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Research Article

Probiotic Bacteria in Wound Healing; An *In-Vivo* Study

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Background: Probiotics are food supplements that benefit the host by improving its intestinal microbial balance. Probiotics are used as diet supplements to prevent diarrhoea and improve lactose tolerance.
Objectives: The present study deals with the isolation of a potent probiotic strain capable of inducing healing properties in rat model.
Materials and Methods: Probiotic VITSAMJ1 was isolated from goat milk using MRS media. The antimicrobial assay was carried out against S. aureus (MTCC 3160) and the wound healing properties were assessed on female Wistar rats. A 1.5 cm^2 subcutaneous wound was induced in the rats, and a probiotic gel formulation was tropically applied onto the wounds. Tissue biopsy was carried out after days 1, 3, 5, 7, 9, and 11. Total leucocyte count and Histopathological analysis were performed after each interval.
Results: VITSAMJ1 can be effectively used for wound healing.
Conclusion: VITSAMJ1 can be effectively used for wound healing.
Keywords: Histopathological studies, Probiotic bacteria, Probiotic gel formulation, Rat model, Wound healing

1. Background

The skin is the largest organ of the integumentary system, which prevents pathogens from entering inside the body. Peptides with antibacterial activity are expressed on epithelial surfaces and provide first line of defence (1). Any disruption in the cellular, anatomic and functional continuity of skin is regarded as a wound. Upon injury, the skin repairs itself by undergoing a cascade of events, which starts by switching on various chemical signals in the body that results into migration, proliferation and differentiation of the immune cells to the wounded area. This cascade facilitates the restoration of the anatomical continuity and function. But these healing processes are slow, increasing the chance of infection (2).

Probiotics are microorganisms that naturally reside within the human body and assist with its normal function and have been historically associated with various dairy products (3). These probiotics can play an important role in respiratory, immunological, and digestive functions and could have a significant effect in alleviating infectious disease in children and adults (4, 5). These probiotics affect the intestinal microflora balance, thus increasing the resistance to infection, inhibiting the growth of harmful bacteria, and promoting food digestion (6, 7). Probiotics-derived products, including bacterial supernatants, have been studied for their wound-healing and antiviral properties as they are believed to be effective remedies for allergies, common cold, and to reduce the risk of colon cancer and cholesterol levels (8). In the present study, goat milk was used as a source of probiotics with high immunomodulatory activity (9).

2. Objective

Development of a probiotic gel formulation for wound-healing treatment.

3. Materials and Methods

3.1. Chemicals and Reagents

All the chemicals used in the study were of the highest purity. MRS media and Muller Hinton Agar were obtained from Himedia laboratory, pudicherry, India. Glycerol and diethyl ether were obtained from SRL laboratories, Mumbai, India. *Staphylococcus aureus* was obtained in lyophilized form from Microbial Type Culture Collection (MTCC), Chandigarh.

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3.2. Sample Collection

To isolate probiotic bacteria, goat milk (Tellicherry goats) was collected from cattle shed in Katpadi, Tamil Nadu, India (12.98°N 79.13°E). The samples were collected aseptically in sterile bottles, which were kept in an ice-box and transported immediately to the laboratory.

3.3. Isolation and Identification of Probiotic Bacteria

1 mL of milk sample was homogenized with 9 mL of sterile distilled water. Serial dilutions were performed and aliquots (100 µL) of 10^{-5} dilutions were spread on MRS agar plates and were incubated at 30 °C for 24 h under aerobic conditions (10, 11). Obtained colonies were selected and purified on MRS agar plates. Morphological and Biochemical analyses, including sugar fermentation profile and gas production in MRS broth, were carried out according to Bergey's Manual (12).

3.4. Growth Kinetics and Mass-Multiplication Studies of the Effective Isolate

Seed culture of the effective isolate VITSAMJ1 was prepared (overnight bacterial cultures contained 1.5×10^8 CFU.mL^{-1}) and 1.5mL was inoculated in 150mL of MRS broth. O.D was obtained at 600 nm every 2 h before reaching the stationary phase. For mass multiplication, 350 mL of media was prepared in an erlenmeyer flask and 2% of obtained seed culture was inoculated and incubated in shaking condition at 37 °C for 4 days. After incubation, the broth cultures were centrifuged at 10,000 rpm for 10min. Supernatant was collected aseptically for the preparation of probiotic gel formulation (13).

3.5.Antibacterial Activity of the Supernatant

Antibacterial activity of the VITSAMJ1 supernatant was tested against *Staphylococcus aureus* (MTCC No-3160) by well-diffusion method. Seed cultures of pathogen *S.aureus* were prepared and swabbed on Muller Hinton agar plates. Cell-free supernatants of the effective isolate VITSAMJ1 were obtained by centrifugation at 10000×g for 10min at 4 °C. 50 µL of obtained bacterial supernatant was added to the wells. A well containing only distilled water was considered as the negative control and Vancomycin (100 mg.L^{-1}) served as the positive control. After aerobic incubation for 24 h at 37 ºC, inhibition zones were measured (14).

3.6. Preparation of the Probiotic Gel

For the preparation of 25 g of probiotic gel, 25 mL supernatant of the effective isolate VITSAMJ1 was mixed with 7 g of glycerine (emulsifying agent) and 18 g of glycerol (15). The use of animals was approved by the VIT University ethical clearance community. 18 female Wistar rats, aged 6 to 8 weeks, weighing 120 g were used for the study. The animals were housed under normal light, room temperature, and humidity and were fed with autoclaved food.

3.7. Induction of Wound and Drug Administration

The rats were anaesthetized with diethyl ether. After shaving the dorsal area, an open-excision-full-length wound, approximately 1.5x1.5 cm long and 3mm deep, was made with a sterilized scalpel. After the wounding process, each mouse was housed in a sterilize cage and was given autoclaved food and distilled water in order to prevent bacterial infection. The animals were separated into three groups - the negative control, the positive control, and the experimental - for the days 1, 3, 7, 9, and 11. The negative control rats were treated with glycerine and glycerol, the control rats were untreated, and the experimental rats were treated with the probiotic gel formulation. Six hours after the wounding process, the wounds in the control and the experimental groups were treated topically twice a day. The rats were sacrificed at days 1, 3, 5, 7, 9, and 11 after wound induction. Paraffin-embedded sections were prepared from the wounded area and were sent for histopathological analysis (16).

3.8. Whole WBC Count

Blood were collected at days 1, 3, 5, 7, 9, and 11 from the wounded rats for WBC count. Heamocytometer was used for the analysis and blood was drawn up to the 0.5 mark and WBC diluting fluid was added up to the 11 mark. The blood samples were loaded into the neubauer chamber for WBC count.

Cells/(µL)=(Number of cells in 1 large square)/(Volume factor (0.1)) X Dilution factor

3.9. Histopathological Studies

Mice were euthanized on days 1, 3, 5, 7, 9, and 11, biopsy sites were excised, fixed in 10% formalin, and preserved in 1mL of 1x PBS and processed for routine histology. The section was stained with haematoxylin – eosin and photographed with a bright-field Olympus microscope (17).

4. Results

4.1. Isolation and Identification of the Probiotic Bacteria

A total of 4 isolates with distinct cellular and morphological characteristics were obtained from the MRS agar plates, namely VITSAMJ1, VITSAMJ2, VITSAMJ3, and VITSAMJ4. The colony morphology

was distinguished on the basis of visual identification and all the isolates were mucoid in nature with smooth surfaces. The *Lactobacilli* strain VITSAMJ1 was initially identified by their ability to grow on the selective MRSA, gram-positive rod shaped, and catalase-negative phenotype.

4.2. Growth Kinetics Studies

Absorbance readings were measured at every 2 h interval at 600 nm and growth curve was plotted, it was observed that the effective isolate VITSAMJ1 was able to achieve stationary phase 16 h past the inoculation **(Fig. 1)**.

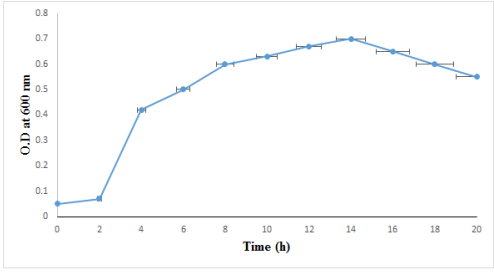

Fig. 1 Growth kinetic studies

4.3. Antibacterial Activity of the Supernatant

S. aureus has long been recognized as one of the most important bacterial pathogens in humans. It is the most common cause of skin and soft tissue infections. The VITSAMJ1 supernatant was tested for its antibacterial activity against *S.aureus* (MTCC 3160) and a zone of inhibition of 22 mm was observed, indicating the inhibitory effect of bacterial supernatant on the growth of *S. aureus* **(Fig. 2)**.

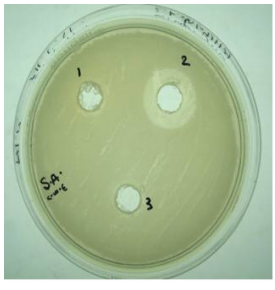

Fig. 2 Antibacterial activity of the supernatant against S. aureus

4.4. Induction of Wound and Drug Administration

The probiotic gel formulate was applied tropically twice a day on the rat model. Wound contraction was determined from the difference between initial induction and the final healing and the results were obtained for days 1, 3, 5, 7, 9, and 11 **(Fig. 3)**. The rats treated with the probiotic gel formulation depicted improved wound healing process from the 3rd day onwards, resulting in a reduction in the wounded area and, ultimately the time required for full recovery.

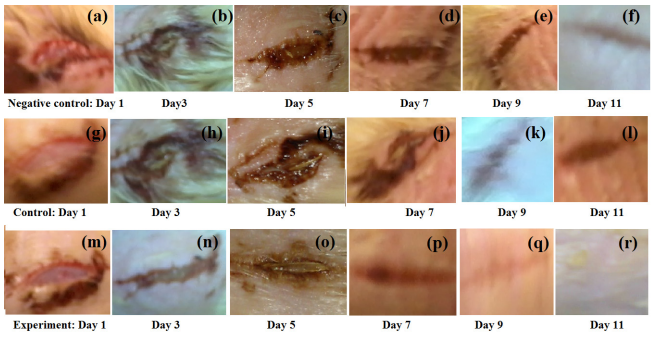

Fig. 3 Photographic representation of wound contraction of different days (a-f) Negative control rats- treated with glycerine and glycerol, (g-l) Control rats – untreated and (m-r) Experimental rats treated with probiotic gel

4.5. Total WBC Count

WBC count was measured using a haemocytometer on days 1, 3, 5, 7, 9, and 11 for the negative control, the positive control, and the experimental groups **(Fig. 4).** It was observed, that as compared to the positive and the negative controls, the experimental rats showed a marked difference in leucocyte levels. By the 3rd da,y the leucocyte counts of the wounded rat treated with the probiotic gel formulation from the effective isolate VITSAMJ-1 was much higher (11,000 uL^{-1}), as compared to the control groups (7,000 uL^{-1}). Leucocyte count reached its highest peak (15,000 uL^{-1}) around the 5th day, as compared to the control (9,000 uL^{-1}). After reaching its peak count, the leucocyte count dropped back to its basal levels.

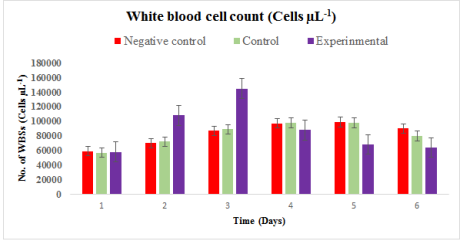

Fig. 4 Graph showing the total number of WBC in negative control, control and experimental mice on various days on the process

4.6. Histopathologicl Studies

Tissue samples from the rats were preserved in 1ml of 1XPBS. The sections were stained with Haematoxylin-Eosin and photographs were taken with a bright-field olympus microscope **(Fig. 5)**. Tissue section of the rats treated with the probiotic gel formulation showed more influx of macrophages and neurophiles from the first day onwards, as compared to the negative control and the positive control groups. Full-tissue recovery was observed by the 11th day in the experimental group, depicting the effectivness of the probiotic gel formulation.

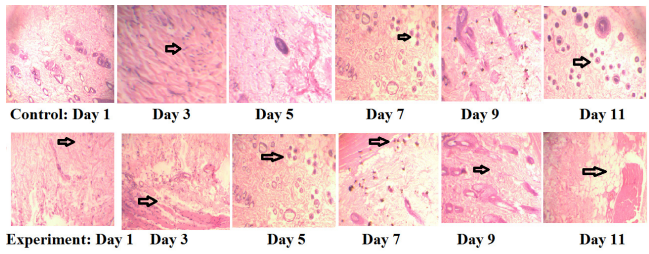

Fig. 5 Photographic representation of skin samples of rat on day 1,3,5,7,9 and 11 on control (that didn't received any treatment) and Experimental (treated with probiotic gel) stained with haematoxylin-eosin and photographed with Olympus microscope

5. Discussion

In the present study, probiotic bacteria was isolated from goat milk, which is known to possess high immunomodulatory activity (9). A total of four gram positive cocci and endospore positive isolates, namely VITSAMJ, VITSAMJ2, VITSAMJ3, and VITSAMJ4, were obtained from the MRS agar plates. Antimicrobial assay was performed by agar-well-diffusion method and the obtained isolates were checked for their antagonistic activity against various pathogens like *P.aeruginosa* and *S.aureus* (14). Agar-well-diffusion method has been routinely used to test anti-microbial susceptibility. The zone of inhibition around the wells directly relates to the efficiency of the bioactive compounds that act against the tested pathogenic bacteria. VITSAMJ1 showed the maximum of 22 mm zone of inhibition against *S.aureus*. Similar results were observed in *Lactobacillus* supernatants in MRS broth against *S. aureus* strain. (18, 19). The efficiency of the inhibitory activity of the four isolates was checked. VITSAMJ1 exhibited more inhibitory activity after 24 h of incubation. Consequently, VITSAMJ1 was selected for further studies. Mass multiplication was performed and the secondary metabolites obtained from the effective isolate VITSAMJ1 was used for the preparation of a probiotic gel formulation (15). An open-excision-full-length wound was made on the dorsal surface of the rat and the VITSAMJ1 supernatant (in gel form) was administered on day 1 after wound induction till the wound was healed completely. The wounded area was maximum on day 1, similar to previous studies (16, 17 and 20). The wounded area was found to be healing and reducing in size as compared to the other two control groups, showing the ability of the secondary metabolites of probiotic isolate VITSAMJ1.

Our results indicate that the VITSAMJ1 supernatants potentiate inflammation. We demonstrated that the VITSAMJ1 supernatants acts as potent chemo attractant or a regulator for the movement of PMNs and macrophages. The whole WBC count was measured over a period of 11 days in the wounded rat. When compared to the positive and the negative controls, the isolates showed a marked difference in leucocyte levels. The results of this study showed that in the groups treated with the effective isolate VITSAMJ1 supernatants, the total leucocyte count was significantly higher than the negative control group, suggesting an improved wound-healing process from day 3 onwards. Studies have demonstrated the effectiveness of the *Lactobacillus* supernatant to promote inflammatory response during tissue repair in rodents by applying subcutaneous injections of the *Lactobacillus* supernatant formulation into the ears of rats, which leads to angiogenesis and improves the process of wound healing (21, 22, 23-24). The wounded sections were also analysed with Haematoxylin- Eosin staining. In the probiotic treated wound a higher level of neutrophil and macrophage migration was observed, indicating faster wound-healing capacity, peaking at day 5. By day 11, the treated wounds showed nearly full regeneration of skin tissue. Previous reports suggest that probiotic strains induce the production of protective cytokines that enhances regeneration and inhibits apoptosis in epithelial cells (25-29). On a contrary, the controls showed lower levels of leucocyte migration. As compared to the probiotic-treated wounds, the controls did not show similar skin tissue regeneration. Interestingly, no infection was found in the experimental groups. Probiotics prevent infection in wounds by an antimicrobial mechanism that involves secretion of antimicrobial peptides, inhibition of bacterial invasion, and inhibit pathogenic bacterial adhesion to epithelial cells (30, 31). This finding suggests that application of the probiotic gel reduces the time required for a wound to heal.

6. Conclusion

This study has shown the effectiveness of probiotic bacteria in inducing the reduction wound size and wound closure. There was no significant difference observed in all the control groups. Animals treated with the probiotic gel showed better wound healing compared to the control groups. Hence, the supernatant obtained from the effective bacterial isolate could be used in the development of an ointment for topical wound healing.

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