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Full Length Research Paper

Study of cutaneous wound healing in rats treated with *Lactobacillus plantarum* on days 1, 3, 7, 14 and 21

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Probiotics are microorganisms in the gastrointestinal tract that help the immune system and reduce inflammation. The beneficial effects of these bacteria on ulcer healing have been proved but little research has been done in the field of cutaneous wound healing. Therefore, the purpose of this experiment is to study the effect of *Lactobacillus plantarum* on cutaneous wound healing. Some strains of *Lactobacillus* isolated from traditional dairy products of Iran are investigated for exopolysaccharide (EPS) production applying the phenol-sulfuric acid method. *L. plantarum* was selected for its high exopolysaccharide and mucoid coloniese production. Wistar male rats divided into 3 groups; Experimental, control and negative control (n = 5) and a full-thickness wound (1.5 × 1.5 cm) was made on the back of each rat (45 rats in 3 groups). After 24 h of injury, two groups, control and experimental, were treated by eucerin, and eucerin contained *L. plantarum*, respectively, but the negative-control group did not receive anything. On days 1, 3, 7, 14 and 21, rats were killed and wound samples were collected for histological and statistical studies. *L. plantarum* significantly decreased wound area as compared to other groups and increased wound healing. Histological study on day 3 showed significant increase in the number of neutrophils and significant increases in macrophages and fibroblasts ($p < 0.001$). Also significant reduction in neutrophils, macrophages and fibroblasts numbers was observed on day 21 ($p < 0.001$). The current study presented a significant decrease in inflammation and an acceleration of wound healing in *L. plantarum* treated rats.

Key words: *Lactobacillus palantarum*, cutaneous wound, probiotic, healing, exopolysaccharide.

INTRODUCTION

Wound is a breach formed in the normal continuvum of the cellular and molecular structure of the body, thereby creating a disruption in the cellular, anatomic and as well as in their functional continuity. Wound healing or wound repair is an intricate process in which the skin or organ or tissue repairs itself after injury (Cordoso et al., 2010; Nilani et al., 2011). Wound caused can be healed by a spontaneous process in the organism through a cascade of events, which starts by switching on various chemical signals in the body; this facilitates the restoration of anatomical continuity and function (Savunen and Viljanto, 1992; Schwartz, 1984). While partial thickness wound heals by mere epithelialization, the healing of full thickness wound which extends through the entire dermis involves more complex well-regulated biological events.

The healing process begins with the clotting of blood and is completed with remodeling of the cellular layers of the skin. The whole process can broadly be classified into 5 overlapping phases, namely, inflammation, granular tissue formation, re-epithelialization, matrix production and remodeling (Janis et al., 2010).

Despite the huge advances in modern medicine, most people in the developing world still rely on traditional and effective knowledge to treat illness and disease. The valued trado-medical practices providing affordable healthcare have been recognized by the World Health

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Organization (WHO) (Olajuyigbe and Afolayan, 2011). Different chemical agents have been used for wound healing but each agent has negative side effects (Weinstein-Oppenheimer et al., 2010; Sasidharan et al., 2010; Khalid et al., 2011; Ahmad et al., 2011). Probiotics are microorganisms that naturally live within the human body and assist with its healthy functioning (Salminen et al., 2010). These "good bacteria" (although some of them are yeasts or other non-bacterial microorganisms) help with tasks including digestion, immune function, nutrient absorption and bowel function (Laws et al., 2001; Yamaguchi et al., 2005). They naturally occur in many foods, migrating either from dirt in which plants were grown or from fermentation (as in yogurt or sauerkraut).

The foundation of good health lives in the digestive system, where countless billions of beneficial microflora protect against harmful pathogens, assist in proper digestion and generally guard the body against foreign invaders that cause disease. If natural gut bacteria are imbalanced or functioning improperly, the body becomes highly susceptible to disease. When such bacteria are balanced, healthy and flourishing, it is very difficult for harmful bacteria and viruses to take hold. Research continues to validate the notion that eating probiotic-containing foods and even supplementing with probiotics helps to promote good health without any negative side effects (Settanni and Moschetti, 2010).

Probiotic, like many other bacteria, are able to produce several types of polysaccharides that are classified according to their location relative to the cell. Those that are excreted outside the cell wall are called exocellular polysaccharides or EPSs. These can form an adherent cohesive layer and are called capsular polysaccharides (Burd and Huang, 2008; Low et al., 1998). The EPSs also can either be loosely attached or be completely excreted into the environment. The bacterial EPSs are not used as energy sources by the producer microorganism. They probably have a protective function in the natural environment, e.g. against desiccation, phagocytosis and predation by protozoa, phage attack, antibiotics or toxic compounds and osmotic stress (Shivanada et al., 2010, Arul et al., 2007; Vander Wal et al., 2009). EPSs also have a role in cell recognition, in adhesion to surfaces and in formation of biofilms facilitating the colonisation of various ecosystems. Also, phosphate groups in EPS are highly regarded in activating macrophages and lymphocytes (Berger et al., 2005; Foligné et al., 2010).

Despite different strains and species, few researches on the effect of these bacteria on cutaneous wound healing have been done. Our group has demonstrated that healing of skin wounds occur faster using probiotics. Since wound contraction and cell regeneration are compatible with each other, we also suggest that *Lactobacillus plantarum* has caused the inflammation stage of the wound to pass by faster to the end. In this paper, we showed a study on the effect of high EPS-production lactic acid bacteria from Iranian traditional cheese on healing process and inflammation factors (Ebrahimi et al., 2011).

MATERIALS AND METHODS

Male Wistar rats weighing 250 to 280 g were housed under normal conditions of light, room temperature and humidity. The dorsal skin was shaved and cleaned with betadine under anesthesia, and one open full-thickness wound that was approximately 1.5 × 1.5 cm long was incised up to the level of subcutaneous adipose tissue by means of a surgical blade. After the wounding process, each mouse was housed in a sterilized cage and given autoclaved food and redistilled water in order to prevent bacterial infection. After 24 h following the wounding, the wounds in the control and experimental groups were treated topically once daily.

The animals were separated into three groups (n = 25), negative control, control and experimental, for the days 1, 3, 7, 14 and 21 (5 rats for each day). Twenty-five rats were kept as negative controls and their wounds left untreated. The second group of 25 rats was used as controls whose wounds were treated with eucerin alone. In the third set of 25 rats, the experimental group, the wound was treated with eucerin contain *L. plantarum*.

In this study, some strains of *Lactobacillus* was isolated from Iran's traditional dairy products by Ebrahimi et al. (2011). The *Lactobacilli* strain was identified by 16S rRNA gene sequencing and showed 98% similarities to *L. plantarum* (Gen Bank accession no GQ423760). So, *L. plantarum* which had been previously approved for the ability of survival and growth and having high EPS production was selected (Ebrahimi et al., 2011).

The *Lactobacillus* was then cultured in an deMan, Rogosa and Sharpe (MRS) agar medium and was incubated for 48 h at 37°C. Following, the bacteria on the surface of the culture were collected with a sterilized kolle handle. In order to prepare the ointment, 10^{10} to 10^{11} CFU/ml bacteria that had been collected every day after a 48 h culture were added to 4 ml of eucerin for each of the 5 mice as a preserver. The culture and eucerin were mixed thoroughly until a uniform income was produced and immediately applied on the wounds in the experimental group. For the control group, however, we used 5 ml of eucerin for each of the 5 mice.

On days 1, 3, 7, 14 and 21 after incision, the remaining wound was measured using a calliper. Subsequently, the area of the wound was calculated from these measurements as a function of time that had passed during the treatment. The degree of contraction was determined from the difference between the initial and final areas of the wounds. Mean values were then calculated. The results were expressed in cm2. The percentage of wound closure was calculated as the following: [(Area of original wound - Area of actual wound)/Area of original wound] × 100. The inside edge of the calipers exactly matched the edge of the wound.

The rats were sacrificed at 1, 3, 7, 14 and 21 days after wounding with ether and the tissues from the wound site include the whole thickness of the skin, and the surrounding skin of the individual animal was removed. These samples were then separately fixed in 10% formalin, dehydrated through graded alcohol series, cleared in xylene and embedded in paraffin wax. Serial sections were stained with haematoxylin/eosin (H&E). Then, sections were used for counting the amount of fibroblasts and inflammation cells such as neutrophils and macrophages. These cells were counted in 100 fields of view in different sections from wound area and numbers were expressed in percentage. This study was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) as in our previous studies.

All values were expressed as the mean ± S.D. Data from diffusion experiments were evaluated using the least squares method and adjusted to the data. Animal group comparisons used ANOVA followed by Post-Hoc Tukey multiple range tests using the Statistical Package for the Social Sciences (SPSS) for Windows.

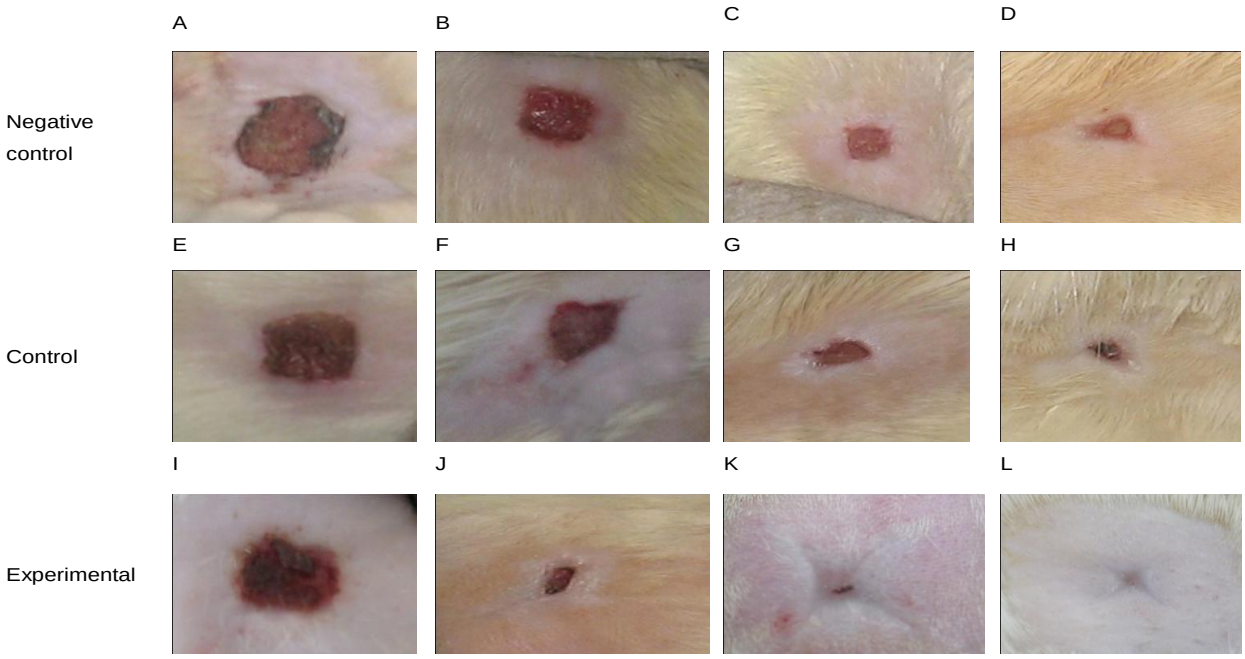

Figure 1. Progress of cutaneous wound healing in negative-control rats untreated, control rats treated with eucerin and experimental rats treated with *L. plantarum.* Wounds of the dorsal skin at 3 (A, E and I), 7 (B, F and J), 14 (C, G and K), 21 (D, H and L) days after incision.

A p-value of ≤ 0.05 was considered significant.

RESULTS

Healing wounds were compared between non treated rats as negative control groups, rats treated with eucerin as control groups and *L. plantarum*-treated rats as experimental groups during the repair process. In contrast, the progress of wound healing in the control and negative control groups appeared to be impaired (Figure 1A to H). In all groups, wounds were covered by a dehydrated wound crust or scab at day 1 after incision, and the scab was almost gone at day 14, revealing a thin residual skin defect (Figure 1K). By 21 days after the incision, the wounds fully healed in all *L. plantarum*-treated rats based on the macroscopic closure of the incision interface and restoration of an epithelial cover (Figure 1L). In most *L. plantarum*-untreated rats, the scab remained until 21 days, or there was a still gapping and red wound field with a scaly surface lacking an epidermal covering (Figure 1D and H). It took over 3 weeks for these aberrant wounds to heal and to be covered with an epidermis (data not shown). The wound's diameter in the experimental group, which was treated with *L. plantarum*, experienced a faster reduction than both the negative control and the control.

In addition, according to the results of measurement of the longitudinal or the perpendicular length of wounds, the wound sizes of negative control and control groups were significantly larger than that of experimental group at all days after the incision (Figure 2).

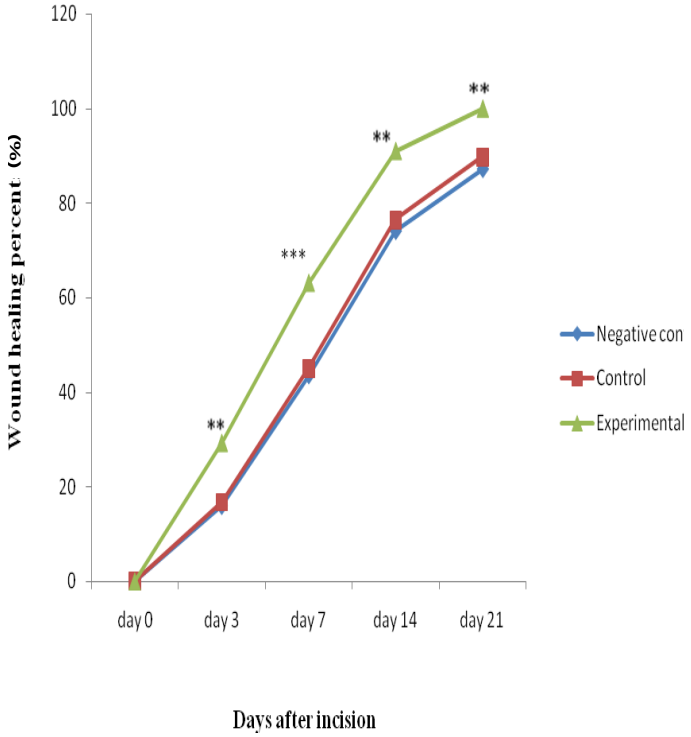

Figure 2. Wound healing percentage in skin lesions. Data represent untreated animals (negative control), animals treated with eucerin (control), and animals treated with *L. plantarum* (experimental). **p < 0.01; ***p < 0.001 as compared with corresponding control and negative control using one-way ANOVA.

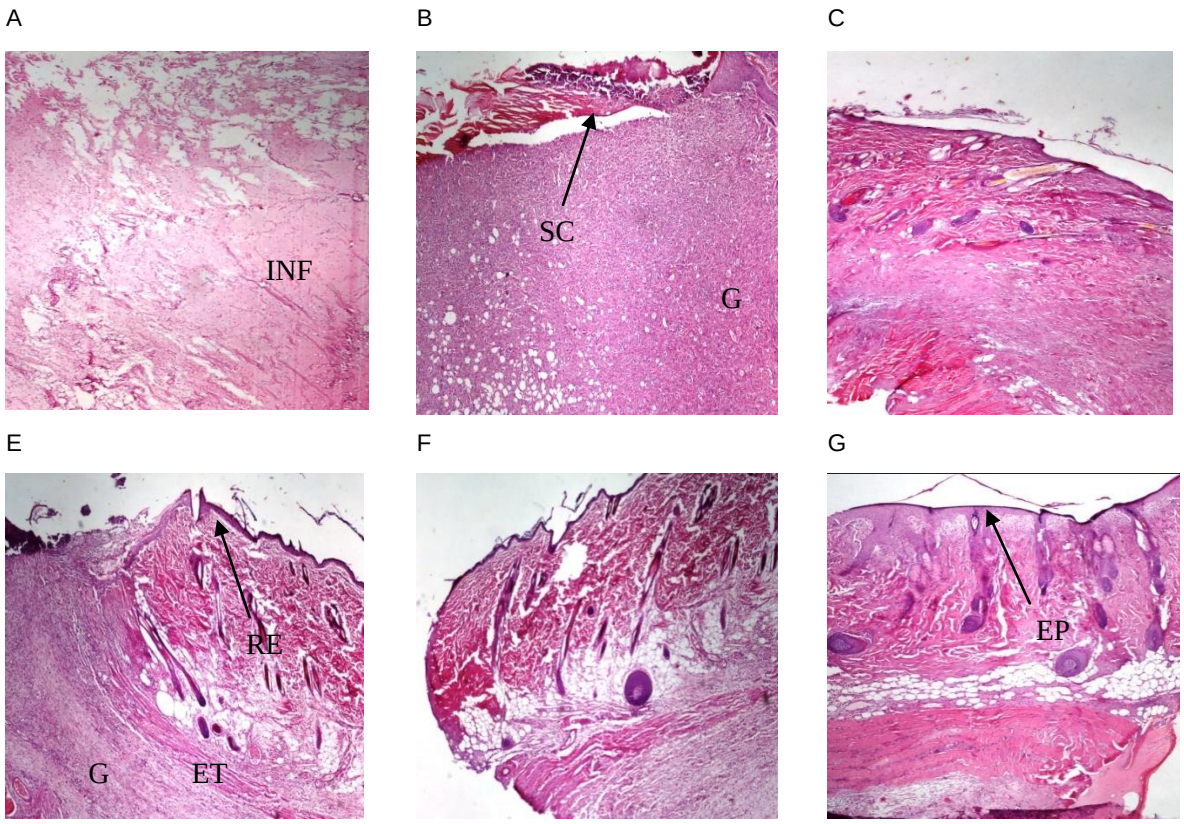

Figure 3. Histological changes during the wound-healing process. Histology of the wound tissue of experimental (D to F), negative-control and control (A to C) groups at day 3 (A and D), day 14 (B and E) and day 21 (C and F) after incision; scab (SC), epithelial tongue (ET), granulation (G), inflammation (Inf) and re-epithelialization (Ep) regeneration edge (Re).

To determine the importance of *L. plantarum* for wound healing, we histologically analyzed full-thickness excisional wounds of all groups at 1, 3, 7, 14 and 21 days after the incision. In this study, although there is an increase in the wound area within the first days of the study, the results are justified since the increase is in compliance with the inflammation phase. At day 3, tissue regeneration from the edges of the wounds of *L. plantarum*-treated rats, artery influence hematopoietic and migration of fibroblasts from deep parts of wounds into scab were observed. The number of inflammation cells was less than zero day (Figure 3D); on the other hand, In some of the superficial and deep parts of the wound, there was diffuse bleeding and inflammatory cells, particularly neutrophils within the loose connective tissue was visible in negative-control and control groups (Figure 3A).

At day 7, the layer of the epidermis grew thick and granular tissue with full cells and artery completely occupied the wound in the experimental group. In the other groups, at day 7, there were no much differences with day 3. Fourteen days after the incision, in wounds of *L. plantarum*-treated rats delicate and numerous strands of collagen still had not achieved complete organization and despite a very small scab, tissue regeneration had better quality (Figure 3E). By contrast, in other groups, re-epithelialization were markedly delayed, still, there was a scab in the center of the wound and granulation tissue remained, this was similar to the quality of experimental group at day 7 (Figure 3B). Finally, at day 21, wounds of *L. plantarum*-treated rats showed complete re-epithelialization and a normal epidermis covered the wound area, collagen fibers were thicker and more dense and some cutaneous annexes, such as sebaceous glands and hair follicles in the center of the scar tissue was formed (Figure 3F). But, the wound of untreated rats still had not been fully restored and inflammatory cells in scar tissue were observed. Re-epithelialization of the wounds showed incomplete and epidermal hyperthickening in the wound area (Figure 3C).

Also, the reduction in the number of neutrophils in the experimental group on days 3 and 7 ($p < 0.001$), 14 and 21 ($p < 0.01$) of the study, was statistically significant when contrasted to the negative control and control groups (Figure 4).

On day 3 of the study, the number of macrophages in the experimental group ($p < 0.001$) showed a significant increase in contrast to the control and negative control groups. Cell counting results also confirmed a significant reduction in the number of macrophages on days 7, 14

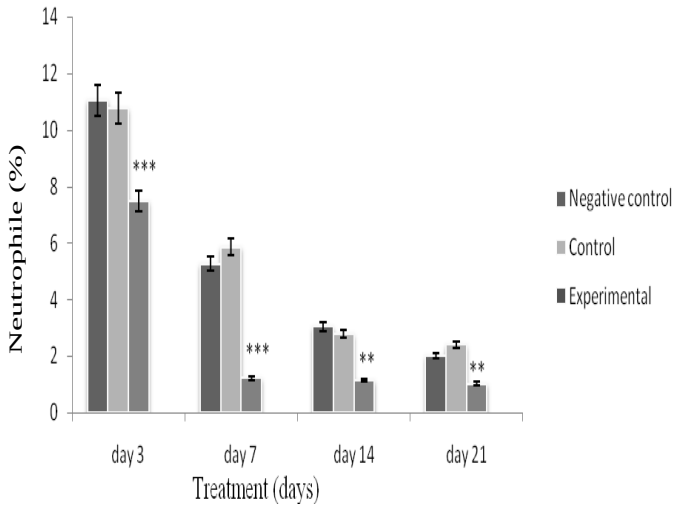

Figure 4. The total amount of neutrophile in negative control, control and *L. plantarum* treated (experimental) at different days. Values are expressed in mean ± SE. **p < 0.01; ***p < 0.001 as compared with corresponding control and negative control using one-way ANOVA.

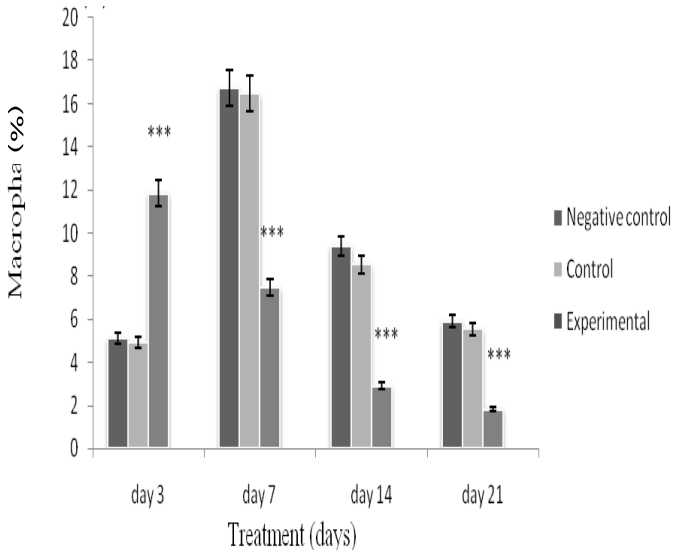

Figure 5. The total amount of macrophage in negative control, control and *L. plantarum* treated (experimental) at different days. Values are expressed in mean ± SE. ***p < 0.001 as compared with corresponding control and negative control using one-way ANOVA.

and 21 (p < 0.001) in the experimental group than the control and negative control groups (Figure 5).

The number of fibroblasts in the experimental group on day 3 (p < 0.01) and 7 (p < 0.001) of this study showed a significant increase in contrast to the control and negative control groups. Cell counting results also confirmed a significant reduction in the number of fibroblasts on days

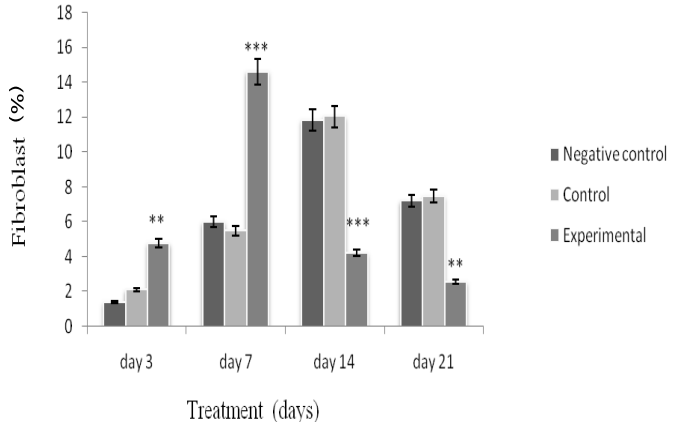

Figure 6. The total amount of fibroblast in negative control, control and *L. plantarum* treated (experimental) at different days. Values are expressed in mean ± SE. **p < 0.01; ***p < 0.001 as compared with corresponding control and negative control using one-way ANOVA.

14 (p < 0.001) and 21 (p < 0.01) in the experimental group than the control and negative-control groups (Figure 6).

DISCUSSION

The clinical treatment of skin loss continues to be a major problem in surgical procedures. A therapeutic agent selected for the treatment of wounds should ideally improve one or more phases of healing without producing deleterious side effects. In the early twentieth century, Metchinkoff (1907) was the first person who proposed that the ingestion of probiotics has beneficial effects on natural micro intestine. The only study on the use of these bacteria in cutaneous wound healing was done by Rodrigues et al. (2004). By using kefir, the study found the bacteria both to decrease inflammation and to restore the wounded area (Rodrigues et al., 2004).

In this study, we have shown that wound healing was markedly accelerated in *L. pelantarum*-treated rats. The process of cutaneous wound healing involves the formation of a matrix rich in fibrin and fibronectin in the wound field, infiltration of neutrophils and macrophages, proliferation of epidermal keratinocytes at the wound edges, formation of granulation tissue and re-epithelialization (Raf, 1991; Qiu et al., 2006; Werner and Grose, 2003). In histological analysis, incisions in the dorsal skin of experimental group showed a marked increase of scab tissue, and the progress from granulation formation to re-epithelialization was accelerate when compared with that in control and negative control groups. These results indicate that *L. plantarum* could play a role in the early stage of the wound healing process.

In terms of coordination, there was no difference between the histometric results and the hypothesized theory

The reduction of the wound size was an effect of a reduction in the wound's inflammation.

Fibroblasts are responsible for synthesis of collagen fibers and areolar connective tissue, and when they penetrate into the areolar connective tissue formed in the tissue regeneration, they cause connective tissue maturation. Since *L. plantarum* stimulates the activity of macrophages and fibroblast cells, these two cells interact with each other, with stimulation of newly built vessels penetrating into the young granulation tissue, improving faster wound healing. After the blood plasma from this process comes in contact with collagen, the clotting reaction in which platelets accumulate and become granulated is initiated (Arul et al., 2007). This phenomenon causes hemeostasis, which attracts inflamed cells to the wound site. These platelets-born are responsible for the production of tissue growth factor and platelet-derived growth factor which play a role of chemical absorbent for neutrophils and monocytes, and are used in the subsequent phases (Boirivant and Strober, 2007).

In relation to this issue, rats of control and negative control groups also exhibit bleeding near the wound site for an extended period of time, even after the surface wound has closed but wounds of the experimental group had a higher strength. Collagen fibers make similarities between the wound after the initial repair tissue before surgery and prevent creating white ugly closure (Price et al., 2008).

Our review is focused on the day 3 which is the inflammation stage of wound healing. We found that the total number of neutrophils in the experimental group was significantly less than both the control group and the negative control group. Tissue pathology studies show a reduction in neutrophil cells in the wound area. Since neutrophil cells are central in the wound's inflammation, an issue associated with an increase in the area's macrophages and the decrease could be the evidence of the positive impact of the product in improving treatment of inflammations (Heidari et al., 2004; Ebrahimi et al., 2009). Since probiotics reduce inflammation (Rea et al., 2008) therefore, this reduction may accelerate the healing process, reduce inflammation and subsequently, re-epithelialization start earlier.

The present investigation describes some unique features of the therapeutic effect of probiotics, specifically in regards to the wound healing process of the dermal layer of rats. In contrast to untreated wounds, wounds treated with *L. plantarum* showed a larger number of infiltrating cells, such as, macrophages, neutrophils and fibroblasts.

Conclusion

The current study presented a significant decrease in inflammation and an acceleration of wound healing in *L. plantarum* treated rats as compared to the control and negative control groups. Further studies are required to develop a detailed mechanism of *L. plantarum* during cutaneous wound healing.

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