

Antibiofilm potential of *Lactobacillus plantarum* spp. cell free supernatant (CFS) against multidrug resistant bacterial pathogens

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Abstract

Biofilm formation is a major determinant factor in development of bacterial infections. In addition, bacteria embedded in a biofilm are more resistant to antimicrobials and thus the ability of bacteria to persist and grow in a biofilm seems to be the major factor for pathogenesis and therapeutic failure. In the current study, a *Lactobacillus plantarum* spp was isolated from Siahmazgi cheese, traditional cheese of Guilan province, Iran, and was identified using morphological, biochemical and molecular identification assays. Antibiofilm potential of the *Lactobacillus plantarum* spp cell free supernatant (CFS) against multidrug resistance *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* was characterized. According to the results, the CFS not only reduced biofilm formation by pathogenic bacteria, but also disrupted preformed biofilms. The CFS remained unaffected by chemicals including EDTA, SDS and Tween 80, and showed stability at high temperatures (80 and 100 °C), as well as a wide range of pH. However, the antibiofilm activity was inhibited after treating with proteinase K. According to these results, *L. plantarum* spp could be regarded as a suitable strain to produce antibiofilm agents which could be used for preventive and therapeutic approaches.

Keywords: Antibiofilm, multidrug resistance, lactic acid bacteria, probiotics

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Introduction

Traditional dairy products have been considered as good sources of lactic acid bacteria (LAB) and ideal vehicles to deliver beneficial bacteria to human gastrointestinal tract. LAB are good candidates to be used as probiotics due to their non-pathogenic nature, good antimicrobial activity and resistance to gastrointestinal condition (1).

Multi-drug resistant bacteria are involved with several community acquired and nosocomial infections as well as foodborne diseases. Biofilm formation is regarded as a major determinant factor in development of infection by pathogenic bacteria (2). Biofilm is a community of microorganisms adhering to each other on a surface which is surrounded by a matrix of extracellular polymers. Bacteria embedded in a biofilm are more resistant to antimicrobials due to the reduction of antimicrobial penetration, slower bacterial metabolic state as well as easier exchange of resistance genes among cells (3). The resistant nature of biofilm is a matter of great concern for global health care system and many studies have been conducted to explore novel, natural and effective antibiofilm agents.

Fermented milk products contain different types of LAB which could be able to inhibit many pathogenic and spoilage microorganisms. Antagonism is attributed to the release of a variety of functionally active compounds including organic acids, exopolysaccharides and bacteriocins which are naturally released into the bacterial growth medium. These compounds have antibacterial and antibiofilm potentials which are able to inhibit bacterial infectivity. Several studies have reported antibiofilm potential of bacteriocines produced by LAB bacteria (4-5). Moreover, some studies reported that a number of exopolysaccharides isolated from commercial fermented milk were capable of interfering with the adhesion of several enteric pathogens (6-7).

The LAB from traditional cheese may produce bioactive compounds with antibiofilm activity and could be used for prevention of bacterial infections and food spoilage (4). However, the stability of these bioactive compounds under unfavorable conditions needs to be characterized. Thus, the current work was conducted to investigate and characterize antibiofilm potential of the cell free supernatant (CFS) of *Lactobacillus plantarum* spp, isolated from Siahmazgi cheese, a traditional cheese from province

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of Guilan, Iran, against multi-drug resistant *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, isolated from clinical infections.

Materials and methods

Microbial strains and culture conditions

Pathogenic bacteria including *S. aureus*, *E. coli* and *P. aeruginosa* were isolated from clinical specimens including urine, blood and respiratory secretions. Bacterial isolation was performed between February to June, 2017, from patients referring to the clinical laboratories of Rasht city, Iran. The patients had an age range of 3-68 year old. Bacterial identification was performed using biochemical assays. At first, Gram staining was performed and then, differential assays including coagulase, catalase, growth in manitol-salt agar and etc., for Gram positive isolates, and Motility, Indole and H₂S production, MR-VP, citrate utilization, sugar fermentation in TSI medium and oxidase assays, for Gram negative isolates were performed. All the isolates were stored at -70 °C in Tryptic Soy broth (TSB) (QUELAB/UK) containing 20% glycerol for subsequent analysis.

Antibiotic resistance profile of the isolates were determined using disk diffusion method and each strain which showed resistance to at least three antimicrobial classes was regarded as multi-drug resistant strain (8).

L. plantarum spp was isolated from Siahmazgi cheese and identified using biochemical and molecular assays. Briefly, cheese samples were collected aseptically, stored at 4 °C and transferred immediately to the laboratory. The samples were mixed and homogenized in 0.85% sterile NaCl solution (1:10 w/v) on a shaker (600 rpm) for ten minutes. Homogenized samples were diluted serially and cultured on MRS agar (Merck GmbH, Germany). After incubation at 37 °C for 48 h, the Gram positive, Catalase negative, non-motile rods were purified. Molecular identification of *L. plantarum spp* was performed by amplification and sequencing of the 16S rRNA gene of the isolate using the universal primers of 27 F: 5'-AGAGTTTGATCATGGCTCAG-3' and 1492 R: 5'-TACGGTTACCTTGTTACGACTT-3' (9). Then, *L. plantarum spp* was grown in MRS broth till stationary phase and bacterial cells were removed by centrifugation at 8000 × g for 15 min. Then, the cell free supernatant was filtrated through a 0.45 µm pore size syringe filter (Biofil/China) and stored at 4 °C until use.

In vitro biofilm assay

Biofilm formation potential of microbial pathogen was investigated in 96-well micro-titer plates, according to the method described previously (10). Briefly, optical density of the isolated strains, grown in Luria Broth (LB) medium, was adjusted to 1.0 at 600 nm (OD₆₀₀) and the cultures were diluted 1:100 in fresh LB. Then, 100 µL of the diluted cultures were added to each well and the plates were incubated at 37 °C for 48 h under static condition. Then, the plates were washed three times using sterile phosphate buffered saline (PBS) and 125 µL of 0.1 % crystal violet solution was added to the wells. After incubation for 15 min at room temperature the plates were washed and then left to dry. Finally, 125 µL of 30% acetic acid was added to solubilize the dye and the OD₅₅₀ was recorded using micro-plate reader (Bio-Rad, USA). The OD values were considered as biofilm formation level for each strain. Finally, ten isolates of each pathogen with the highest biofilm formation potential were used for antibiofilm assays.

Anti-adhesive activity of the cell free supernatant

Anti-adhesive potential of the CFS from *L. plantarum spp* against multi-drug resistant pathogens was investigated using the method reported by Dusane et al., (11). Briefly, bacterial pathogens were allowed to adhere for 4h in micro-titer plates and then were incubated with 100 µL of the CFS from *L. plantarum spp* for 2 h at 37 °C. After incubation, the wells were aspirated and washed with PBS and biofilm formation was quantified as described above.

Disruption of preformed biofilms

Biofilms of the pathogenic bacteria were allowed to form in micro-titer plates for 24h. Then, the medium was discarded from each well, rinsed with PBS and 100 µL of the CFS was added to each well. The plates were incubated for a further 24 h at 37 °C. After incubation, the medium was discarded and biofilm formation in each well was quantified using crystal violet method as described above (12).

Determination of physicochemical properties of *L. plantarum* CFS

In order to determine thermal stability, the CFSs was incubated at 25, 37, 50, 75 and 100 °C for 30 min. In addition, stability of CFS at different pH was investigated in

a range between 4 to 10. Lactic acid was used to reduce the pH and a 1 M NaOH solution was used to increase the pH. MRS broth with pH adjusted to different values were used as controls. In another experiment, CFS was prepared and treated with different chemicals (10 mg/mL) including Tween 80, SDS and EDTA for 30 min at 30 °C. Finally, antibiofilm activity of the CFSs treated to different physicochemical conditions against clinical isolates of *P. aeruginosa* was determined and compared with controls.

Enzymatic characterization of antibiofilm potential of CFS
 In order to evaluate enzymatic susceptibility of antibiofilm compounds produced by *L. plantarum*, the CFS was treated with pepsin, proteinase K and lipase at the concentration of 0.5 mg/mL. The tubes were incubated for 4 h at 37 °C and then, antibiofilm activity of the CFSs against clinical isolates of *P. aeruginosa* were determined. MRS broth containing different enzymes with the mentioned concentration were used as controls.

Statistical analysis

The assays were performed in triplicates and the data were statistically evaluated using ANOVA and p values less than 0.05 were considered statistically significant (SPSS software, USA).

Results

Biofilm inhibition of *L. plantarum* CFS

Anti-adhesive potential of *L. plantarum* CFS against bacterial pathogens, which were able to produce strong biofilm, was investigated. According to the results, biofilm formation was significantly reduced when *P. aeruginosa* strains were treated with the CFS while the least antibiofilm activity was observed against *E. coli* strains. The attachment of *P. aeruginosa* strains to microtiter plates was significantly inhibited (71-89%), with the mean value of 76.8%. biofilm formation by *S. aureus* strains was inhibited in a range of 49-66% (mean value = 58.6) and biofilm inhibition of 52.2% was observed when *E. coli* strains were exposed to CFS. Biofilm inhibitory potential of the CFS against bacterial pathogens was presented in Fig 1 a and b.

Disruption of preformed biofilms

In our study, preformed biofilms of all bacterial pathogens were significantly disrupted by *L. plantarum* CFS ($P < 0.05$). However, similar biofilm disruption levels was observed for all bacterial pathogens. Biofilms of *P. aeruginosa*, *S. aureus* and *E. coli* in microtiter plates were disrupted to 46.6%, 41.8% and 37.0%, respectively (Fig. 2).

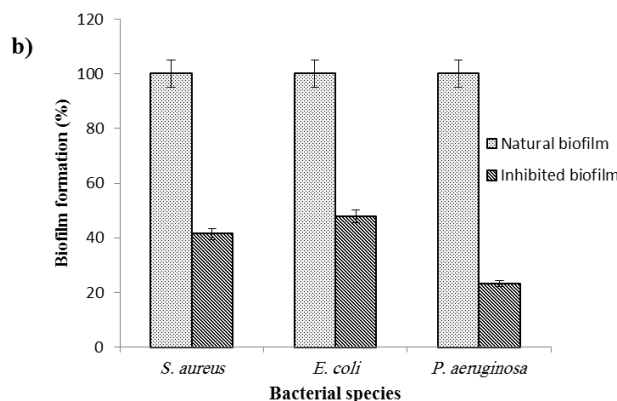


Figure 1 a) Biofilm inhibition by the CFS from *L. plantarum* spp. 1: natural biofilms produced by bacterial pathogens, 2: inhibited biofilms, 3: negative controls. **b)** Effect of the CFS from *L. plantarum* spp on biofilm formation of pathogenic bacteria.

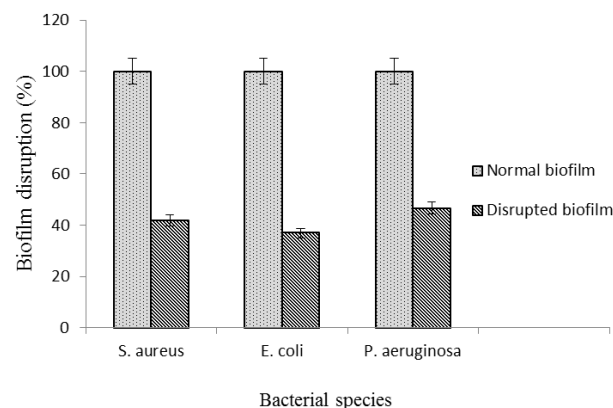


Figure 2 Disruption of preformed biofilms of bacterial pathogens by the CFS from *L. plantarum* spp.

Effect of different physicochemical conditions on antibiofilm activity of CFS

According to the results, *L. plantarum* CFS remained stable after incubation at 30 °C at pH 4.0 and 6.0. However, the antibiofilm activity partially reduced at pH 8.0 and 10.0. In addition, incubation at different temperatures revealed heat stable nature of the CFS. Antibiofilm activity of the CFS against pathogenic bacteria did not reduce significantly when was incubated at 25, 37, 50 and 75 °C. However, a

partial reduction of antibiofilm potential was observed when the CFS was incubated at 100 °C for 30 min (Fig 3 a and b). Moreover, treating the CFS with different chemicals including SDS, tween 80 and EDTA did not significantly affect antibiofilm activity of the CFS.

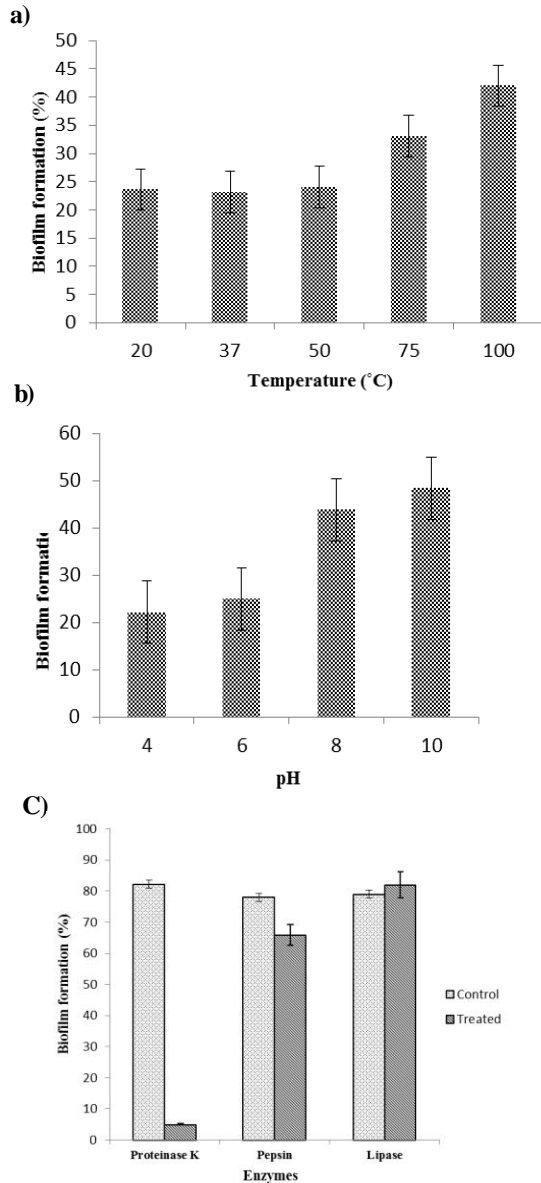


Figure 3 Effect of different physicochemical treatments on antibiofilm activity of CFS from *L. plantarum* spp against pathogenic *P. aeruginosa* strains. a) Temperature, b) pH, c) Chemicals.

Effect of different enzymes on antibiofilm activity of CFS
Incubation of *L. plantarum* CFS with proteinase K for 4 h at 37 °C, totally inhibited antibiofilm potential of the CFS, while incubation with pepsin partially reduced antibiofilm activity to 44.1%, compared to the control. In addition, treating of the CFS with lipase insignificantly affected its antibiofilm activity (Fig 3c).

Discussion

In this study we isolated a *L. plantarum* spp from Siahmezezi cheese, a traditional cheese which is produced in the north of Iran, and determined its antibiofilm potential against multidrug resistance bacterial pathogens.

Occurrence of *L. plantarum* in cheese microbiota appears to be a common trait of a variety of milk cheeses (4). Isolation of *L. plantarum* strains from traditional cheese has been reported previously and have been shown to be able to produce a variety of antimicrobial compounds (13).

Ability to adhere to different surfaces and biofilm formation have been highlighted as important features associated to bacterial pathogens. In addition, biofilm formation is regarded as an important factor associated to the increased antibiotic resistance characteristic of bacterial pathogens. Traditional dairy products have been considered as good sources of LAB and ideal vehicles to deliver probiotic bacteria to human (1). LAB produce a variety of secondary metabolites which have antimicrobial and anticancer activities. The secondary metabolites are released in bacterial growth medium and so, the CFS could exhibit different biological activities. CFS from LAB contain a variety of biologically active compounds including exopolysaccharides and proteins. Our results showed CFS from *L. plantarum* spp strongly inhibited biofilm formed by all bacterial pathogens studied in this work. The highest antibiofilm activity was observed against *P. aeruginosa*, a bacterial pathogen which is involved with a variety of human infections. Several virulent factors are associated with bacterial initial adhesion to surfaces, which is followed by production of extracellular polymeric substances and result in biofilm formation. Antibiofilm activity of *L. plantarum* CFS could be associated to inhibition of both steps. Antibiofilm activity of the released exopolysaccharides derived from bacterial CFS has been reported, previously (6). Although, the molecular mechanism of biofilm inhibition by the CFS was not investigated in our work, it was possible that inhibition of biofilm was mediated by these exopolysaccharides via modification of bacterial cell surfaces and thus prevention of initial attachment and/or acting as signal molecules which down-regulate expression of the genes involved with biofilm formation. Biofilm formation consists of initial bacterial adherence to the surface, via bacterial surface adhesions, followed by multiplication and production of extracellular polymeric matrix. Biofilm inhibitory potential of bacterial CFS could be associated to repression of the genes involved with initial adhesion and chemotaxis. Kim and Kim (6) reported that released exopolysaccharide (r-

EPS) produced from probiotic bacteria reduced biofilm formation of enterohemorrhagic *E. coli* O_{157:H7} via repression of the curl production genes, including *crl*, (2.0-fold), *csgA* (1.8-fold), and *csgB* (1.7-fold,) and chemotaxis gene *cheY* (2.2-fold). Inhibition of *cheY* is also associated with biofilm inhibition in *P. aeruginosa* (14). In addition, functionally active compounds of bacterial CFS could act as signaling molecules which down-regulate expression of the biofilm modulating and/or quorum sensing genes (15). Biofilm formation is regulated by quorum sensing in a variety of bacterial species. Biofilm formation has been reported to be strongly affected by inhibition of quorum sensing signaling pathway in *S. aureus* (16). Thus, inhibition of biofilm in *S. aureus* by *L. plantarum* CFS could be associated with quorum sensing inhibition. However, further investigations needs to be performed to confirm this finding.

Bacterial pathogens exhibited different level of susceptibility to *L. plantarum* CFS. The difference could be associated to the difference in the cell surface characteristics, different signaling pathways as well as diversity of adhesions associated with each pathogen. Biofilm inhibitory potential of local isolates of LAB has been reported. Sadri et al., (17) reported a moderate antibiofilm potential of CFS from *L. casei* and *L. acidophilus* which inhibited adhesion of uropathogenic *E. coli* strains by 46.7 and 25.3%, respectively, which were less than antibiofilm activity of *L. plantarum* strain isolated in our study. The difference could be associated to the different characteristics of both LAB bacteria and microbial pathogens. In addition, the CFS from *L. casei* showed strong biofilm inhibitory potential against *P. aeruginosa* (18) (up to 87%) which was in accordance to our results. Aminnezhad and Kasra-Kermanshahi (18) associated antibiofilm activity of the CFS to the modulation of quorum sensing signaling pathway of bacterial pathogen.

Disruption of preformed biofilm by *L. plantarum* CFS was also investigated. Our results showed that the CFS disrupted bacterial biofilms efficiently. However, there was not a significant difference in the biofilm disruption level among the bacterial pathogens. Disruption of the preformed biofilms are involved with digestion of the extracellular compounds which are produced by biofilm forming cells which cause cell aggregation. It seems that several digestive enzymes are released in the CFS and could be associated with disruption of the preformed biofilm. Similar levels of biofilm disruption against pathogenic bacteria by *L. plantarum* CFS could be associated to the similar nature of polymeric matrix produced by bacterial pathogens. In

addition, lower antibiofilm activity of *L. plantarum* CFS against preformed biofilms compared to the free cells, is evidence that cells in a biofilm are more resistant to antimicrobial agents compared to free-floating cells (19). Physicochemical characterization of *L. plantarum* CFS at different conditions, showed the CFS is stable at a wide range of temperatures and pH. However, the CFS showed better antibiofilm potential at low pH. Accordingly, we suppose the higher antibiofilm activity at low pH could be attributed to the higher solubility of antibiofilm compounds, better folding of proteins and/or enhanced binding and blocking of adhesions of bacterial pathogens. Higher antimicrobial activity of *L. plantarum* CFS at low pH has been reported previously by several authors (20-21). In addition, antibiofilm potential of *L. plantarum* CFS did not significantly reduced when was exposed to high temperatures. Heat stability of the CFS is a good feature which could be used in food industries.

Antibiofilm activity of the CFS showed good stability in presence of different chemicals including EDTA, SDS and tween 80. High stability of the CFS in presence of the mentioned chemicals indicates its stable antibiofilm potential to be used as biopreservatives and pharmaceutical compounds.

Antibiofilm potential of *L. plantarum* CFS was completely inhibited by Proteinase K. This finding has been reported previously for bacteriocines produced by *L. plantarum*, indication the role of bacteriocines in biofilm inhibition of the CFS from *L. plantarum* spp (22). In addition, antibiofilm activity of the CFS was not strongly affected by the lipase which shows the antibiofilm activity is not dependent on the lipid compounds of the CFS.

Conclusion

In this study a *L. plantarum* spp was isolated from traditional cheese of province of Guilan and antibiofilm potential of the *L. plantarum* CFS against clinical isolates of *P. aeruginosa*, *S. aureus* and *E. coli* was investigated. This work showed that traditional dairy products are good sources of beneficial bacterial strains and bioactive compound released by these strains could be employed in food and pharmaceutical industries for prevention of bacterial infections and contamination. However, purification and identification of the functionally active compounds with antibiofilm activity from *L. plantarum* CFS, characterization their functionality at *in vivo* condition and evaluation of the exact molecular mechanisms involved with biofilm inhibition, were the limitations of this study which need to be focused in future works.

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Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contribution

Study design and data interpretation was performed by Dr. H. Zamani and all authors were involved with the experimental assays, data acquisition and drafting of the manuscript.

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