Interference of Lactobacillus casei with Pseudomonas aeruginosa in the treatment of infected burns in Wistar rats

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**ABSTRACT**

**Introduction**

Tissue damage can result from physical, chemical, and biological burns. Depending on the type and duration of tissue repair, it can be divided into chronic and acute types. Burn wounds are recognized as a type of tissue damage (1, 2). They are usually classified into three degrees: The epidermal and dermal layers of the skin are involved in second-degree burns. These burns are commonly accompanied by blisters and fluid buildup-related abdominal pain and are also extremely sensitive to touch (3, 4).

Severe ulcers, which can be categorized as burn wounds, require an average of three weeks to heal. Due to the high prevalence of burns in both developing and developed countries, burn trauma has been recently taken into consideration. The trauma associated with burns, especially when excision and grafting of burn eschar are contraindicated, may lead to various complications, including wound infection. On the other hand, wound healing has been always a health concern. Several medications and ointments, such as silver sulfadiazine, have been used for burn wounds, although they have several limitations and side effects (5, 6).

**Materials and Methods:** In this study, the anti-adhesion activity of L casei was examined by the glass slide method, and inhibitory substances in the cell-free supernatant (CFS) were quantified by high-performance liquid chromatography (HPLC). Following the induction of second-degree wounds, multidrug-resistant (MDR) P. aeruginosa was injected subcutaneously and directly on the burn. The animals were divided into four groups. The supernatant of L. casei was sprayed for treatment every day and wound healing was examined.

**Results:** Based on our findings, the supernatant of L. casei showed considerable anti-adhesion effects on P. aeruginosa. HPLC analysis indicated that the inhibitory effect of this supernatant can be due to four main organic acids including lactic acid, acetic acid, citric acid, and succinic acid. The effect of treatment on fibroblastic cells showed that the treated group by supernatant of L. casei had more fibroblastic cells compared with the non-treated group. Moreover, this supernatant increased the rate of fibroblastic cells, re-epithelialization in the wound area, and the largest thickness of the epidermis and dermis layers.

**Conclusion:** The present findings showed that L. casei supernatant significantly reduced inflammation and could be used to treat P. aeruginosa infection in second-degree burns.

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Animal models of burn wounds are essential for the evaluation and improvement of therapeutics for burn wounds. Several animal models, including mouse models, are extensively used to study the burn wound healing process. Burns can be mimicked in experimental animal models to evaluate the patterns of burn wound healing. The selection of an appropriate method to induce burns, besides the use of constant water temperature to cause the same burn in all specimens, is crucial (15, 16).

This study aimed to evaluate the antimicrobial and macroscopic effects of L. casei supernatant as a topical treatment for burn wounds associated with P. aeruginosa infection.

Materials and Methods
Microorganisms
Pathogenesis bacteria
The P. aeruginosa colony was identified from hospitalized burn patients in Firoozgar Hospital of Tehran via Gram staining, as well as standard biochemical tests, including oxidase test, catalase test, Indol test, Methyl Red and Voges Proskauer (MR-VP) test, motility test, citrate test, and fermentation of sugar types (TSI) and color production were done (17, 18).

Probiotics bacteria
L. casei PTCC 1608 was the probiotic bacteria used in the experiments prepared from the Persian Type Culture Collection of the Iranian Research Organization for Science and Technology. The bacterium was cultured anaerobically for one day at 37 °C in the liquid MRS medium. The CFS was prepared by centrifugation of an overnight culture of probiotic bacteria filtered with a 0.2 μm pore size filter (8, 19).

Biofilm formation
Glass slide method
To investigate P. aeruginosa adhesion on the glass slide, the overnight culture of P. aeruginosa (1 ml; 10^8 CFU/ml) was inoculated into a flask, containing 100 ml of sterile brain-heart infusion (BHI) broth, supplemented with 1% sucrose. The glass slides were washed in distilled water twice after cleaning detergent solution, then dried and transferred to flasks then autoclaved at 121 °C for 15 min. After that, the flasks were transferred to a rotational incubator (100 rpm) at 35 °C for 18–20 hr. Then, the glass slides were taken off from the flasks and rinsed twice with 10 ml of PBS solution to eliminate unattached cells. The glass slides were stained with 2% crystal violet for 5 min, rinsed, exposed to the air, and a snapshot was taken by an optical microscope with a digital camera (22).

Anti-adhesion activity
The effect of lactobacilli CFS on the P. aeruginosa adherence was determined with a glass slide method. To investigate P. aeruginosa adhesion on the glass slide, 1 ml of overnight cultured P. aeruginosa (10^8 CFU/ml) was inoculated into a flask containing 100 ml of sterile BHI broth supplemented with 1% sucrose and one slide with and without 10 ml L. casei supernatant. The glass slides were washed in distilled water after cleaning detergent solution, then dried and transferred to flasks then autoclaved at 121 °C for 15 min. After that, the flasks were transferred to a rotational incubator (100 rpm) at 35 °C for 18–20 hr. Then, the glass slides were taken off from the flasks and rinsed twice with 10 ml of PBS solution to eliminate unattached cells. The glass slides were stained with 2% crystal violet for 5 min, rinsed, exposed to the air, and a snapshot was taken by an optical microscope with a digital camera (22).

High-performance liquid chromatography (HPLC) analysis
Organic acids were quantified by the HPLC method. L. casei strains were cultured in MRS broth medium for 24 hr. CFS was prepared by centrifugation of an overnight culture of probiotic bacteria and filtered with a 0.2 μm pore size filter. The filtrate was then re-cultured for 72 hr in the MRS broth medium to make sure that it is sterile. Twenty µl of the filtrate was injected into the HPLC system. Chromatographic separation was achieved by reverse phase column chromatography (C18 column) through an aqueous mobile phase. The UV absorbance was recorded at 60 °C. Quantification of probiotic supernatants was performed on an Agilent 1100 series HPLC system (23, 24).

Animals
In the present study, 30 healthy intact male Wistar rats were used. The experimental animals were purchased from the Pasture Institute of Tehran, Iran. The animals were randomly divided into 5 groups (n=6) and housed in a standard animal room (temperature 20–22 °C, 12-12 hr dark-light cycle, 37% humidity). They had free access to water and food pellets before and during the experiments at all times (25, 26).

Burn Wounds
The study protocol was approved by the Animal Ethics Committee of Islamic Azad University, Qom branch. The rats’ backs were shaved immediately before burning, then they were anesthetized by intraperitoneal injection of ketamine. A cone-shaped metallic device, made of iron with a diameter of 1 cm was used for the creation of burn wounds. The skin was for 5 sec heated by a heated metal in boiling water. Acetaminophen oral solution was administrated to the rats for enhancing the pain thresholds (27).

Groups and induction of second-degree burn wounds
After induction of the second-degree wounds, MDR P. aeruginosa was dropped on the burn surfaces with 200–300 CFU (day 0). The animals were divided into four groups (n=6) as follows: the negative control (no drugs were used in this group), control (silver sulfadiazine 1% treatment as a gold standard treatment), L. casei...
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supernatant treatment groups. The supernatant was sprayed for treatment every day. The groups were observed for 28 days (28, 29).

Wound measurement area (Ruler method)
Assessment of the wound tissue healing process was done through visual inspection and wound size measurements (width and length) through a disposable ruler at the same time per week (30-32).

The assessment of wound healing process was applied through two formulas:

1) Open wound area (% OWA):

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\% \text{ OWA} = \frac{(\text{WAn})}{\text{WAo}} \times 100
\]

(WAn wound area size per week and WAo wound area size on day zero)

2) Wound healing rate:

Wound healing rate = \((\text{wound area size on day zero - wound area size on day X}) / \text{wound area size on day zero} \times 100

(X= on days 7, 14, 21, and 28)

Histopathological study
Histopathologic follow-up examinations were used for the skin samples taken from each group on the 7th, 14th, and 21st days. The following processes were applied:
The biopsy specimens were fixed in 10% formaldehyde followed by paraffin embedding. Then, the samples were stained using Hematoxylin-Eosin (H&E) staining techniques.

In the next step, the histopathologic examination was done under an Olympus optical microscope and performed by 2 pathologists blinded to the experimental groups. Finally, inflammation, re-epithelialization, neovascularization, the occurrence of granulation tissue, and collagen accumulation were considered in these specimens via the scoring system used for histopathologic examination, as previously described (33, 34).

Statistical analysis
The collected data were analyzed through one-way ANOVA and Tukey tests by using SPSS software version 23.0 (SPSS, Chicago, IL). P-value<0.05 was considered as significant level.

Results
Antibiotic susceptibility and Biofilm formation
Out of all P. aeruginosa isolates one of them was selected with multi-drug resistance. After that, P. aeruginosa biofilm was identified using glass slides and SEM methods (Figures 1 and 2).

Anti-adhesion activity
The glass slide method proved the presence of L. casei supernatant dramatically reduced the process of attachment (Figure 3).

High-performance liquid chromatography (HPLC) analysis
HPLC analysis indicated that supernatant inhibitory effect can be due to the main organic acids, including lactic acid, acetic acid, citric acid, and succinic acid. Figure 4 shows the presence of different organic acids using HPLC analysis.

Burn wound healing
The effect of treatments on fibroblastic cells showed that the group treated by supernatant of L. casei had the most number of fibroblastic cells compared with the

Figure 1. Pseudomonas aeruginosa biofilm formation on the glass slide

Figure 2. Scanning electron microscopy (SEM) of Pseudomonas aeruginosa biofilm cells

Figure 3. Pseudomonas aeruginosa adhesion on the glass slide. 1: Control group (in the absence of Lactobacillus casei supernatant). 2: Experimental group (in the presence of L. casei supernatant)
non-treated group. Also, the mean number of fibroblastic cells indicated that there was a significant difference in the mean number of fibroblastic cells between the group treated by the supernatant of \textit{L. casei}, sulfadiazine, and non-treated group on day 28 (Figure 5). The results from the assessment of the weekly wound surface area are shown in Table 1.

**Histopathological study**

The results of the burn wound healing process by

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**Table 1. Area measurement via ruler based method**

| Treatment          | Test results | Days 7 | Days 14 | Days 21 | Days 28 |
|--------------------|--------------|--------|---------|---------|---------|
| Control            | Wound measurement area percentage | 100    | 49      | 9       | 4       |
|                    | Wound healing rate percentage     | 0      | 51      | 91      | 96      |
| \textit{Lactobacillus casei} | Wound measurement area percentage | 25     | 9       | 0       | 0       |
|                    | Wound healing rate percentage     | 75     | 91      | 100     | 100     |
| \textit{Silver sulfadiazine} | Wound measurement area percentage | 100    | 25      | 4       | 0       |
|                    | Wound healing rate percentage     | 0      | 75      | 96      | 100     |

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**Figure 4.** Presence of different organic acids analyzed by HPLC. LA: lactic acid, AA: acetic acid, CA: citric acid, SA: succinic acid

**Figure 5.** The appearance of the burn wound area in treated groups with (supernatant of \textit{L. casei} and Silver sulfadiazine) at Wistar rats on; A. 21 days and B. 28 days
sectioning and H&E staining on the burn day 28 showed the largest thickness of the epidermis and dermis layers in the treated group with L. casei compared with another group (silver sulfadiazine) indicating the high efficiency of the supernatant of L. casei (P<0.05) (Figure 6).

Discussion

Bacterial adhesion to host cells is one of the essential steps in the development of infections (34, 35). It can be classified into two major categories, that is, non-specific and specific (35, 36). Non-specific adhesion is reversible and occurs by physiochemical forces. In this type of adhesion, mobile bacteria move toward chemoattractants, such as glucose and oligopeptides, and grow on surfaces. Chemotaxis regulates and prepares bacteria-host cell interactions (35, 36). On the other hand, specific adhesions are irreversible and occur when specific bacterial adhesions (e.g., capsules, pili, and some exopolysaccharides) interact with host cell receptors, which contribute to initial bacterial infections. Today, researchers believe that by preventing bacterial-host cell interactions, infections can be avoided (37-39).

Due to scarcity of data on the consumption of antibiotics, the prevalence of MDR bacteria is on the increase, which may be threatening for human health; therefore, researchers are seeking new safe alternative treatments (40). According to many studies, probiotic bacteria, especially lactobacilli and their secondary metabolite products, can be proper candidates for controlling and preventing infections (41, 42). In the present study, the antimicrobial and anti-adhesive effect of L. casei supernatant against P. aeruginosa in burn wounds was investigated.

There are various analytical techniques for the measurement of organic acids, such as titrimetric methods, gas chromatography, colorimetric assays, and enzymatic methods (43). The HPLC method is a separation method for the quantitative measurement of organic acids within a short period, which requires the minimum sample volume for separating one or more organic acids (44). In the present study, the HPLC analysis showed that the supernatant of L. casei contained high concentrations of lactic acid, acetic acid, citric acid, and succinic acid. These results showed that the increase in organic acids, especially lactic acid, may account for the remarkable antagonistic activity of L. casei.

In the current study, the antagonistic effects of probiotic Lactobacilli against P. aeruginosa were evaluated. The results showed that the L. casei supernatant had a good inhibitory effect on P. aeruginosa growth. Vuotto et al. (2014) reported that probiotic culture and their supernatant were able to inhibit P. aeruginosa burn infections (45). One of the main properties of probiotic Lactobacilli which makes them so potent to antagonize pathogenic bacteria is the production of antimicrobial substances (46, 47). Some studies have demonstrated that the antibacterial compounds of probiotic Lactobacillus can interact with the bacterial cell membrane by reducing the pH of the environment, denaturing proteins, and finally, eradicating pathogens through the physiology and morphology of the cytoplasmic membrane of bacteria and leakage of its content (42).

Today, several distinct surface Lactobacilli proteins are predicted to enhance binding to the surface of pathogenic bacteria, which inhibits pathogen attachment and colonization (48). As described in other studies, probiotic bacteria through coaggregation with pathogenic bacteria can prevent their growth and attachment to host cells, as confirmed in the present study (47). Since the anti-adhesive effects of probiotic bacteria are vital for preventing the primary stage of infection, the anti-adhesive activity of L. casei was investigated in the current study. Researchers have highlighted the anti-adhesive role of probiotic bacteria, but the mechanisms of their action remain undetermined (45, 48). A study (2019) demonstrated that P. aeruginosa colonization and adhesion were inhibited by Lactobacillus plantarum supernatant (49). Also, Bienenstock et al. (2013) demonstrated that probiotic bacteria prevent pathogenic bacteria attachment due to competition for specific host cell receptors (50).

In the current study, macroscopic and microscopic assessments of the wound area, treated with the supernatant of L. casei, indicated a greater recovery than
the control group, especially on days 21 and 28 after burning induction. The pathological findings revealed that *L. casei* significantly accelerated the repair process in full-thickness wounds on day 21. Our experimental results are consistent with the findings of some previous studies, in which the positive role of topical application of probiotic bacteria in the wound healing process was documented.

Researchers (2017) evaluated the effect of local treatment of second-degree burns with *L. acidophilus* on male rats. Their results showed that the treatment of burn wounds with *L. acidophilus* could accelerate the recovery of second-degree burn wounds (27). Besides, they found that probiotic bacteria exerted beneficial effects on different aspects of the wound healing process, such as the reduction of the inflammatory response and acceleration of granulation tissue formation and re-epithelialization.

According to one study (2012), the use of kefir grains for the treatment of thermal burns led to the faster recovery of ulcers, compared with treatment with silver sulfadiazine (28).

**Conclusion**

With the increasing incidence of MDR *P. aeruginosa* in hospitals and clinics, new treatments are necessary for preventing the pathogen’s growth without inducing greater resistance. These results indicated that the supernatant can be successfully and conveniently used for treating the mouse model of burn wounds. They are easy to use, cost-effective, and safe for use by humans and they also can be used as alternatives to chemical drugs.

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**Ethical Approval**

This study was approved by the Islamic Azad University, Qom branch Ethical Committee (approval ID: IR.IAU.QOM.REC.1397.021).

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

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