



Evaluation of the Simultaneous Effects of *Lactobacillus delbrueckii* and *L. lactis* on Biofilms of Isolates from Chronic Ulcer Infections with Multiple-drug Resistance

Kobra Tarajian¹, Hossein Fazeli¹, Pezhman Beshkar² and Vajihe Karbasizade^{1,*}

¹Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

²Laboratory Hematology and Blood Banking, Cellular and Molecular Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

*Corresponding author: Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. Email: vajihekarbasizade@gmail.com

Received 2022 April 24; Revised 2022 October 30; Accepted 2022 October 31.

Abstract

Background: Bacterial biofilm is a major barrier to chronic wound healing. Therefore, the prevention of biofilm formation has an effective role in accelerating the healing of these wounds. Today, probiotics' anti-biofilm and antibacterial activity have been proven, and bacteriotherapy by probiotics is a new strategy for treating chronic ulcer infections.

Objectives: The present study aimed to investigate the synergistic effects of *L. delbrueckii* and *L. lactis* on biofilms of bacterial agents isolated from these ulcers in the human plasma biofilm model (hpBIOM).

Methods: This study examined 82 specimens of chronic ulcer biofilms and identified bacterial isolates using phenotypic and molecular methods. After preparing the hpBIOM, 50 μ L of each probiotic (10^9 CFU/mL) was added in two doses separately and simultaneously. After 24 hours, 1 mL of bromelain (0.1 g/mL) was added to the complex and incubated at 37°C for two hours. Then, the surviving bacterial cells were counted by serial dilutions.

Results: Among 119 bacterial isolates, *Staphylococcus aureus* (19%), *Escherichia coli* (17.0%), and *Pseudomonas aeruginosa* (14%) were the most common bacterial isolates. *Lactobacillus delbrueckii* showed anti-biofilm activity against multiple-drug resistance pathogens, *Staphylococcus*, *P. aeruginosa*, and *Klebsiella pneumoniae*. Although *L. lactis* had anti-biofilm activity against these three pathogens, its effect was less than that of *L. delbrueckii*. The two probiotics did not have any synergistic effect on the biofilms of the isolates.

Conclusions: The results of the present study emphasized the potential of probiotics in destroying biofilms of isolates with multiple-drug resistance; however, their simultaneous use for this purpose requires further investigation.

Keywords: Ulcer, Biofilm, Drug Resistance, Probiotics, *Lactobacillus delbrueckii*, *L. lactis*

1. Background

Chronic ulcers do not improve within 1 - 3 months (1). In such ulcers, several factors usually delay the ulcer healing process. These factors include endocrine disorders (diabetes), vascular problems, immune system dysfunction, infection, and bacterial biofilm formation (2). The most important chronic ulcers encompass diabetic foot ulcers, venous leg ulcers, pressure ulcers, and surgical site infections (3). Ulcer healing has four successive stages, including homeostasis, inflammation, proliferation, and regeneration, and chronic ulcers often remain in the inflammation stage (4). Studies indicate that 70% of chronic ulcers have biofilms, while only 6% of acute ulcers, which have timely healing, form biofilms (5). Bacterial resistance to antimicrobials in the biofilm phase is a major global issue (6, 7). By providing special conditions, biofilms en-

able the horizontal transfer of antibiotic-resistance genes and virulence factors among bacteria, thereby minimizing the effectiveness of antibiotics and antiseptics and causing the overuse of antibiotics and the spread of multiple-antibiotic resistance. Hence, reducing the bacterial load is a key parameter in the ulcer healing path (8).

Many studies have examined the antimicrobial activity of probiotics through the production of bacteriocins, lactic acid, hydrogen peroxide, and other antimicrobial molecules (9). In this regard, *Lactobacillus lactis* has a special place due to the production of nisin bacteriocin, and several studies have demonstrated its antibacterial function (10). *Lactobacillus delbrueckii* also competes with pathogenic bacteria by producing lactic acid and propionic acid and decreasing pH, thus inhibiting their growth (11). It is worth noting that bacteria in the biofilm exhibit high resistance to antimicrobial agents compared to

the planktonic form; hence, it is necessary to consider the physiological status of the ulcer and the presence of bacteria in the biofilm to evaluate the inhibitory effect of probiotics. Besser et al. overcame this challenge by designing a novel *in vivo* like human plasma biofilm model (hp-BIOM) and studied the anti-biofilm activity of *L. plantarum*, *Bifidobacterium lactis*, and *Saccharomyces cerevisiae* on clinically isolated biofilms (12). The hpBIOM-aided evaluation of the anti-biofilm potential of other probiotics provides valuable information for the biofilm treatment of chronic ulcers by bacteriotherapy.

2. Objectives

Since there was no information about the effects of two probiotics, *L. lactis*, and *L. delbrueckii*, on inhibiting and destroying biofilms of chronic ulcers separately and simultaneously, the present study examined the simultaneous effects of the two probiotics on the inhibition of pathogens isolated from chronic ulcer biofilms with multiple-drug resistance (MDR) in hpBIOM.

3. Methods

3.1. Specimen Collection

From July 2020 to February 2021, 82 specimens of chronic ulcer biofilms were collected from patients who visited the Khanevadeh and Al-Zahra Hospitals in Isfahan. Inclusion criteria were as follows: The presence of chronic ulcers lacking timely healing, the presence of biofilm in the ulcer, and consumption of at least a period of antibiotics. Ulcers were first cleaned with sterile normal saline; then, biopsy specimens were obtained from ulcer biofilms with forceps and transferred to a laboratory in a nutrient transfer medium for microbial examination.

3.2. Isolation and Identification of Bacterial Isolates

The specimens were cultured on MacConkey and blood agar (QuE-Lab, Italy) and incubated at 37°C for 24 hours. After isolation, the isolates were identified using Gram staining and conventional diagnostic tests. Finally, a universal primer was used to amplify the 16S rRNA gene for molecular identification, and the PCR product was sent to Zist Exir Company for sequencing (13).

3.3. Antibiotic Susceptibility of Isolates

The antibiotic resistance pattern of the isolates was determined using the Kirby-Bauer method according to CLSI standards for the following antibiotics: Imipenem (10 µg), amoxicillin-clavulanic acid (10/20 µg), ciprofloxacin (5 µg), ceftioxin (30 µg), ceftriaxone (30 µg), cefepime

(30 µg), cefotaxime (30 µg), Co-trimoxazole (1.25/23.75 µg), penicillin (10 µg), clindamycin (2 µg), gentamicin (10 µg), and ampicillin (10 µg) (BD BBL, Canada). The susceptibility of the isolates to colistin and vancomycin was examined using E-test, and the corresponding minimum inhibitory concentration (MIC) values were determined. After determining the antibiotic resistance pattern, three isolates, namely *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*, with multiple-antibiotic resistance, were selected for further analysis.

3.4. Preparation of Probiotics

Two probiotics, *L. lactis*, and *L. delbrueckii*, were isolated from the industrial yogurt starter of Hansen Company. After confirmation of phenotypic identity using biochemical tests, the probiotics were separately cultured in MRS broth at 37°C and 100-150 rpm in a shaker incubator for 48 hours.

3.5. Human Plasma Biofilm Model (hpBIOM) Preparation

The hpBIOM was prepared based on the method proposed by Besser et al. (14). Plasma and buffy coats were collected from donors. First, the buffy coat was centrifuged at room temperature and 3,000 rpm for 30 minutes to remove residual red blood cells. The platelet-rich plasma and buffer coat were then fused and shaken continuously in a test tube at 100 rpm at 22°C. For 1 mL of plasma, 1 mL of the bacterial isolate was added at a concentration of 0.5 McFarland (1.5×10^8), and then 18.27 µL of CaCl₂ was added, mixed well, and quickly transferred to 12-well culture plates. The plates were incubated for two hours at 37°C and 50 rpm to form polymerized plasma and disks of bacterial biofilm. After preparing the biofilm disk, 100 µL of suspension of each probiotic with a turbidity of 10⁹ CFU was added to the top of the disk, and the second dose of probiotic was added with the same amount and turbidity after two hours and was then incubated at 37°C. To investigate the simultaneous effects of *L. lactis* and *L. delbrueckii* on the biofilms of isolates, 50 µL of each probiotic with a turbidity of 10⁹ CFU was added to the disk in two doses with an interval of two hours.

3.6. Biofilm Dissolution and Bacterial Growth Count

For enzymatic digestion of plasma, 1 mL of bromelain (b4882 Sigma-Aldrich) at a concentration of 0.1 g/mL in PBS was added to the wells two and 24 hours after adding the second dose of probiotic, followed by incubation at 37°C for two hours. To count the surviving pathogenic bacterial cells, serial dilutions were prepared from 100 µL of solution of each well and inoculated on MRS TSA agar. Then, the plates were incubated at 37°C overnight. Finally, colony counting was performed.

3.7. Field Emission-Scanning Electron Microscopy (FE-SEM)

In the present study, the interaction of *L. delbrueckii* probiotic with *P. aeruginosa* isolates and the simultaneous interaction of *L. delbrueckii* and *L. lactis* with *P. aeruginosa* after 24 hours were investigated using field emission-scanning electron microscopy (FE-SEM).

3.8. Statistical Analysis

Data were expressed as mean \pm SD. The experiments were performed in triplicate to evaluate the anti-biofilm activity of probiotics for each plasma donor. The Mann-Whitney test was used to compare the results between treatment and control groups, and $P < 0.05$ was considered significant. GraphPad Prism™ software version 9 was used.

4. Results

The present descriptive cross-sectional study examined 82 patients, including 56 (68.3%) men and 26 (31.7%) women with chronic ulcers who visited Khandevadeh and Al-Zahra Hospitals in Isfahan. Among them, the frequency of bedsores was 18.3%, diabetic ulcers 63.4%, arterial ulcers 6.1%, and venous ulcers 12.2%. Most patients (41.5%) were in the age range of 61 - 70 years. The duration of ulcer involvement was two to three months in most patients (31.7%). According to Fisher's exact test results, the frequency distribution of the cause of ulcers was not statistically different between different age groups ($P = 0.986$).

4.1. Bacterial Isolates

Among 82 biofilm specimens, 47 (57.3%) and 35 (42.7%) were monomicrobial and polymicrobial, respectively. There were 33 specimens containing two bacteria, and two specimens had three bacteria. The frequency of Gram-negative and Gram-positive isolates was 65.5% and 34.5%, respectively. Among Gram-negative bacteria, *Escherichia coli* (17%), *P. aeruginosa* (14%), *Proteus mirabilis* (10%), *K. pneumoniae* (8%), and *Acinetobacter baumannii* (6%) were predominant. *Staphylococcus* species (80.5%) were the predominant pathogen among Gram-positive bacteria. *Staphylococcus aureus* (19%) and coagulase-negative staphylococci (9%) were the predominant pathogens among Gram-positive bacteria (Figure 1).

4.2. Antibiotic Resistance Patterns of Isolates

Table 1 presents the antimicrobial resistance patterns of Gram-positive and Gram-negative isolates. All staphylococcal isolates were resistant to penicillin, of which 27% were methicillin-resistant *S. aureus* (MRSA), and resistance to ciprofloxacin was 63.6%; also, vancomycin was the most

effective antibiotic against Gram-positive bacteria. The highest resistance to imipenem was observed in *E. coli* isolates (75.0%). The percentage of colistin resistance was 29.5% in *P. aeruginosa*. Among Gram-negative isolates, *K. pneumoniae* had high resistance to cefotaxime (66.6%), ceftriaxone (55.5%), colistin (33.3%), and co-trimoxazole (77.7%). On the contrary, the most effective antibiotics against Gram-negative bacteria were cefepime and ceftioxin. In general, 59.9% of the isolates were MDR. The MDR percentages among Gram-positive and Gram-negative isolates were 61% and 59%, respectively.

4.3. Interaction of *Lactobacillus delbrueckii* with Biofilms of MDR Isolates

The study of the anti-biofilm activity of *L. delbrueckii* against three MDR pathogens, *S. aureus*, *P. aeruginosa*, and *K. pneumoniae*, indicated the significant effect of this probiotic against all three pathogens, but *L. delbrueckii* could not completely remove pathogenic isolates. However, it significantly inhibited bacterial growth in plasma biofilm over four hours against three pathogens in donor 1 ($P < 0.05$) (Figure 2A-C). In donors 1 and 2, significant anti-biofilm activity was observed against *S. aureus* during 24 hours, and it was accompanied by an average \log_{10} reduction of 2.36 CFU/mL. The same result was also observed for *P. aeruginosa*, and a \log_{10} reduction of 1.43 CFU/mL was detected within 24 hours (Figure 2A and B). In donor 3, no decrease in *K. pneumoniae* biofilm was observed by *L. delbrueckii*; however, there was a significant decrease in donor 1, after four and 24 hours, compared to the control group ($P < 0.05$). The antibacterial activity of *L. delbrueckii* was higher in donor 1 than in the other two donors (Figure 2C).

4.4. Interaction of *Lactobacillus lactis* with the Biofilm of Pathogenic Isolates

Lactobacillus lactis resulted in a significant reduction in the growth of the three pathogens after 24 hours of incubation in donor 1. The antimicrobial activity of *L. lactis* against *S. aureus* and *P. aeruginosa* had the same pattern. That means no change was observed in hpBIOMs in donors 2 and 3, and a \log_{10} reduction of 0.74 - 0.88 CFU/mL was detected in the biofilm of pathogens in donor 1. In the case of *K. pneumoniae*, a \log_{10} reduction of 0.47 CFU/mL was observed in all three donors after 24 hours (Figure 3D-F).

4.5. Simultaneous Effects of *Lactobacillus lactis* and *Lactobacillus delbrueckii* on Biofilm in hpBIOM

The present study examined the simultaneous interaction of two probiotics, *L. lactis*, and *L. delbrueckii*, against three MDR isolated from clinical specimens using donor

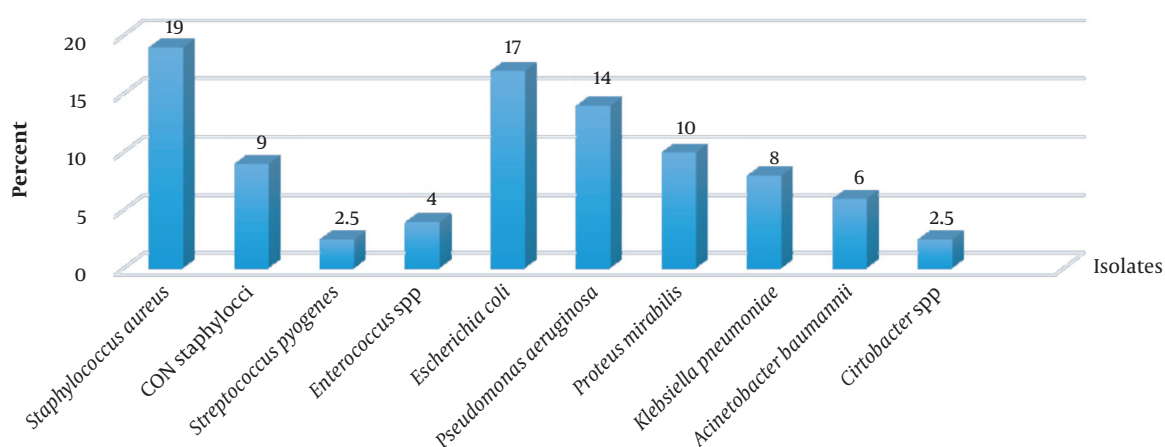


Figure 1. Frequencies of bacterial isolates from biofilms of chronic ulcers; CON, coagulase-negative.

Table 1. Antibiotic Resistance Patterns of the Most Frequent Bacterial Isolates from Chronic Ulcer Biofilm^a

| Type of Antibiotic | <i>S. aureus</i> , N = 22 | CON Staphylococci, N = 11 | <i>E. coli</i> , N = 20 | <i>P. aeruginosa</i> , N = 17 | <i>P. mirabilis</i> , N = 12 | <i>K. pneumoniae</i> , N = 9 | <i>A. baumannii</i> , N = 7 |
|--------------------|---------------------------|---------------------------|-------------------------|-------------------------------|------------------------------|------------------------------|-----------------------------|
| PEN | 22 (100.0) | 11 (100.0) | NT | NT | NT | NT | NT |
| CLI | 13 (59) | 7 (63.6) | NT | NT | NT | NT | NT |
| VAN | (0.0) | (0.0) | NT | NT | NT | NT | NT |
| AMC | 5 (23) | 4 (36.3) | 3 (15.0) | 5 (29.5) | 2 (16.6) | 2 (22.2) | 2 (28.5) |
| FEP | NT | NT | 3 (15.0) | 1 (5.8) | 2 (16.6) | 2 (22.2) | 1 (14.3) |
| CTX | NT | NT | 7 (35.0) | NT | 5 (41.6) | 6 (66.6) | NT |
| FOX | 6 (27) | 3 (27) | 3 (15.0) | NT | 1 (8.3) | 1 (11.1) | NT |
| CRO | NT | NT | 7 (35.0) | 9 (53.0) | 6 (50.0) | 5 (55.5) | 3 (43.0) |
| CIP | 14 (63.6) | 7 (63.6) | 14 (70) | 11 (64.7) | 9 (75.0) | 7 (77.7) | 5 (71.4) |
| CST | NT | NT | 4 (20.0) | 5 (29.5) | 2 (16.6) | 3 (33.3) | 1 (14.3) |
| GEN | 5 (23) | 2 (18.2) | 6 (30.0) | 11 (64.7) | 6 (50.0) | 5 (55.5) | 3 (43.0) |
| IPM | NT | NT | 15 (75.0) | 13 (76.4) | 8 (66.6) | 8 (88.8) | 5 (71.4) |
| SXT | 4 (18.2) | 3 (27) | 12 (60.0) | 10 (59.0) | 8 (66.6) | 7 (77.7) | 3 (43.0) |
| AMP | NT | NT | 13 (65.0) | NT | 8 (66.6) | 6 (66.6) | NT |

Abbreviations: CON staphylococci, coagulase-negative staphylococci; PEN, penicillin; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin; AMC, amoxicillin-clavulanic acid; FEP, cefepime; CTX, cefotaxime; FOX, cefoxitin; CRO, ceftriaxone; CIP, ciprofloxacin; CLI, clindamycin; CST, colistin; GEN, gentamicin; IPM, imipenem; NT, not tested.

^a Values are expressed as No. (%).

plasma, with better antibiofilm activity. Two probiotics simultaneously prevented the formation of biofilms by all three pathogens within 24 hours compared to the control group ($P < 0.05$) (Figure 2). The highest anti-biofilm activity of two probiotics was simultaneously observed on *P. aeruginosa* with a log₁₀ reduction at 1.47 CFU/mL and 0.83 CFU/mL in the first and second donors, respectively. However, comparing the inhibitory effects of the two probiotics

simultaneously and alone demonstrated that they did not synergize the antibiofilm activity of each other.

4.6. Findings of FE-SEM

Field emission-scanning electron microscopy (FE-SEM) was used for a more accurate analysis of the anti-biofilm activity of *L. delbrueckii* on *P. aeruginosa*. A comparison of control and treatment images revealed the influence of *L. del-*

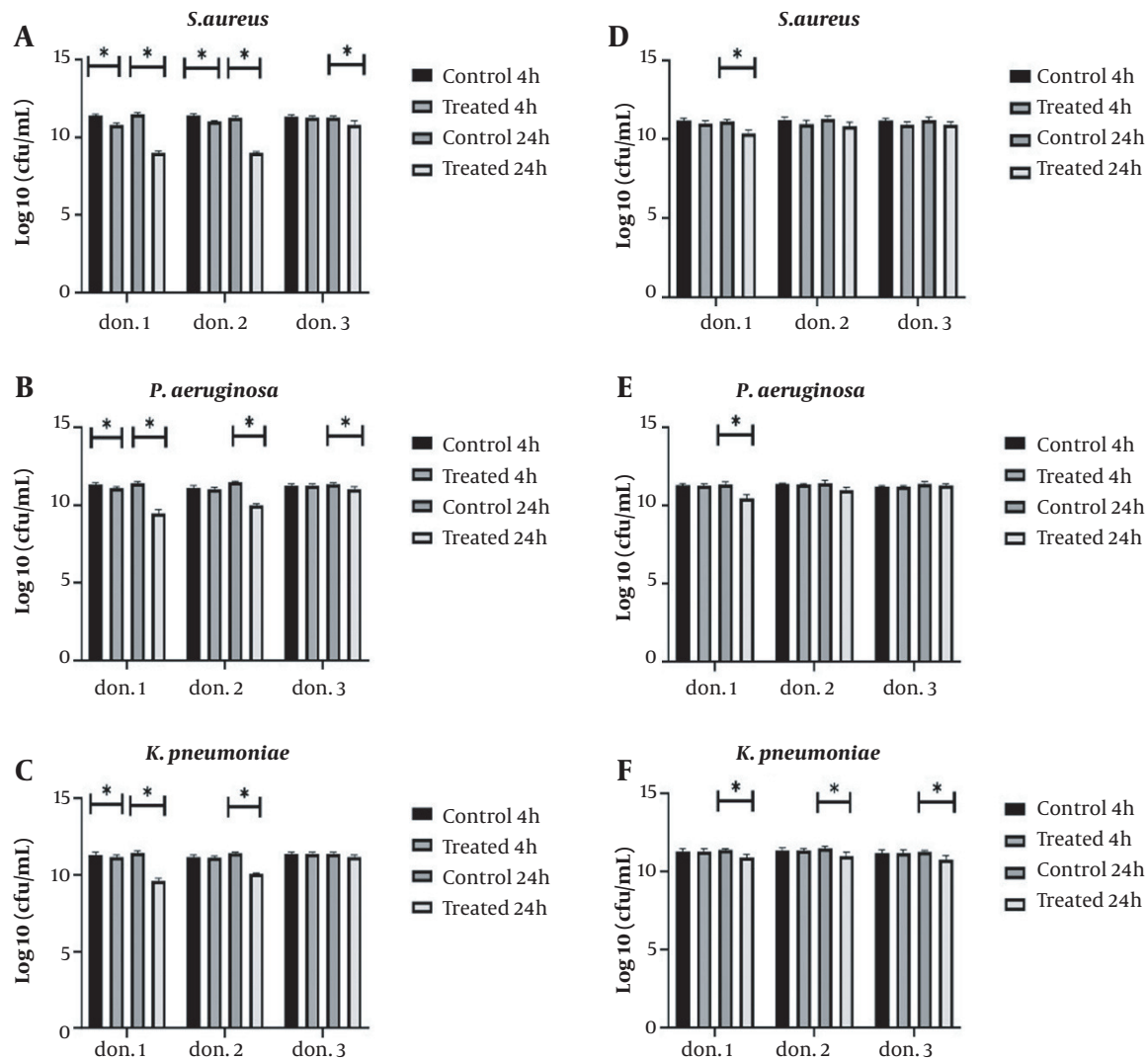


Figure 2. Simultaneous interaction of *Lactobacillus delbrueckii* and *L. lactis* against three MDR isolated from clinical specimens. All tests were repeated three times (*: $P < 0.05$).

brueckii on hpBIOM. The number of *P. aeruginosa* cells was significantly reduced, and the matrix was covered largely by *L. delbrueckii* (Figure 4A and B). The FE-SEM image of the simultaneous interaction of two probiotics indicated the formation of exopolysaccharides by *L. delbrueckii*, among which *L. lactis* was obvious and replaced *P. aeruginosa* cells (Figure 4C).

5. Discussion

Chronic ulcers have become a major challenge worldwide by increasing economic costs and impacting patients' quality of life (15). In the present study, similar to

other studies, the most common bacteria isolated from chronic wounds were Gram-negative bacteria belonging to the *Enterobacteriaceae* family (16). Among them, *E. coli* was the most common Gram-negative bacterium. In contrast, Wong et al. reported *P. aeruginosa* as the most common Gram-negative bacterium in chronic wounds (17). The frequency of MRSA strains in our study was consistent with Ralph's findings (18). In the present study, like Silva et al., the frequency of MDR bacterial isolates was high (19). Based on the findings of this study, the resistance of Gram-negative isolates to fluoroquinolones (ciprofloxacin) and carbapenem (imipenem) was increasing.

The biofilm formation by bacteria is a non-endogenous

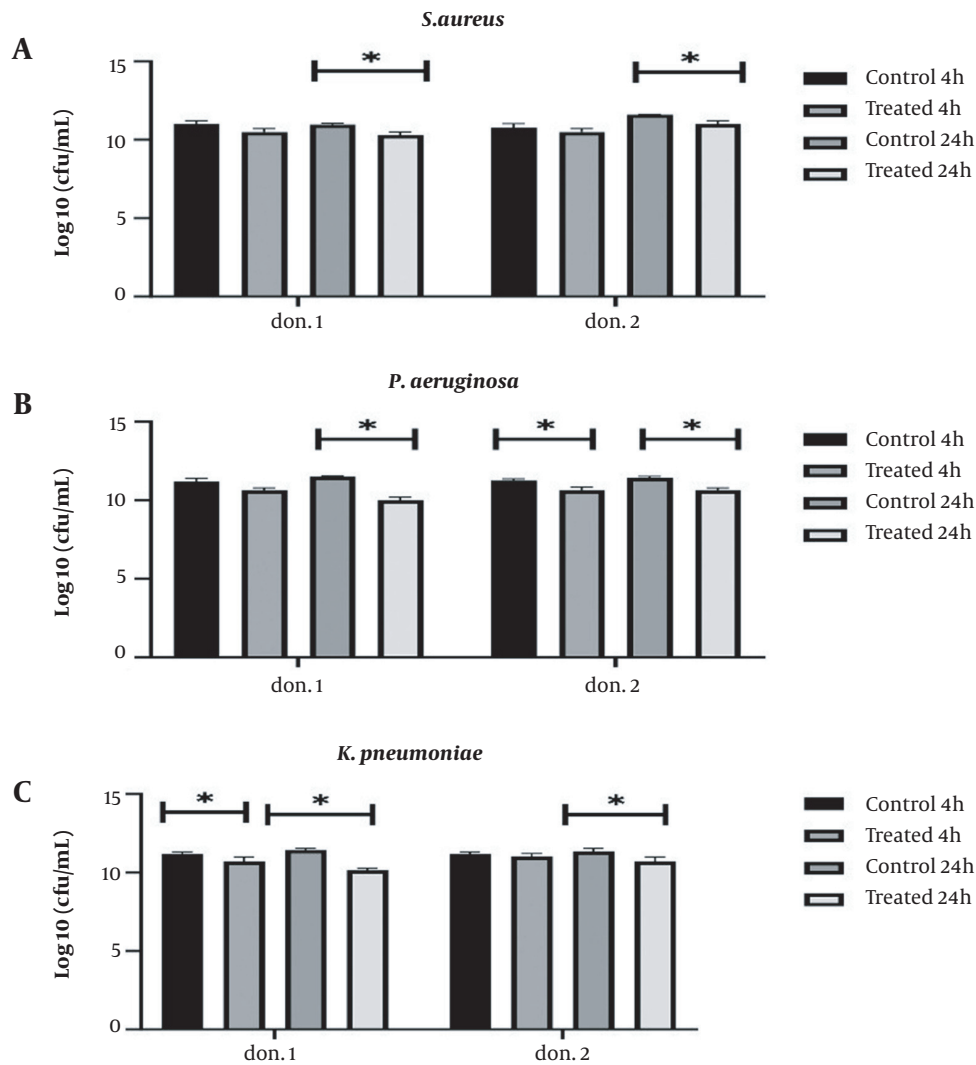


Figure 3. Antimicrobial activity of *Lactobacillus delbrueckii* and *L. lactis* in hpBIOM. A, B and C, *L. delbrueckii* significantly inhibited the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in all three donors within 24 hours but did not affect *Klebsiella pneumoniae* biofilm in the third donor. *L. delbrueckii* showed the best anti-biofilm activity in the first donor plasma; D, E and F, antimicrobial activity of *L. lactis* against *S. aureus* and *P. aeruginosa*, except for *K. pneumoniae*, was donor-plasma dependent. All tests were repeated three times (*: $P < 0.05$).

reason making chronic ulcer healing difficult. Bacteria in polysaccharides of the biofilm matrix have phenotypic resistance compared to the planktonic form and genetically acquire their resistance to antimicrobials by horizontal transfer of resistance genes in the biofilm (20). This study examined the anti-biofilm activity of two probiotics, *L. lactis*, and *L. delbrueckii*, on three MDR pathogens isolated from chronic ulcers in hpBIOM. This study used this model since hpBIOM largely mimics the human ulcer biofilm. In the present study, *L. delbrueckii* showed significant anti-biofilm activity against the three pathogens during 24

hours of incubation. *Lactobacillus* supernatant contains exopolysaccharides and biosurfactants that inhibit bacterial biofilms (21, 22). Kim et al. reported that *L. acidophilus* exopolysaccharides could inhibit *E. coli* enterohemorrhagic biofilm in the microplate. It was also reported that the biosurfactants produced by *Lactobacillus* RC-14 considerably reduced *S. aureus* attachment to surgical implants (23).

The cell-free supernatant and whole-cell of *L. delbrueckii* had stronger antimicrobial activity on *S. aureus* than on *P. aeruginosa* and *K. pneumoniae*. Gram-negative bacteria are more resistant to antimicrobial compounds

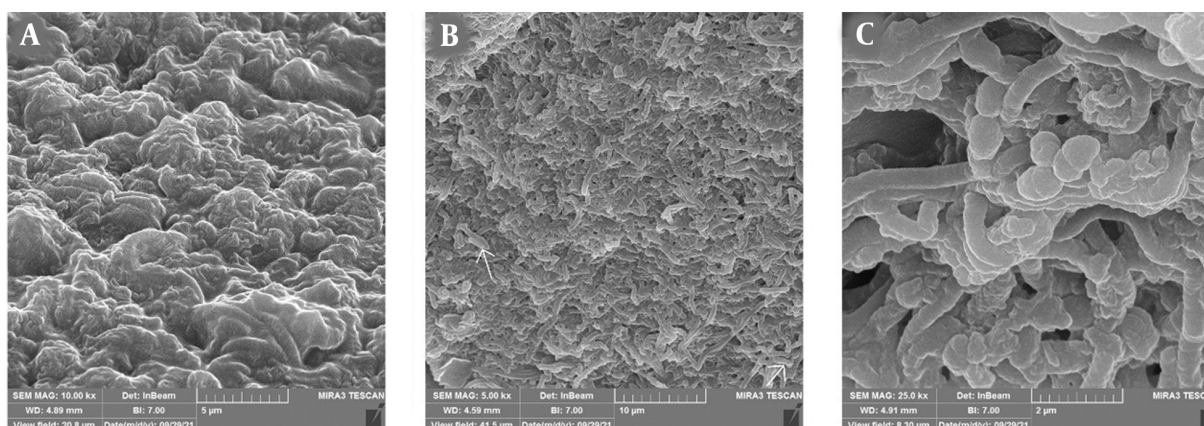


Figure 4. FE-SEM images of simultaneous effects of *Lactobacillus lactis* and *L. delbrueckii* on the growth of *Pseudomonas aeruginosa* in hpBIOM: A, *P. aeruginosa* biofilm after 24 hours that is associated with high production of exopolysaccharide; B, *P. aeruginosa* after exposure to *L. delbrueckii*, the surface of which is covered with microcolonies containing probiotics and a very small number of *P. aeruginosa* (arrow); C, *P. aeruginosa* biofilm in simultaneous exposure to *L. lactis* and *L. delbrueckii*, where a high density of two probiotics was visible.

in supernatants due to an outer membrane in their wall (24). Furthermore, *P. aeruginosa* can develop resistance to antimicrobial agents in the cell-free supernatant. *Pseudomonas aeruginosa* can form a dense biofilm that increases its resistance to antimicrobials in the supernatant. In the present study, *L. lactis* exhibited a lower antimicrobial effect than *L. delbrueckii*. Nisin bacteriocin is an antimicrobial compound produced by *L. lactis* and has strong antimicrobial effects against Gram-positive bacteria (25). However, nisin-like bacteriocins are also produced by some *L. lactis* strains that affect Gram-negative pathogens, including *Salmonella typhimurium*. Kuwano et al. reported that nisin Z made the cytoplasmic membrane of Gram-negative and Gram-positive bacteria permeable (26). The low anti-biofilm activity of *L. lactis* in this study was probably due to a lack of optimal production of nisin since its production was influenced by environmental factors, such as pH, incubation temperature, and carbon source (27, 28).

Unlike Besser's study, none of the probiotics could eliminate the pathogens (12). The difference was probably due to the types of probiotics used and the MDR of pathogens. The MDR bacteria use resistance mechanisms like efflux pumps that play a crucial role in their resistance to antimicrobial agents in the supernatant (29). The results of FE-SEM studies indicated that probiotics in hpBIOM in competition with pathogenic species prevented the biofilm formation by pathogenic bacteria through competitive removal, spatial inhibition, and colonization. Chan et al. found that the lipoteichoic acid (LTA) in the cell wall of *Lactobacillus* prevented the binding of uropathogenic bacteria to epithelial cells (29).

For the first time, this study examined the simulta-

neous effect of the two probiotics on the biofilm of MDR pathogens in hpBIOM. The results demonstrated the non-synergistic effects of *L. lactis* and *L. delbrueckii* bacteria on the biofilm of pathogens. The anti-biofilm activity decreased compared to the situation in which only a probiotic was used. According to studies, synergistic interactions often occur between probiotics when there is a common metabolite between the two probiotics. Somkuti and Steinberg investigated the synergistic effect on pediocin production by *L. delbrueckii*. *Lactobacillus delbrueckii* metabolizes lactose to glucose and galactose by producing β -galactosidase in the medium and provides the substrate for *Pediococcus acidilactici* (30).

Another study indicated a synergistic interaction between lactic acid bacteria (LAB) and *Saccharomyces cerevisiae*. Yeast increased the growth of *L. sanfranciscensis* by producing the growth factor and carbon dioxide in the dough, but the growth of *L. delbrueckii* by yeast depended on the carbon source in the dough. In the present study, the reduction of anti-biofilm activity in the simultaneous presence of two probiotics was probably due to the competition between the two types of probiotics for food and, consequently, a decrease in the production of antimicrobial agents. The donor-specific antibacterial activity of probiotics was a significant finding of the present study, which was consistent with Besser's study. The finding highlights the need to investigate donors' immunological characteristics and their effect on the anti-biofilm activity of LAB in future studies.

5.1. Conclusions

This study showed that *L. delbrueckii* and *L. lactis* have antibacterial activity against the biofilms of MDR isolates from chronic wound infection. However, their synergistic effects on these isolates' biofilm were insignificant. Therefore, more research is necessary to find probiotics with significant synergistic effects. Since the anti-biofilm activity of probiotics in this model was affected by donor plasma, conducting detailed immunological studies in this field indicates the prospect of using probiotics *in vivo*.

Footnotes

Authors' Contribution: V. K.: Study concept and design; K. T. and P. B.: Data collection, processing, and performing experiments; V. K. and H. F.: Research supervision; V. K., H. F. and K. T.: Data analysis and manuscript drafting.

Conflict of Interests: There are no personal financial interests. Stocks or shares in companies: No; consultation fees: No; patents: There are no patents; personal or professional relations with organizations and individuals (parents and children, wife and husband, family relationships, etc.): No; unpaid membership in a government or non-governmental organization: No; are you one of the editorial board members or a reviewer of this journal? No.

Ethical Approval: This research was approved by the Ethics Committee of Isfahan University of Medical Sciences (ethics code: IR.MUI.MED.REC.1399.133). Link: ethics.research.ac.ir/IR.MUI.MED.REC.1399.133.

Funding/Support: This study was supported by the Department of Microbiology, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran, Grant no.399024.

References

1. Werdin F, Tennenhaus M, Schaller HE, Rennekampff HO. Evidence-based management strategies for treatment of chronic wounds. *Eplasty*. 2009;**9**: e19. [PubMed ID: 19578487]. [PubMed Central ID: PMC2691645].
2. Fonder MA, Lazarus GS, Cowan DA, Aronson-Cook B, Kohli AR, Mamelak AJ. Treating the chronic wound: A practical approach to the care of nonhealing wounds and wound care dressings. *J Am Acad Dermatol*. 2008;**58**(2):185–206. [PubMed ID: 18222318]. <https://doi.org/10.1016/j.jaad.2007.08.048>.
3. Rahim K, Saleha S, Zhu X, Huo L, Basit A, Franco OL. Bacterial contribution in chronicity of wounds. *Microb Ecol*. 2017;**73**(3):710–21. [PubMed ID: 27742997]. <https://doi.org/10.1007/s00248-016-0867-9>.
4. Mihai MM, Preda M, Lungu I, Gestal MC, Popa MI, Holban AM. Nanocoatings for chronic wound repair-modulation of microbial colonization and biofilm formation. *Int J Mol Sci*. 2018;**19**(4):1179. [PubMed ID: 29649179]. [PubMed Central ID: PMC5979353]. <https://doi.org/10.3390/ijms19041179>.
5. Gajula B, Munnamgi S, Basu S. How bacterial biofilms affect chronic wound healing: A narrative review. *International Journal of Surgery: Global Health*. 2020;**3**(2): e16. <https://doi.org/10.1097/gh9.000000000000016>.
6. Snyder RJ, Bohn G, Hanft J, Harkless L, Kim P, Lavery L, et al. Wound biofilm: Current perspectives and strategies on biofilm disruption and treatments. *Wounds*. 2017;**29**(6):S1–17. [PubMed ID: 28682297].
7. Harika K, Shenoy VP, Narasimhaswamy N, Chawla K. Detection of biofilm production and its impact on antibiotic resistance profile of bacterial isolates from chronic wound infections. *J Glob Infect Dis*. 2020;**12**(3):129–34. [PubMed ID: 33343163]. [PubMed Central ID: PMC7733422]. https://doi.org/10.4103/jgid.jgid_150_19.
8. Kadam S, Shai S, Shahane A, Kaushik KS. Recent advances in non-conventional antimicrobial approaches for chronic wound biofilms: Have we found the 'chink in the armor'? *Biomedicines*. 2019;**7**(2):35. [PubMed ID: 31052335]. [PubMed Central ID: PMC6631124]. <https://doi.org/10.3390/biomedicines7020035>.
9. Fijan S, Frauwallner A, Langerholc T, Krebs B, Ter Haar Nee Younes JA, Heschl A, et al. Efficacy of using probiotics with antagonistic activity against pathogens of wound infections: An integrative review of literature. *Biomed Res Int*. 2019;**2019**:7585486. [PubMed ID: 31915703]. [PubMed Central ID: PMC6930797]. <https://doi.org/10.1155/2019/7585486>.
10. Tavares LM, de Jesus LCL, da Silva TF, Barroso FAL, Batista VL, Coelho-Rocha ND, et al. Novel strategies for efficient production and delivery of live biotherapeutics and biotechnological uses of *Lactococcus lactis*: The lactic acid bacterium model. *Front Bioeng Biotechnol*. 2020;**8**:517166. [PubMed ID: 33251190]. [PubMed Central ID: PMC7672206]. <https://doi.org/10.3389/fbioe.2020.517166>.
11. Devi M, Rebecca LJ, Sumathy S. Bactericidal activity of the lactic acid bacteria *Lactobacillus delbrueckii*. *J Chem Pharm Res*. 2013;**5**(2):176–80.
12. Besser M, Terberger J, Weber L, Ghebremedhin B, Naumova EA, Arnold WH, et al. Impact of probiotics on pathogen survival in an innovative human plasma biofilm model (hpBIOM). *J Transl Med*. 2019;**17**(1):243. [PubMed ID: 31345229]. [PubMed Central ID: PMC6659307]. <https://doi.org/10.1186/s12967-019-1990-4>.
13. Greisen K, Loeffelholz M, Purohit A, Leong D. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *J Clin Microbiol*. 1994;**32**(2):335–51. [PubMed ID: 7512093]. [PubMed Central ID: PMC2630334]. <https://doi.org/10.1128/jcm.32.2.335-351.1994>.
14. Besser M, Dietrich M, Weber L, Rembe JD, Stuermer EK. Efficacy of antiseptics in a novel 3-dimensional human plasma biofilm model (hp-BIOM). *Sci Rep*. 2020;**10**(1):4792. [PubMed ID: 32179838]. [PubMed Central ID: PMC7075952]. <https://doi.org/10.1038/s41598-020-61728-2>.
15. Brown MS, Ashley B, Koh A. Wearable technology for chronic wound monitoring: Current dressings, advancements, and future prospects. *Front Bioeng Biotechnol*. 2018;**6**:47. [PubMed ID: 29755977]. [PubMed Central ID: PMC5932176]. <https://doi.org/10.3389/fbioe.2018.00047>.
16. Guan H, Dong W, Lu Y, Jiang M, Zhang D, Aobuliximu Y, et al. Distribution and antibiotic resistance patterns of pathogenic bacteria in patients with chronic cutaneous wounds in China. *Front Med (Lanshan)*. 2021;**8**:609584. [PubMed ID: 33816517]. [PubMed Central ID: PMC8010674]. <https://doi.org/10.3389/fmed.2021.609584>.
17. Wong SY, Manikam R, Muniandy S. Prevalence and antibiotic susceptibility of bacteria from acute and chronic wounds in Malaysian subjects. *J Infect Dev Ctries*. 2015;**9**(9):936–44. [PubMed ID: 26409734]. <https://doi.org/10.3855/jidc.5882>.
18. Krumkamp R, Oppong K, Hogan B, Strauss R, Frickmann H, Wiäfe-Akten C, et al. Spectrum of antibiotic resistant bacteria and fungi isolated from chronically infected wounds in a rural district hospital in Ghana. *PLoS One*. 2020;**15**(8): e0237263. [PubMed ID: 32764812]. [PubMed Central ID: PMC7413558]. <https://doi.org/10.1371/journal.pone.0237263>.
19. Silva V, Marcoleta A, Silva V, Flores D, Aparicio T, Aburto I, et al. [Prevalence and susceptibility pattern of bacteria isolated from infected

- chronic wounds in adult patients]. *Rev Chilena Infectol.* 2018;**35**(2):155-62. Spanish. [PubMed ID: 29912253]. <https://doi.org/10.4067/s0716-10182018000200155>.
20. Mendoza RA, Hsieh JC, Galiano RD. The impact of biofilm formation on wound healing. In: Hakan Dogan K, editor. *Wound healing-current perspectives*. London: IntechOpen; 2019. <https://doi.org/10.5772/intechopen.85020>.
 21. Barzegari A, Kheyrolahzadeh K, Hosseiniyan Khatibi SM, Sharifi S, Memar MY, Zununi Vahed S. The battle of probiotics and their derivatives against biofilms. *Infect Drug Resist.* 2020;**13**:659-72. [PubMed ID: 32161474]. [PubMed Central ID: PMC7049744]. <https://doi.org/10.2147/IDR.S232982>.
 22. Meroni G, Panelli S, Zuccotti G, Bandi C, Drago L, Pistone D. Probiotics as therapeutic tools against pathogenic biofilms: Have we found the perfect weapon? *Microbiol Res.* 2021;**12**(4):916-37. <https://doi.org/10.3390/microbiolres12040068>.
 23. Kim Y, Oh S, Kim SH. Released exopolysaccharide (r-EPS) produced from probiotic bacteria reduce biofilm formation of enterohemorrhagic *Escherichia coli* O157:H7. *Biochem Biophys Res Commun.* 2009;**379**(2):324-9. [PubMed ID: 19103165]. <https://doi.org/10.1016/j.bbrc.2008.12.053>.
 24. Salminen S, von Wright A. Current probiotics - safety assured? *Microb Ecol Health Dis.* 1998;**10**(2):68-77. <https://doi.org/10.1080/089106098435287>.
 25. Song AA, In LLA, Lim SHE, Rahim RA. A review on *Lactococcus lactis*: from food to factory. *Microb Cell Fact.* 2017;**16**(1):55. [PubMed ID: 28376880]. [PubMed Central ID: PMC5379754]. <https://doi.org/10.1186/s12934-017-0669-x>.
 26. Kuwano K, Tanaka N, Shimizu T, Nagatoshi K, Nou S, Sonomoto K. Dual antibacterial mechanisms of nisin Z against Gram-positive and Gram-negative bacteria. *Int J Antimicrob Agents.* 2005;**26**(5):396-402. [PubMed ID: 16226432]. <https://doi.org/10.1016/j.ijantimicag.2005.08.010>.
 27. Guinane CM, Piper C, Draper LA, O'Connor PM, Hill C, Ross RP, et al. Impact of environmental factors on bacteriocin promoter activity in gut-derived *Lactobacillus salivarius*. *Appl Environ Microbiol.* 2015;**81**(22):7851-9. [PubMed ID: 26341205]. [PubMed Central ID: PMC4616941]. <https://doi.org/10.1128/AEM.02339-15>.
 28. Dimov S, Ivanova P, Harizanova N. Genetics of bacteriocins biosynthesis by lactic acid bacteria. *Biotechnol Biotechnol Equip.* 2005;**19**(sup2):4-10. <https://doi.org/10.1080/13102818.2005.10817270>.
 29. Chan RC, Reid G, Irvin RT, Bruce AW, Costerton JW. Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. *Infect Immun.* 1985;**47**(1):84-9. [PubMed ID: 3917428]. [PubMed Central ID: PMC261473]. <https://doi.org/10.1128/iai.47.1.84-89.1985>.
 30. Somkuti GA, Steinberg DH. Pediocin production in milk by *Pediococcus acidilactici* in co-culture with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. *J Ind Microbiol Biotechnol.* 2010;**37**(1):65-9. [PubMed ID: 19844751]. <https://doi.org/10.1007/s10295-009-0648-2>.