Influence of Lactobacillus acidophilus and Lactobacillus plantarum on wound healing in male Wistar rats - an experimental study

Abstract
Context and Aim: Probiotics have been documented with various pleiotropic effects other than improving general gut health, but the potential benefits of strain-specific Lactobacillus on wound healing are unknown. Hence, the objective of the study is to evaluate and compare the wound healing property of Lactobacillus acidophilus and Lactobacillus plantarum on various wound models in male Wistar rats. Subjects and Methods: Excision wound, resutured incision wound, and dead space wounds were inflicted under light thiopentone anesthesia in male Wistar rats (n = 6, in each group). The rats received one of the Lactobacillus orally as per their weight for a period of 10 days in resutured incision (assessed by wound breaking strength) and dead space wounds (granuloma dry weight, histopathology of granulation tissue, and biochemical hydroxyproline estimation), whereas in excision wounds, treatment was monitored by planimetry. Data were expressed as mean ± standard error of mean and analyzed by ANOVA followed by Tukey’s multiple post hoc test. P < 0.05 was considered as statistically significant. Results: L. acidophilus showed a significant difference (P < 0.05) in all the three models, namely, enhanced wound contraction and decreased days for complete epithelization in excision wound; increased breaking strength in resutured incision wound; increased granuloma dry weight and cellular infiltration in granulation tissue with marked increase in collagen content indicating wound healing. Conclusions: The study suggests that the wound healing activity of L. acidophilus if could be extrapolated to clinical situations may decrease dosage and duration of treatment and can be a potential adjuvant to reduce hospitalization with efficient recovery after injury and sustained good health.

Keywords: Dead space wound, excision wound, Lactobacillus acidophilus, Lactobacillus plantarum, resutured incision wound

Introduction
Wound healing is a natural curative response to tissue injury. Healing consists of a series of cellular events that promotes resurfacing, reconstitution, and restoration of the tensile strength of wounded skin.[1] Healing is an organized process, generally explained in terms of four overlapping characteristic phases: hemostasis, inflammation, proliferation, and maturation.[2] During hemostasis, platelets play a crucial role in clot formation. Debridement of injured tissue takes place during the inflammatory phase by inflammatory cells. Epithelialization, angiogenesis, and fibroplasia occur during the proliferative phase. In the meantime, granulation tissue forms and the wound begins to contract. Eventually, during the maturation phase, collagen forms tight cross-links with each other and with protein molecules, increasing the strength of the scar.[3] The injuries destroy the physical skin barrier that usually prevents the invasion of microorganisms and consequently provides novel sites for bacterial colonization, infection, and clinical sepsis. Delayed healing may result in loss of function or poor cosmetic outcome.[3] It is proved that healing environment may be manipulated to enhance or to accelerate the healing process.

Probiotics are defined as “live microorganisms which when administered in adequate amount confer a health benefit to the host.”[4] Probiotics have been reviewed extensively for years mainly on improving general gut health. There are increasing evidences to suggest that the functioning of the immune system can be modulated by bacteria in the gut at both systemic and mucosal level.[5] Probiotics have been documented to show various pleiotropic effects such as lowering blood

Access this article online
Website: www.ijabmr.org
DOI: 10.4103/ijabmr.IJABMR_329_16
Quick Response Code:
cholesterol levels, antihypertensive effects, providing therapeutic benefit in inflammatory bowel disease and Helicobacter pylori infection, reduce allergic reactions, prevent dental caries, reduce risks of cancers, and exhibit immunomodulatory effects which are health-promoting effects, beyond gut well-being.[5-7] Various new mechanisms by which probiotics exert their beneficial effects have been acknowledged and it is now clear that significant differences exist between different probiotic species and strains leading to the selection of organisms in a more rational manner to treat a specific disease.[5]

Recently, a new concept “gut–brain–skin axis” hypothesis, as proposed by Arck et al.[8] suggested that modulation of the microbiome by consumption of probiotics can exert profound beneficial effects, for example, on skin inflammation and skin homeostasis. Even few increasing evidences said that host immune response can be elicited by bacterial compounds such as cell wall fragments, their metabolites, and dead bacteria followed by improvement in skin’s barrier function. This concept is supported by some evidence revealing that peptide-containing cells in skin, brain, and gut are connected by a common embryonic origin.[4] Furthermore, the comorbidity of inflammatory bowel and skin diseases may strengthen the link between the gut and skin.[5] A study provided a historical perspective to the current investigations and clinical implications of the gut-brain-skin connection in acne.[9] Stokes and Pillsbury hypothesized that Lactobacillus acidophilus cultures can act as remedy in stress-induced alteration of normal intestinal microflora, increased intestinal permeability, and systemic inflammation.[10]

Few experimental studies explained that lactic acid bacteria have increased the number of T-lymphocytes, CD4+ cells, and antibodies and have increased the secretion of immunoglobulin A, phagocytosis, and respiratory macrophages, as immediate response. They also stimulated the lymphocyte proliferation, the natural killer cell activity, the production of interleukins (ILs), interferon (IFN), and tumor necrosis factors (TNFs).[11] Cytokines have emerged as an important mediators of wound-healing events. Research work done to assess the immunomodulatory effect of Lactobacillus species on the cytokine profiles and proliferative response of human peripheral blood mononuclear cells concluded that all Lactobacillus strains tested were capable of inducing the production of IL-1b, IL-10, IFN-α, and TNF-α.[12] These cytokines are necessary to stimulate angiogenesis, act as mitogen for fibroblasts, and promote synthesis of collagen.[1,2]

The association of symbiotic gut organisms with various physiological and disease processes has been the focus of intense research over recent years. Increasing demand for probiotic usage further boosts the urge to understand the role of these bacteria on cutaneous wound healing. Lactobacillus plantarum and L. acidophilus are the most predominant Lactobacillus species in the gut, and several strains of these species are considered as probiotics. There is no much data available on the comparative wound healing capacities of these two organisms. Hence, our aim is to study the effect of L. plantarum and L. acidophilus on various cutaneous wound models, namely, resutured incision, excision, and dead space wounds in male Wistar rats.

Subjects and Methods

Source of data

Animals

Healthy male Wistar rats weighing 150–200 g procured from central animal research facility of the institute were housed individually under standard environmental conditions, fed with pellet rodent diet and water ad libitum and were allowed to acclimatize for a week. The study was approved by Institutional Animal Ethics Committee. The animals were starved overnight before the day of experimentation and divided into control and treatment groups (n = 6) for each wound model. All the wounding procedures were carried out under thiopentone anesthesia with aseptic precautions. Experiments were conducted according to the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines for use and care of experimental animals.[13]

The drugs required for this experiment were obtained from pharmaceutical companies. The test drugs were administered orally in their therapeutic equivalent doses (1 × 10⁶ colony forming units [CFU]/g) as calculated with the help of conversion table devised by Paget’s and Barnes.[14] Drugs were administered once in 24 h for 10 days, to study their effect on dead space wound and resutured incision wound healing, while they were administered once in 24 h till complete closure of excision wound.

Wound models

• Wound models were prepared through following procedure:[15-18] Excision wounds were made by excising the full thickness circular skin approximately 2.5 cm diameter (approximately 500 mm²) from the nape of the neck and animals were placed in individual cages. Wound healing was assessed by tracing the raw wound margin on polythene paper to determine the area on wounding day, followed by 4th, 8th, 12th, 16th day and subsequently on every alternate day till complete closure of wound (falling of the scab without any raw area). Similarly, scars were traced on complete epithelization to assess wound contraction by noting scar size and shape. The wound healing was expressed as percentage closure of the original wound area (on day 0) (percentage closure = 1−Aₙ/A₀ × 100 [Aₙ is wound area on corresponding days and A₀ is wound area on day zero])

• Resutured incision wounds were inflicted by silk
sutures 1 cm apart on two 6 cm long paravertebral parallel incisions under anesthesia. Sutures were removed on the 8th day, and breaking strength of the wounds was measured on the 11th day by using continuous water flow technique. The breaking strength was expressed as the minimum weight of water necessary to bring about gaping of the wound. Three readings were taken on each wound, and the mean of six such readings in each animal was used for statistical analysis. Subsequently, animals were sacrificed by overdose of an anesthesia.

- Dead space wounds were inflicted by implanting sterile cotton pellets (10 mg) and cylindrical grass piths (25 mm × 3 mm) subcutaneously in the groin and axilla randomly. The granulation tissues were dissected out after sacrificing the animal on the 11th day and were dried at 60°C overnight to record the constant dry weight, expressed as mg/100 g body weight. One of the granulation tissues over the grass piths was opened and trimmed to a rectangular piece for estimation of hydroxyproline content colorimetrically. The other granulation tissues grown on the grass pith were preserved in 10% formalin for histopathological studies to evaluate the effect of Lactobacillus on collagen formation.

Statistical analysis

Data were expressed in terms of mean and standard error of mean. The one-way ANOVA was applied to find out the comparison of three study groups followed by Tukey’s multiple post hoc procedure used for pairwise comparison. The dependent t-test was performed to find out the significance between the time points. Analysis was done by using SPSS 20.00 version (IBM). The statistical significance was set at 5% level of significance (P < 0.05).

Results

The mean percentage closure of excision wound is significantly higher in L. acidophilus group as compared to L. plantarum and control at day 4, 8, 12, 16, and 18 (P < 0.05).

Similarly, there is no significant difference between control group as compared to L. plantarum with mean percentage closure of excision wound at each time point (P > 0.05) [Table 1 and Figure 1].

Further, the comparison of three treatment groups with respect to change in mean percentage closure of excision wound from day 4 to day 8, day 4 to day 16, and day 4 to day 18 showed significant difference (P < 0.05) and day 4 to day 12 failed to show the significant difference (P > 0.05) [Table 2 and Figure 2].

Similarly, the mean time complete epithelization is significantly smaller in L. acidophilus as compared to control and L. plantarum (P < 0.001). However, the mean scar area showed similar findings. When groups were compared, the control and L. plantarum groups showed no significant difference (P > 0.05). L. acidophilus group showed significant and higher wound breaking strength, granuloma dry weight, and hydroxyproline content as compared to L. plantarum and control (P < 0.001), but when L. plantarum is compared with control, no significant difference was found (P > 0.05) [Table 3 and Figure 3].

Table 1: Comparison of three groups (control, Lactobacillus acidophilus, and Lactobacillus plantarum) with respect to percentage closure of excision wounds at day 4, day 8, day 12, day 16, and day 18

| Groups              | Day 4 Mean±SEM | Day 8 Mean±SEM | Day 12 Mean±SEM | Day 16 Mean±SEM | Day 18 Mean±SEM | Pair-wise comparisons by Tukey’s multiple post hoc procedures |
|---------------------|----------------|----------------|-----------------|-----------------|-----------------|-------------------------------------------------------------|
| Control             | 18.57±1.86     | 52.50±2.88     | 80.75±2.88      | 87.12±1.34      | 93.30±1.52      |                                                             |
| LA                  | 35.25±0.85     | 61.52±2.41     | 96.60±1.01      | 99.33±0.45      | 100.0±0.00      |                                                             |
| LP                  | 21.63±0.47     | 54.95±1.45     | 86.50±1.43      | 90.15±0.91      | 94.82±0.66      |                                                             |
| F                   | 53.8400        | 4.0271         | 18.57±1.86      | 42.8556         | 13.5821         |                                                             |
| P                   | 0.0001*        | 0.0398*        | 0.0001*         | 0.0001*         | 0.0004*         |                                                             |

Pair-wise comparisons by Tukey’s multiple post hoc procedures:

- Control versus LA: P=0.0002*
- Control versus LP: P=0.2060
- LA versus LP: P=0.0002*

*P<0.05; F-ANOVA test. LA: Lactobacillus acidophilus; LP: Lactobacillus plantarum; SEM: Standard error of mean

Figure 1: Comparison of three groups (control, Lactobacillus acidophilus, and Lactobacillus plantarum) with respect to percentage closure of excision wounds at day 4, day 8, day 12, day 16, and day 18
The microscopic study of section of granulation tissue (dead space wound) was carried out arbitrarily quantifying the amount of granulation tissue and collagen content in different groups. There was increase in granulation tissue, with increased cell infiltration with lymphocytes, macrophages, and moderate increase in collagen content corroborating increase in granuloma dry weight and hydroxyproline content in \textit{L. acidophilus}-treated group as compared to that of control indicating wound healing.

**Discussion**

Wound healing is said to be complete only when the disrupted surfaces are firmly knit by collagen; there is obliteration of dead spaces and the functions have been restored to normal.\cite{1} In the present study, wound healing properties of \textit{L. acidophilus} and \textit{L. plantarum} were analyzed and the results clearly indicate that \textit{L. acidophilus} significantly ($P < 0.05$) enhanced wound healing in all the three cutaneous wound models and supports the popular long-standing medical traditions of consuming food-grade organism in fermented beverages and active yogurt.

Various medical approaches and therapeutic interventions can affect the different phases involved in the wound healing cascade. The comparison of three treatment groups with respect to change in mean percentage closure of excision wound from day 4 to day 8, 12, 16, and 18 showed significant difference ($P < 0.05$) indicate that \textit{Lactobacillus} has accelerated the normal healing cascade with an orderly process of hemostasis and fibrin deposition which leads to an inflammatory cell cascade, characterized by neutrophils, macrophages, and lymphocytes within the tissue\cite{19} (histopathological reports) which is then followed by proliferation of fibroblasts and collagen deposition; finally, remodeling by collagen cross-linking and scar maturation.\cite{20}

The present study showed that the mean percentage closure of excision wound was significantly more ($P < 0.05$) in the

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**Table 2: Comparison of three groups (control, \textit{Lactobacillus acidophilus}, and \textit{Lactobacillus plantarum}) with respect to changes in percentage closure of excision wounds from day 4 to day 8, day 12, day 16, and day 18**

| Groups   | Day 4-8 | Day 4-12 | Day 4-16 | Day 4-18 |
|----------|---------|----------|----------|----------|
| Control  | 33.93±2.71 | 62.18±2.93 | 68.55±1.26 | 74.73±1.78 |
| LA       | 26.27±1.83 | 61.35±0.99 | 64.08±0.98 | 64.75±0.85 |
| LP       | 33.32±1.41 | 64.87±1.88 | 68.52±1.20 | 73.18±0.96 |

| Percentage of change in control | 182.76, $P=0.0001^*$ | 334.92*, $P=0.0001^*$ | 369.21*, $P=0.0001^*$ | 402.51*, $P=0.0001^*$ |
| Percentage of change in LA     | 74.52*, $P=0.0001^*$ | 174.04*, $P=0.0001^*$ | 181.80*, $P=0.0001^*$ | 183.69*, $P=0.0001^*$ |
| Percentage of change in LP     | 154.01*, $P=0.0001^*$ | 299.85*, $P=0.0001^*$ | 316.72*, $P=0.0001^*$ | 338.29*, $P=0.0001^*$ |
| $F$                             | 4.2868 | 0.7725 | 4.9537 | 18.0286 |
| $P$                             | 0.0337* | 0.4794 | 0.0223* | 0.0001* |

**Pair-wise comparisons by Tukey’s multiple post hoc procedures**

| Control versus LA                | $P=0.0467^*$ | $P=0.9574$ | $P=0.0385^*$ | $P=0.0003^*$ |
| Control versus LP                | $P=0.9757$ | $P=0.6441$ | $P=0.9998$ | $P=0.6691$ |
| LA versus LP                     | $P=0.0694$ | $P=0.4773$ | $P=0.0400^*$ | $P=0.0009^*$ |

*$P<0.05$, *$P$-Applied paired $t$-test and $F$-ANOVA test. LA: \textit{Lactobacillus acidophilus}; LP: \textit{Lactobacillus plantarum}; SEM: Standard error of mean
L. acidophilus treatment group as observed on 4th, 8th, 12th, 16th, and 18th postwound day; we also noticed that there is significant decrease in mean scar area (34.17 ± 0.70) and mean epithelization time (16.67 ± 0.42) with P < 0.001 as L. acidophilus may regulate host inflammatory responses and serve to minimize collateral tissue injury. This rapid wound healing attribute of L. acidophilus was not a generic feature of consuming any bacterium, as similar dosage levels of another bacteria L. plantarum did not significantly impact the wound healing process.

The L. acidophilus group showed stellate shaped scars compared to oval shaped in control group, which could be explained on the basis of increased wound contraction due to enhanced granulation tissue formation as observed in dead space wound model. Wound contraction depends on the myofibroblast located at wound margins, its connection to components of the extracellular matrix, and myofibroblast proliferation.[1] The wound healing activity of L. acidophilus treatment group was also confirmed by comparatively increased granuloma formation (dry weight of cotton pellet), and the presence of collagen was estimated by hydroxyproline content with mean value (10.38 ± 0.44) showing significant result (P < 0.001). Collagen is an important component in all phases of wound healing. Synthesized by fibroblasts, they give integrity and strength to all tissues and play a major role, especially in the proliferative and remodeling phases of repair;[19,21] which was further corroborated by histopathological study of granulation tissue.

The objective of the present study was not planned to probe into the wound healing mechanism of Lactobacillus (probiotics) but to know the exact type of lactic acid bacteria responsible for the beneficial of wound healing. Based on earlier reports, it could be hypothesized that L. acidophilus has multiple diverse influence on host and can modulate humoral, cellular, and nonspecific immunity.[6] It is difficult to say how exactly immune system recognize and respond to a Gram-positive bacteria (Lactobacillus). Previous study done on burn wound showed that L. acidophilus can influence antibacterial activity by secreting antimicrobial peptides, inhibit bacterial invasion, can block bacterial adhesion to epithelial cells which is proved to be beneficial in burn wound cases to reduce complications.[22]

Lew et al. explained in a review article that Lactobacillus metabolize carbohydrates to produce lactic acid as predominant end products (50%–85%) which modulate growth factors and will trigger proliferative phase and also help in the remodeling of epidermal and dermal cells.[4] Although L. plantarum in previous study[23] showed marked increase of scab tissue and accelerated healing process from granulation formation to epithelization, in the present study, it failed to produce similar effect (P > 0.05) which appears to be controversial and need further investigations in this regard.

We now report an improvement of skin homeostasis by oral administration of Lactobacillus in an experimental wound model. This provides a clue on the existence of gut-brain-skin axis through which the alteration of gastrointestinal microbiota would work toward cutaneous wound healing. This concept of gut-brain-skin is supported by few published articles such as the stress-induced premature termination of active hair growth, and hair follicle regression on skin is significantly counteracted by treatment with Lactobacillus.[24,25] This concept of probiotics may reduce the dose, frequency, or duration of each treatment.

The above observations raise the interesting prospect that feeding of just right kind of bacteria may exert profound beneficial effect on skin homeostasis, skin inflammation, and wound healing. We provide experimental evidence to support long-standing medical tradition of consuming food-grade organisms which can give potential to check harmful bacteria and virus and reduce hospitalization without any harmful effects.
Conclusion

The present study revealed that *L. acidophilus* promotes wound healing and may support the hypothesis for the relationship between gut-brain-skin axis. If could be extrapolated to clinical situation, *L. acidophilus* can be a potential adjuvant to reduce hospitalization with efficient recovery after injury and sustained good health.

Acknowledgment

The authors would like to thank Dr. Nayana K Hashilkar, Dr. Urmila Kagal, and Dr. Netravati B Angadi for their support in the study. The authors specially acknowledge with thanks to all the postgraduates and staff members of Department of Pharmacology, J.N.Medical College, Belagavi.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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