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Comparison of the effects of *Lactobacillus plantarum* versus imipenem on infected burn wound healing

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Abstract

Background: Infection of burn wounds is one of the most important problems in the world. *Lactobacillus plantarum* is known for burn wound healing because of the immunomodulatory and anti-microbial roles. This study was performed to compare the effects of *L. plantarum* and imipenem – alone and in combination – on infected burn wound healing.

Methods: Burn wounds were experimentally induced on 50 rats in three test groups (germ and supernatant of *L. plantarum*) and two control groups (n=10 each) and were inoculated with *Pseudomonas aeruginosa*. During a 14-day period, wounds in all groups were daily treated topically. The data were analyzed using one-way analysis of variance followed by Tukey–Kramer and LSD. A p-value of < 0.05 was considered as statistically significant.

Results: The mean size of the wound on day 14 after the treatment in the probiotic group was significantly lower than the control and the supernatant treated groups ($p<0.05$). The percentage of wound healing was significantly higher in the probiotic pellet treated group compared to the imipenem and the supernatant groups (by Anova test: 69.58%, $p=0.022$). The mean leukocyte count in the probiotic pellet group (12110) and supernatant group (13650) was significantly higher than the imipenem group (7670) ($p=0.002$ and 0.001, respectively). Wound cultures revealed that the percentage of cases where the pathogens had no growth was significantly different among the comparison groups. In all three test groups, *P. aeruginosa* was completely eliminated in comparison to the positive control group ($p<0.05$).

Conclusion: The results of our study showed that *L. plantarum* and its by-products promote wound healing and can be used as an alternative to antibiotics to treat ulcer infections caused by resistant bacteria.

Keywords: *Lactobacillus plantarum*, Imipenem, Burn, Wound healing

Conflicts of Interest: *None declared*
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Introduction

Burns are a major cause of many psychological, physical, and economic injuries (1-4). The frequency and rates of antibiotic resistance among pathogenic bacterial populations can be attributed to the widespread use of antibiotics leading to nosocomial and community-acquired infections (5-7).

Although burn wound surfaces are sterile at the time of a thermal injury, these wounds finally become colonized

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↑What is "already known" in this topic:
Some *Lactobacillus* strains and their products have significant inhibitory activities against multidrug resistant clinical isolates.

→What this article adds:
This study showed that *L. plantarum* and its by-products promote wound healing and can be used as an alternative to antibiotics to treat ulcer infections caused by resistant *P. aeruginosa*.

with microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa, fungal* pathogens particularly *Candida* spp. and *Herpes simplex virus* (8). *P. aeruginosa*, as a major opportunistic human pathogen, carries antimicrobial resistance properties that make it difficult to treat infected burn wounds and is considered as the main cause of death in burned patients (8-11) Therefore, it is essential to find new ways to control drug-resistant *P. aeruginosa* infections.

Previous studies have shown that the intestinal microflora ceases the growth and adherence of pathogenic bacteria to enterocytes. (12, 13) The World Health Organization (WHO) has promoted the use of gastrointestinal microflora, termed *probiotics*, aiming at achieving health benefits in the host (14, 15).

Lactobacillus plantarum 299v (*L. plantarum 299v*), a Gram-positive facultative anaerobic or microaerophilic bacterium, which was first isolated from the human intestine, uses mannose-specific adhesions that help it to compete with Gram-negative and positive bacteria for receptor sites and nutrients in the mucosal membrane (16-18). In addition, they secrete antibacterial substances such as lactic acid, benzoic acid, hydrogen peroxide, and bacteriocins (19, 20) that contribute to the inhibition of forming pathogenic bacterial colonies (21). Moreover, production of short-chain fatty acids (SCFAs) by *L. plantarum 299v* lowers the intestinal pH, creating an unfavorable environment for the growth of pathogens (21, 22). This strain has been reported to have antibacterial activity against several potential pathogenic microorganisms including *P. aeruginosa, Listeria monocytogenes*, *Escherischia coli* and *Enterococcus faecalis* (23-25). Some *Lactobacillus* strains and their products have significant inhibitory activities against multidrug-resistant clinical isolates of *P. aeruginosa* and are also effective in the local treatment of burn infections (25, 26). *L. plantarum299v* is able to survive in severe environments, even in the presence of antibiotics (26-28). These properties make this strain a superior probiotic compared to other commensal bacterial strains.

The main goal of this research was to compare the effects of *L. plantarum* in the form of bacterial cell pellet, supernatant and combination of both, as well as the use of imipenem, as therapeutic strategies for infected burn wounds of rats.

Methods

Clinical isolates: Thirty clinical samples of burn wounds were collected from patients of hospitalized in Motahari Hospital, Tehran, Iran. The samples were transferred to the microbiology unit of Antimicrobial Resistance Research Center to investigate the presence of *P. aeruginosa* using conventional microbiological methods (29).

Antimicrobial Susceptibility Testing: Antibiotic sensitivity patterns of *P. aeruginosa* isolates were investigated by disk diffusion(dd) method using Mueller Hinton agar medium based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2017). Antibacterial susceptibility of *P. aeruginosa* strains was evaluated for eight different antibiotic disks (Mast Group Ltd., Merseyside, UK). Antibiotic disks included Ceftazidime (30 µg), Ciprofloxacin (30 µg), Gentamicin (10µg), Amikacin (30 µg), Imipenem (10 µg), Cefipime (30 µg) and Tetracycline (30 µg). *P. aeruginosa* ATCC 27853 was used for quality control in the study. Results were interpreted as susceptible, intermediate, or resistant according to the standards. The antibiotic-resistant strains were selected for the *in-vivo* step in this study.

In this research, the bacteria were cultured on Mueller-Hinton agar with 0.5 McFarland standards. Then the discs placed on the medium and were incubated at 37°C for 16 hours.

Preparation, cultivation, and selection of probiotic bacteria: In this study, different strains of probiotics were obtained from the Probiotic Research Center of Alborz University of Medical Sciences, Karaj, Iran and DSM. (26). The commercial probiotic strains used in this study were *Bacillus coagulans (DSM1), Bifidobacterium bifidum (DSM20456) and L. plantarum 299v (DSM9843)*. The local *Lactobacillus* strains included *L. salivarius* strain ES1, *L. reuteri* strain ES10 and *L. salivarius* strain ES8. The *Lactobacillus* strains were grown anaerobically on De Man, Rogosa and Sharpe (MRS broth at 37°C for 48 h (30). After that, they were transferred to the MRS agar medium. Then, they were standardized based on 0.5 McFarland standards (1.5×10^8cfu / ml) and were kept at 4°C.

Antimicrobial activity of probiotic strains against pathogens: The inhibitory activity of the probiotic strains against *P. aeruginosa* was evaluated using the disk diffusion method as described by NCCLS (31). The selected resistant *P. aeruginosa* was examined using probiotic coated disks. For this purpose, a blank disk (6 mm in diameter) was inoculated with 20 µl probiotic suspension (1.5×10^8 cfu/ ml bacteria) and was placed on a nutrient agar medium and was incubated at 37°C for 16 h. Afterward, the probiotic strain with the longest inhibitory diameter was selected for the next step (Fig. 1 and Fig. 2).

Preparation of probiotic supernatant: In this section, the supernatant was prepared by centrifuging of probiotic suspension at 4000 rpm for 10 minutes. The centrifuged suspension was filtered through a sterile 0.22 µ-pore-size filter unit. Finally, the cell-free supernatant (CFS) and precipitated cells of probiotics were collected and kept at 4°C until use (32, 33).

Animals: This experimental study was carried out in the Animal Laboratory of Iran University of Medical Sciences with ethic committee code IR.IUMS.REC1393.24557. Fifty male adult Wistar rats of similar age (8-10 weeks) and weight (200-250 gr) were maintained under controlled conditions of light (12h light/dark photoperiod), room temperature (32 ± 2°C) and relative humidity (60–70%). They were kept in polyethylene boxes with enough appropriate space and free access to food and water that were refreshed every day.

Induction of wounds and treatment procedure: The animals were anesthetized using 100 mg/kg of ketamine and 10 mg/kg of xylazine (34) injected to intraperitoneal space, then the hair on the dorsal areas was

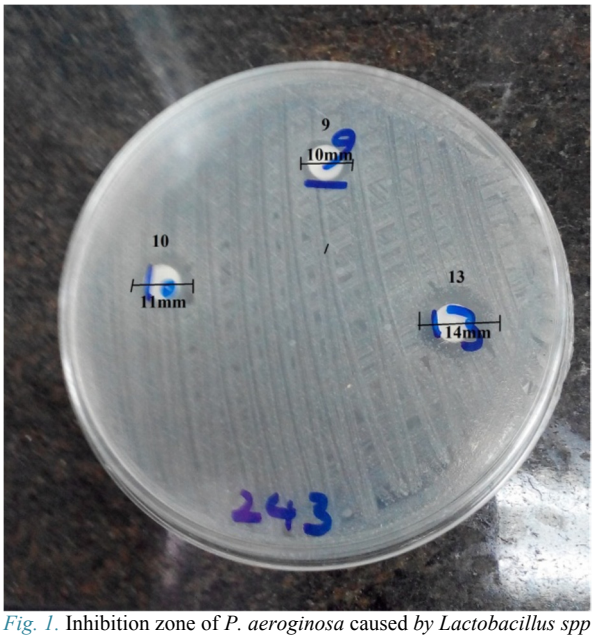

Fig. 1. Inhibition zone of *P. aeroginosa* caused *by Lactobacillus spp* by disk diffusion method. Disk 8: *L. Plantarum299v* (16mm), disk 9: *L. salivarius* ES1 (0mm), Disk 10: *Bacillus Coagulans* (0mm), disk 11: *L. reuteri* ES10 (0mm), disk 12: *L. salivarius* ES7 (0mm), disk 13: *Bifidobacterium bifidum* (0mm).

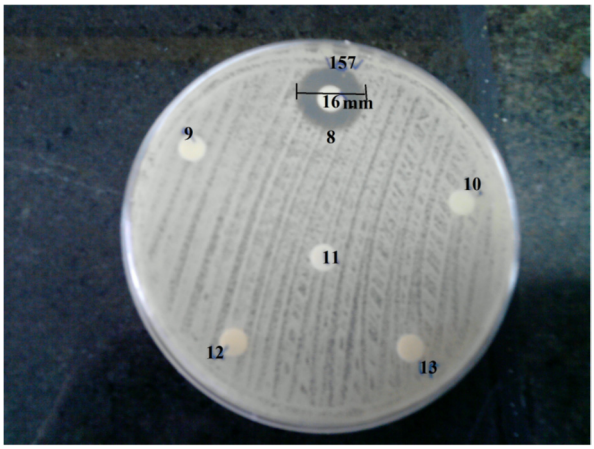

Fig. 2. Inhibition zone of *P. aeroginosa* caused by *Lactobacillus spp* by disk diffusion method. Disk 9: *L. salivarius ES1* (10mm), Disk 10: *Bacillus Coagulans* (11mm), disk 13: *Bifidobacterium bifidum* (14mm).

shaved, disinfected with ethanol 70% and were then exposed to a hot steel rod with a temperature of 95°C and a diameter of 2 cm. Thereafter, the wounds were covered with sterile gauze. Twenty-four hours later, the wounds were inoculated with 1 ml of *P. aeruginosa* (1.5×10^8 CFU/ml). All rats were divided into 5 groups (n=10 each) 24 h after the induction of infection randomly. Burn wounds in all groups were topically treated with a eucerin ointment containing different compositions daily for 14 days. The treatment applied on each group are described as: 0.9% NaCl (negative control group), imipenem (positive control group), the cells of probiotics (test group 1), supernatant of probiotics (test group 2) and combination of germ and supernatant of probiotic (test group 3) (Table 1).

Table 1 shows the different formulations used in each group of rats. Ten rats in each group were studied (total population = 50).

Table 1. Animals in different groups treated with different formulations

Groups (n = 10)	Interventions
Negative control	Eucerin ointment +0.9% NaCl (1g/1ml): Without treatment
Positive control	Eucerin ointment + imipenem (1g/1mg); imipenem (50mg/1kg)
Test 1	Eucerin ointment + probiotic cell pellet ($1g/10^8$)
Test 2	Eucerin ointment + supernatant of probiotic (lg/1ml)
Test 3	Eucerin ointment + (probiotic cell pellet + supernatant of probiotic) (10^8/1g/ml)

Evaluation of the wound healing process: All animals were sacrificed by the use of high doses of ether anesthetics on the 14th day of the treatment and evaluations were conducted as follows:

Morphological assessment of the wounds: Burn wounds were evaluated 24 h after induction of burn (before initiation of treatment: day 0), on the 7th and 14th day of treatment by measuring the area of the wounds with the naked eye with a ruler (35). The percentage of wound recovery was computed according to the following formula (1).

$$(1)\ \frac{\textbf{wound area on first day } (mm^2) - \textbf{wound area on last day } (mm^2)}{\textbf{wound area on first day } (mm^2)} \times 100$$

Burn wound hematological assessment: Blood samples were collected by heparin-coated tubes. Leukocytes were counted on days 0, 7, and 14 using a cell counter device (Cell Counter Refreshed Sysmex kx2).

Burn wound bacterial infection assessment: On days 0, 7, and 14, the surface layers of the lesions were removed by a wet sterile swab in all animals and were cultured on blood agar medium and then were incubated at 37 °C. The cultures were assessed for *p. aeruginosa* after 24 h using common laboratory tests (35).

Statistical analysis: The data was analyzed by SPSS software, version 20.0. Comparison between the groups were done using one-way analysis of variance (ANOVA) followed by Tukey–Kramer and LSD (post Hoc tests). A p-value < 0.05 was considered as statistically significant.

Results

Antibiotic Susceptibility Testing: The resistance patterns of 30 strains of *P. aeruginosa* to some of the antibiotics were investigated. The frequency of the resistance of *P. aeruginosa* isolates against the tested antibiotics (number/percentage) are as follows: Ceftazidime (30/100), Gentamicin (28/93.4), Amikacin (28/93.4), Imipenem (30/100), Cefipime (30/100), Ciprofloxacin (30/100) and Tetracycline (88/60). All of the tested strains showed resistance to ceftazidime, imipenem, cefepime and ciprofloxacin. These results reflect the low susceptibility of tested strains to other tested antibiotics. Of 30 samples, the one with the highest antimicrobial resistance was selected for animal assay. The resistance was examined based on the diameter of the inhibition

Table 2. Antimicrobial activity of some bacterial probiotics against resistant *P. aeruginosa*

Bacteria	Mean of inhibitory zone (mm)
Bacillus Coagulans (DSM1)	10
Bifidobacterium bifidum	14
L. Plantarum299v (DSM9843)	16
L. salivarius ES1	10
L. reuteriES10	10
L. salivariusES7	0

zone.

In-vitro effects of probiotic bacteria on *P. aeruginosa*: The antibacterial activity of *Bacillus coagulans* (*DSM1*), *Bifidobacterium bifidum* and *L. plantarum299v (DSM9843)* as commercial probiotic strains, and also local *Lactobacillus* strains including *L. salivarius ES1*, *L. reuteri ES10*, *L. salivarius ES7* was tested against resistant *P. aeruginosa* (Table 2).

The antimicrobial activity of probiotic strains was studied based on the inhibition zones (total population of *P. aeroginosa* = 30). The mean of the inhibitory zones was measured for every *P. aeroginosa* strain (Table 2).

The results showed that *L. Plantarum299v* had the highest inhibitory diameter (16mm) against selected resistant *P. aeruginosa* compared to other probiotics (Fig. 1 and Fig. 2).

Evaluation of wound healing process in rats: After the treatment procedure, the wound healing process in all animals was investigated and the following factors were taken into consideration on the 14th day of treatment:

Survey of morphology of wound: Wound sizes were measured twenty-four hours after the induction of burn and also on the 7th and 14th days of the treatment with the naked eye (Fig. 3 and Fig. 4).

A description of groups is presented here. Negative

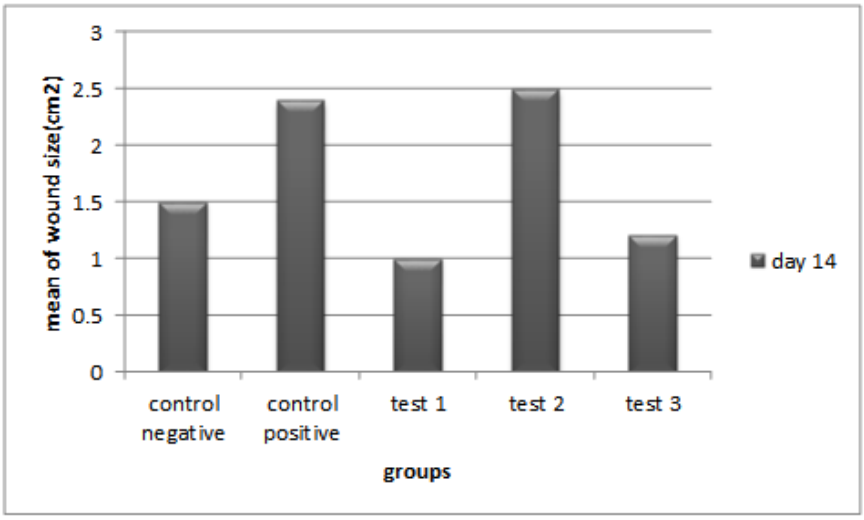

Fig. 3. Mean size of burn wounds in 5 groups (n = 10 each) on day 14 (post treatment).
Test 1, negative control: (P =0.001)*; test 1, positive control: (P=0.000); test 1, test 2: (P=0.03). Statistical method of ANOVA followed by Tukey as post Hoc test.

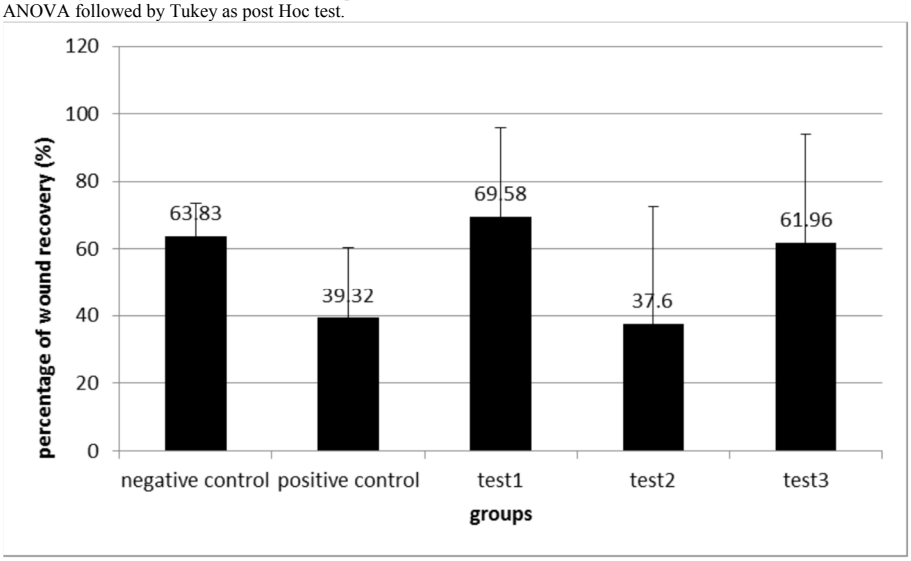

Fig. 4. The percentage of wound recovery after injury induction on the 14th day of treatment in 5 groups (mean ± SD).
The percentage of wound recovery surveyed by the statistical method of ANOVA (F; 3.16, df: 4, sig: 0.022*) followed by LSD as Post-Hoc test. Test 1, positive control: (P=0.014)*; test 1, test 2: (P=0.01)*; test 3, test 2 (P=0.04) *; negative control, positive control: (P=0.04) *.
Groups: negative control; without treatment, positive control ;antibiotic, Test 1; probiotic cell pellet, Test 2; supernatant of probiotic, Test 3; probiotic cell pellet + supernatant of probiotic

Control: treatment by eucerin ointment + NaCl. Positive control: treatment by eucerin ointment + imipenem antibiotic. Test-1: treatment by eucerin ointment + probiotic cell pellet. Test-2: treatment by eucerin ointment + supernatant of probiotics. Test-3: treatment by eucerin ointment + probiotic cell pellet + supernatant of probiotics.

The mean size of the wounds in the test-1 group was significantly lower than the negative and positive control groups on day 14 after the treatment; P-values were 0.001 and <0.001, respectively. Also, the results showed that on the same day, the mean size of the wounds in the probiotic cell pellet group was significantly lower than the supernatant group (P=0.003).

Then, the percentage of wound recovery was computed by comparison of the status of the wound on the first and the last day (Fig. 4). The percentage of wound recovery was calculated by ANOVA (P=0.022) followed by LSD and Post-Hoc test, and the results were compared in all groups.

According to Figure 4, the results showed that the percentage of wound recovery in test-1 group was higher than the positive control (P=0.014) and test-2 groups (P=0.001) compared to others. This percentage in test-3 group was higher than the test-2 group (P=0.004). The percentage of wound recovery in negative control was higher than the positive control group (P=0.004).

Hematological assessment of the burn wounds: The number of leukocytes was counted 24 h after the induction of burn and also on the 7th and 14th day of the treatment. The mean leukocyte count for each group are demonstrated in Figure 5.

The mean number of white blood cells (WBCs) for the test groups 1, 2 and 3 were compared to the control groups on days 0, 7, and 14. Results indicated that there was a significant increase in the mean number of WBCs for test-1 group, which was higher than the mean of the positive control group on day 14 (P=0.002). In addition, the number of WBCs was significantly higher in the test-2 group compared to the positive control group on day 14 (P=0.001) (Fig. 5).

Wound culture: Burn wounds of all animals were sampled to be checked for the possible presence of *P. aeruginosa*, the results of which are presented in Table 3.

The number of samples which were negative for *P. aeruginosa* growth was calculated for each rat separately on the last day of the treatment (total population of animals = 50). On day 14, *P. aeruginosa* was completely eliminated in all the test groups except for the positive control group. In the test-3 group, *P. aeruginosa* growth was not observed on day 7 in addition to day 14 of treatment.

The results showed that the percentage of cases with no P. aeruginosa growth the percentage of the absence of pathogen growth have significant differences among the other comparison groups (Table 3). The number of cases with no P. aeruginosa growth on days 7 and 14 was calculated by χ^2 statistical method, and the results were Chi-square= 29.20, and Chi-square= 28.69 (P<0.001).

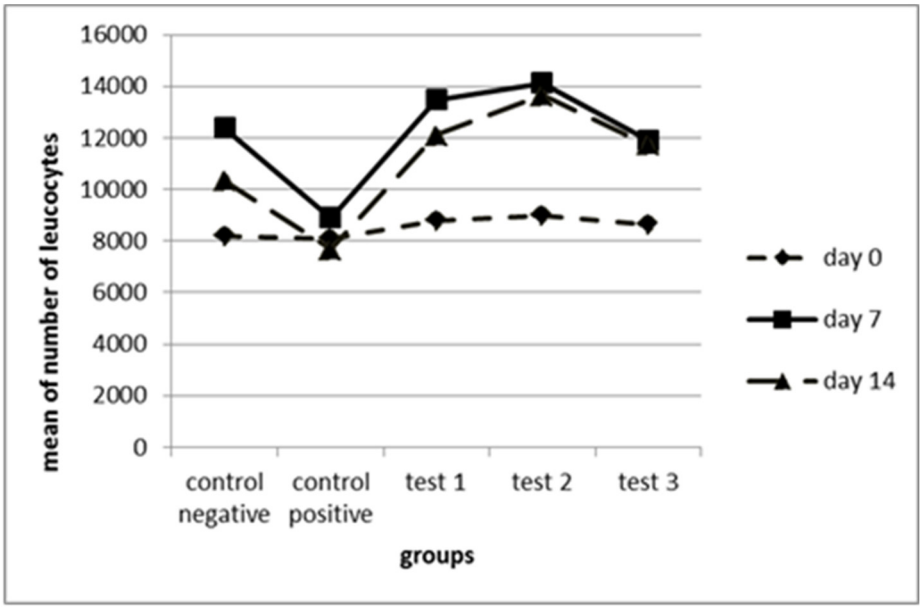

Fig. 5. The mean number of leukocytes in 5 groups (n = 10 each) on days 0, 7 and 14.
Test 1, positive control: (P=0.02) *; test 2, positive control: (P=0.001)*. Statistical method of ANOVA followed by Tukey as post Hoc test.

Table 3. The percentage of cases with no *P. aeruginosa* growth in each group after injury induction on 7th and 14th day of treatment

	The number of cases with no *P. aeruginosa* growth (%)	
Groups	Number on day 7	Number on day 14
Negative control	40	100
Positive control	0	20
Test 1	50	100
Test 2	30	100
Test 3	100	100

Discussion

Pseudomonas aeruginosa is an important cause of burn wound infections and because it is resistant to many antibiotics, making this microorganism a serious therapeutic problem (35-37). In several previous studies, *P. aeruginosa* isolates exhibited full or intermediate resistance to antimicrobial agents (38-41).

In addition, *Pseudomonas* spp. develop resistance to new antimicrobial agents faster than other gram-negative bacteria (42); therefore, in this study, *P. aeruginosa* was used to infect the burn wounds.

All of *P. aeruginosa* strains in our research were resistant to imipenem, cefepime, ciprofloxacin, ceftazidime. In our study, the microbial growth inhibitory activities of six potent probiotic strains were evaluated against *P. aeruginosa*. Among them, *L. plantarum 299v* was selected based on the diameter of the inhibitory zone for further study. It has been reported that *L. plantarum* is more functional in eliminating infections because of secreting short-chain fatty acids (43), bacteriocins (20), and other by-products (42).

Probiotics are considered to be useful microorganisms that involved in the regulation of the host's inflammatory and immune responses. Therefore, *L. plantarum299v* may be helpful for the treatment of burn wound infections. (36, 44, 45) In addition, *Lactobacillus* spp. helps phagocytes avoid apoptosis caused by pathogens (46, 47).

In this study, in order to evaluate the microbial growth inhibitory effects of probiotics against *P. aeruginosa*, burned-rat models were used. After the induction of burn and inoculation of *P. aeruginosa*, burn wounds were treated with three different types of treatment protocols, including probiotic cell pellet, probiotic supernatant, and a combination of probiotic pellet and supernatant. Then the results were compared with the control groups.

Reduction of the size of the wound and its recovery rate are symptoms of wound healing and were evaluated in this study. In this research, in the group treated with probiotic pellet, wound healing was significantly better than the control groups on day 14 of the treatment. Also, we found that wound healing was much more pronounced in the probiotic cell pellet recipient group in comparison with supernatant and antibiotic-treated groups.

Moreover, the percentage of wound recovery in this group was significantly higher than the antibiotic-treated and test-2 groups.

It seems that the supernatant contains antimicrobial and anti-inflammatory agents that are produced by the probiotic bacteria. It also seems that the advantage of probiotic pellet over supernatant is due to the presence of the whole cell. Given that the bacterial cell is present, these compounds are constantly produced. Also, probiotics can promote the strength of the immune system and reduce inflammation and accelerate the wound healing process following the agglomeration of lymphocytes, macrophages, and polymorphonuclear in the damaged area (48, 49). In addition, probiotics can increase the collagenesis, hyaluronic acid, and antioxidants, which aid in wound healing and immune responses. On the other hand, the production of organic acids, bacteriocins, hydrogen peroxide, and ethanol by probiotics can help in the reduction of inflammation and improve the wound healing process (35, 50, 51).

In this study, we observed that the number of WBCs increased in test groups on day 7 and 14, in comparison with the antibiotic-treated group. However, this result was statistically significant in the probiotic pellet treated group.

Several studies have demonstrated that *Lactobacillus* strains promote the immune system's capacity by increasing the cells of the innate immune system, including macrophages and neutrophils, which in turn can be associated with early inflammation (52-54).

There are several reports of enhancement of humoral and cellular immune response resulting from the administration of *Lactobacillus* species in animals and humans (55, 56). Furthermore, *Lactobacillus* spp. has been shown to increase the T-cell lymphocyte population in mice. Some probiotics can stimulate a protective immune response by competing against microbial pathogens (57).

In this study, the percentage of cases in which *P. aeruginosa* had no growth was investigated for each group after induction of the injury on the 7th and 14th day of treatment. The results revealed that this percentage was significantly different from those in other comparison groups. In all three test groups containing the probiotic pellet or supernatant of probiotics or pellet with supernatant of probiotics, *P. aeruginosa* was completely eliminated in comparison to the positive control group on day 14 but the group with a combination of pellet and supernatant of probiotics was more effective in the growth inhibition of *P. aeruginosa* compared with two other test groups.

Furthermore, it seems that the metabolites of *L. plantarum299v* could suppress the growth of *P. aeruginosa*. Similarly, other studies have demonstrated that Lactobacilli are able to inhibit the growth of *P. aeruginosa* by different mechanisms, such as the production of bacteriocin (26, 36)

In this research, in all groups of probiotic recipients, *P. aeruginosa* was seen less in the wound in comparison with the antibiotic-treated group. It was remarkable that the antibiotic recipient (imipenem) group was the only group that failed to inhibit the growth of *P. aeruginosa* on day 14 after treatment, while this antibiotic is commonly used in the process of treating burns nowadays. Therefore, this inappropriate use of antibiotics can lead to the prevalence of antibiotic resistance (7). However, further research is needed for this issue.

Conclusion

In general, *L. plantarum* can act as a bio-therapeutic microorganism and may be a good candidate to overcome the growing challenge of nosocomial infections, but the use of probiotics and its by-products in topical wound treatments requires further investigation. The results of this study indicate that *L. plantarum* and its by-products can be used as an alternative to antibiotics to treat ulcer infections caused by resistant bacteria.

Acknowledgments

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Conflict of Interests

The authors declare that they have no competing interests.

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