements of the peptide solution could inhibit biofilm formation by both the strains of *S. marcescens* (Figure 4). When compared with the control, bacteriocin obtained from *L. acidophilus* ATCC 4356 showed a significant inhibitory effect on the biofilm of *S. marcescens* ATCC 13880 and *S. marcescens* ATCC 19180 ($P < 0.05$ and $P < 0.05$, respectively) (Figure 4). Furthermore, bacteriocin from *L. plantarum* ATCC 8014 exhibited a significant reduction in the absorbance on the biofilm of *S. marcescens* ATCC 13880 and *S. marcescens* ATCC 19180 when compared with the control ($P < 0.05$ and $P < 0.05$, respectively) (Figure 4). SEM images demonstrating the effect of BLS produced by *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 on *S. marcescens* strains' biofilm and results from these experiments are summarized in Figures 5a–5f. Figures 5a and 5d show the 24-h biofilm of *S. marcescens* ATCC 19180 and *S. marcescens* ATCC 13880, respectively, as a control. The 24-h biofilms of *S. marcescens* ATCC 19180 and *S. marcescens* ATCC 13880 incubated with exposure to BLS from *L. acidophilus* ATCC 4356 are shown in Figures 5b and 5e, respectively. Figures 5f and 5c show the 24-h biofilms of *S. marcescens*

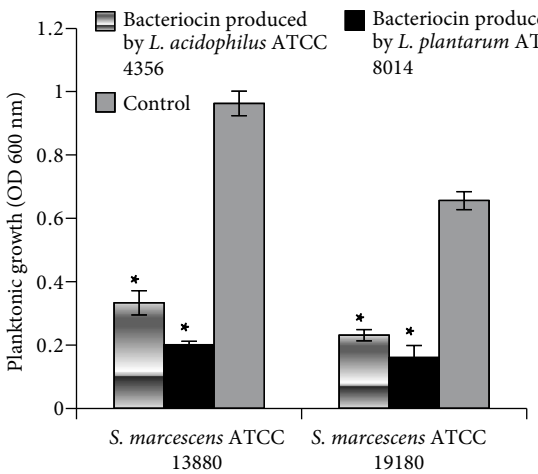

Figure 3. The inhibitory effect of BLS from *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 on planktonic cells of *S. marcescens* strains. Data represent the mean and standard deviation (±SD) of 3 different experiments performed in triplicate. *$P < 0.05$ (Student's t-test).

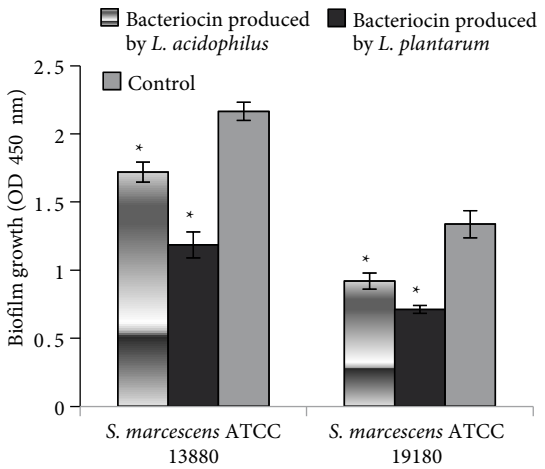

Figure 4. Inhibitory effect of BLS from *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 on *S. marcescens* strains' biofilm. Data represent the mean and standard deviation (±SD) of 3 different experiments performed in triplicate. *$P < 0.05$ (Student's t-test).

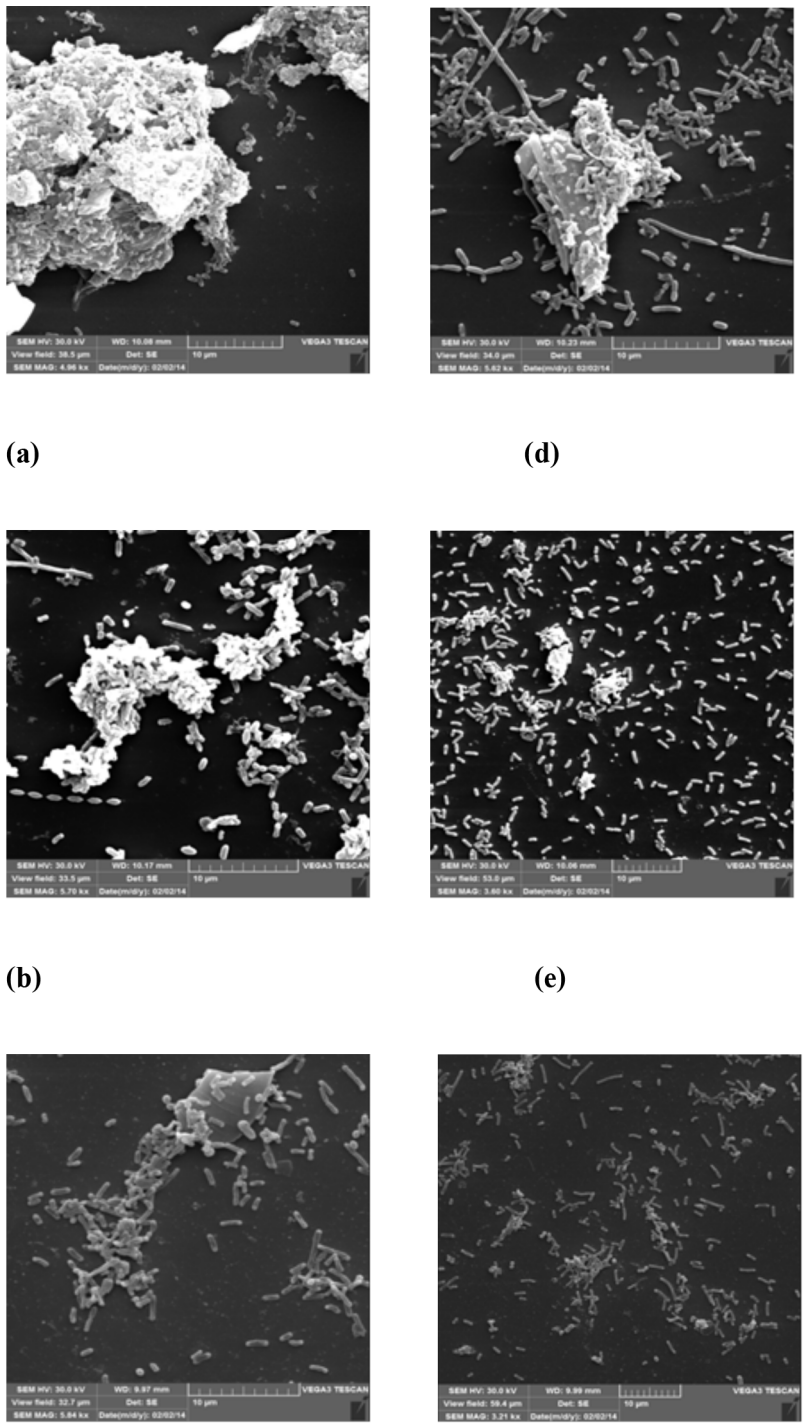

Figure 5. Figures a and d show the 24-h biofilms of *S. marcescens* ATCC 19180 and *S. marcescens* ATCC 13880, respectively, formed on glass slides in MBD enriched with 0.2% glucose and 0.5% casamino acid as a control. Figures b and e show the 24-h biofilms of *S. marcescens* ATCC 19180 and *S. marcescens* ATCC 13880, respectively, incubated with exposure to BLS from *L. acidophilus* ATCC 4356 with 3 replacements during 1 h. Figures f and c show the 24-h biofilms of *S. marcescens* ATCC 13880 and *S. marcescens* ATCC 19180, respectively, incubated with exposure to BLS from *L. plantarum* ATCC 8014 with 3 replacements during 1 h (scale bar = 10 μm).

ATCC 13880 and *S. marcescens* ATCC 19180, respectively, incubated with exposure to BLS from *L. plantarum* ATCC 8014.

4. Discussion

Nowadays, biofilm infections represent a serious global health menace, typically due to the appearance of antibiotic-resistant strains. As biofilm-associated infections are often chronic, their successful prevention and treatment are important topics. The widespread use of antibiotics in the treatment of infections has resulted in increased numbers of antibiotic-resistant bacteria and fewer treatment options, rendering most antibiotics useless (27). Applications of probiotics and their antimicrobial metabolites such as bacteriocins act as alternative antimicrobial strategies in the treatment and prevention of infections (28). In the present study, we investigated the effects of *Lactobacillus* bacteriocins against planktonic cells and biofilms of *S. marcescens* strains. The results showed that the BLS produced by both the strains of *Lactobacillus* played an important role in the antiplanktonic and antibiofilm activity. Although the sensitivity of gram-negative bacteria to bacteriocins produced by lactic acid bacteria is not common (29), *L. acidophilus* and *L. plantarum* have already been reported to produce bacteriocins that are effective against pathogenic bacteria (13,14,30–34).

The *L. plantarum* and *L. acidophilus* supernatants exerted an inhibitory effect on *S. marcescens*. The findings showed that untreated CFS inhibited both the strains of *S. marcescens*, while the neutralized and catalase-treated supernatants of *L. acidophilus* and *L. plantarum* exhibited inhibitory activity against the test bacteria (Figures 1a and 1b). Furthermore, digestion by pepsin and trypsin also had a clear effect on the inhibitory activity of the CFS (Figure 1a and 1b). Upon treatment with proteolytic enzymes, the antibacterial activity against the strains tested disappeared. The susceptibility of the inhibitory compound to digestion by proteases also supported its categorization as bacteriocin. The molecular weight of the *L. plantarum* bacteriocin was estimated to be 63 kDa and that of the *L. acidophilus* bacteriocin was 48 or 68 kDa (Figures 2a and 2b). Similar results have also been reported by Aslim et al. (35), Lauková (36), and Lash et al. (13), who found that the BLS produced by *L. acidophilus* and *L. plantarum* could be antagonistic to numerous gram-negative and gram-positive pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *S. marcescens*, and others (13,35,36).

First, the growth inhibitory effect of BLS was determined on the planktonic cells of *S. marcescens* strains. The BLS obtained from *L. plantarum* ATCC 8014 and *L. acidophilus* ATCC 4356 had a strong inhibitory effect on the planktonic form of *S. marcescens* strains examined (Figure 3). This inhibitory activity was also reported by Lash et al. (13). In addition, the *L. plantarum* and *L. acidophilus* supernatants showed a significant inhibitory influence on gram-negative bacteria, which is an interesting and rare result, because the inhibitory effects of gram-positive strains on gram-negative bacteria are less widespread (14,29,37,38). This inhibition could be partly due to the augmented sensitivity of MODA, which examines the antibacterial activity on the basis of direct antagonism between the *L. plantarum* and *L. acidophilus* supernatants and the test culture in the liquid medium. As such, the inhibitory activity was not affected by the diffusion grade of the composition of the agar medium as in other methodologies.

The different cell wall susceptibilities among the bacteria may be the main cause for the variation in the results. According to a previous study (14), gram-positive bacteria are frequently found to be more susceptible to BLS than gram-negative bacteria. It is well known that the outer membrane present only in gram-negative bacteria plays a significant role as an effective barrier. However, in the present study, the outstanding sensitivity of *S. marcescens* strains towards BLS may possibly be due to membrane penetrability. Similar results regarding the effect of BLS of *L. plantarum* and *L. acidophilus* on the outer membrane permeability in gram-negative bacteria were reported in earlier studies (15,35–36). The findings of the present study revealed that BLS produced from *Lactobacillus* strains showed antibiofilm activity against *S. marcescens* strains (Figures 4 and 5). In contrast to the growth inhibitory activity against planktonic cells, the biofilms of *S. marcescens* strains were less susceptible to BLS because, for inhibiting the biofilm, a peptide solution acting against biofilm should be replaced every 20 min.

Bacteria existing as a biofilm are often more difficult to eliminate when compared with the planktonic form. In addition, bacteria also use quorum sensing (QS) to coordinate the formation of biofilms. QS is a cell–cell signaling mechanism, which is often connected to the establishment of complex communities of bacteria. It has been reported that QS between the bacterial populations leads to the expansion of the biofilms (39). The opportunistic pathogen *S. marcescens* uses QS and nutrient cues to organize the development of biofilms (40). Ramose et al. stated that the *Lactobacillus* supernatants diminished the quorum signals (acyl-homoserin-lactones) produced by *P. aeruginosa* (41). The eradication of *S. marcescens* biofilm in the present study could possibly suggest that BLS has QS inhibitory activity. Another possible explanation for the damage to the biofilms could be the effect of bacteriocins on the cellular membrane instability and penetrability via formation of complex or ionic canals through binding to the receiving particles such as lipids or proteins, leading to the dispersion and loss of ability to form proton driving force (32).

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