Journal of Advances in Microbiology

Volume 23, Issue 7, Page 15-25, 2023; Article no.JAMB.101770
ISSN: 2456-7116

Effects of Bacteriocin Extracted from *Lactobacillus rhamnosus* on Cutaneous Wound Healing in Mice

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2023/v23i7734

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/101770

Original Research Article

Received: 22/04/2023
Accepted: 24/06/2023
Published: 05/07/2023

ABSTRACT

Aim: This study evaluates the effects of bacteriocin produced by *Lactobacillus rhamnosus* on cutaneous wound healing in mice.
Study Design: Randomized experimental design was employed in the study.
Methodology: Probiotic *L. rhamnosus* was isolated from nono milk using MRS agar media and identified based on morphological and biochemical characteristics. The potent bacterium was subjected to bacteriocin production. Antimicrobial activity of the bacteriocin was carried out against wound pathogenic bacteria and wound healing effects of the bacteriocins were assessed on mice. 2 mm cutaneous wound was induced on the mice and crude bacteriocin was topically applied on the wounds. White blood cell count was carried out after days 3, 5, 9, and 14. Histopathological analyses were performed after day 14.
Results: Crude bacteriocin was effective against wound pathogenic bacteria such as *S. aureus*, *P. aeruginosa* and *E. coli*. Topical application of bacteriocin showed a faster and better wound healing when compared to positive control groups in *P. aeruginosa* infected wound model. Histology examination revealed an increase wound healing time in *L. rhamnosus* bacteriocin treated mice.

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The crude bacteriocins demonstrated excellent wound-healing activity in skin excision albino mice model.
Conclusion: *L. rhamnosus* bacteriocin had various beneficial actions on wounds. It improved wound healing, inhibited wound pathogens and increase wound healing time.

Keywords: Bacteriocin; wound healing; wound infection; probiotic; Lactobacillus rhamnosus.

1. INTRODUCTION

"The human skin plays an important role as a barrier between the external environment and internal organs and tissue. It acts as one of the body's first lines of defense against harmful microbes, therefore, any damage or breakage on the surface of the skin as a result of injury, leads to wound" [1]. Wound is a complex micro-environment where infections by bacterial pathogens represent major concerns in patient treatment. Inappropriate caring of the wound followed by dead tissue and moist environment may delay wound healing making the wound prone to infection [2,3]. Management of wound infection has become a growing problem worldwide as they result in high financial costs for treatment and devastating outcomes such as amputation. So, effective treatments are more important than ever in reducing its economic and healthcare challenges [4]. The treatment of wound infection involves the use of antibiotics; however an increasing healthcare problem is the resistance of microorganisms that usually caused wound infections to antimicrobial drugs. Inappropriate use of antibiotics may lead to problems such as drug-specific adverse effects and selection of multidrug-resistant bacteria [5,6]. Therefore, alternatives to the antibiotics are of great interest and antimicrobial peptides such as bacteriocin produced by beneficial microorganisms (*Lactobacillus* species) are an obvious choice.

"Lactobacillus is a gram-positive, catalase negative, non spore forming bacteria with rod–shaped morphology. They are characterized by their ability to produce lactic acid as a by-product of glucose metabolism" [7]. "*Lactobacillus* species has been reported to be a rich source of bacteriocin and other antimicrobial substances" [8]. Recent studies have demonstrated several antimicrobial mechanisms of *Lactobacillus* such as production of inhibitory substances such as bacteriocins, nutrient competition, strengthening the immune system and competition for binding sites [9,10].

"Bacteriocins are antimicrobial peptides or proteins, which are produced and secreted by bacteria for self defense against the growth of closely related bacteria species. They are generally classified as safe and have antimicrobial activity against similar bacteria strains and in rare cases against a broader range of unrelated groups of bacteria" [11]. "Bacteriocins have diverse applications in various fields which have led them to gain a lot of attention. Many studies have shown the beneficial effects of bacteriocins in wound healing using animal studies, such as reducing inflammation, speeding the wound healing process, and strengthening the immune system" [12,13,14]. This is usually achieved through different mechanisms, including the blockage of pathogen adhesion and nutrient competition.

They have a distinct mode of action when compared to traditional antibiotics and are only needed in small amount to kill or inhibit the growth of bacteria which makes them an alternative to antibiotics in the context of antimicrobial resistance [14]. The present study was aimed at evaluating the wound healing effect of bacteriocin produced by *Lactobacillus rhamnosus* WE1-3 using *P. aeruginosa* infected mice model.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Wound Pathogens

Wound swabs of infected wound patients were collected from some selected hospitals in Anambra State. The samples were processed and analyzed by standard Microbiological analysis. Morphological and biochemical identification such as catalase test, oxidase test, citrate test and sugar fermentation test was done based on Bergy's Manual of Systematic Bacteriology [15].

2.2 Isolation of *Lactobacillus* sp. from Nono Milk

The bacteria *Lactobacillus* sp. was isolated from nono milk by pour plate method using De Mann Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, Hampshire, England) media

asdescribed by Guerreiro [16]. Ten-fold serial dilution was performed by adding 1 mL of nono milk to 9 mL of distilled water (from 10^{-1} up to 10^{-8}). After that, 0.1 mL of the last serial dilutions (10^{-8}) was aseptically spread on sterile plates. MRS agar was poured onto it and allowed to set. Plates were then incubated at 37^{o}C under anaerobic conditions using an anaerobic candle jar for 48 hours. After incubation, the single colony of *Lactobacillus* was isolated by observing their colony morphology on MRS agar plates. Well isolated colony was transferred to MRS broth for enrichment of *Lactobacillus* at 37^{o}C

2.3 Identification of *Lactobacillus rhamnosus* from Nono Milk

Lactobacillus sp. isolated from nono milk was identified based on the colony morphology on MRS agar (shape, colour and pigmentation), physiological and biochemical characteristics such as catalase, oxidase and citrate test was carried out as described in the Bergey's Manual of Determinative Bacteriology [15]. Furthermore, carbohydrates fermentation test was conducted by using (API 50 CHL) strip consisting of wells of different carbohydrate substrate as described by Cheesbrough [17]. Glucose, lactose, sucrose and lactose were used in this study as carbohydrate.

2.4 Production of Crude Bacteriocin by *Lactobacillus rhamnosus*

Crude bacteriocin was produced as previously described by Wei [18]. *Lactobacillus rhamnosus* was grown anaerobically in 1000 mL MRS broth for 48 hours at 37°C. After incubation, the broth culture was centrifuged at 5000 rpm for 10 minutes to obtain a cell-free supernatant. The pH of the supernatants was adjusted to 6.5, treated with catalase (5 mg/ml) and filtered through 0.22 µm pore size filters (EMD Millipore, Billerica, MA, USA). Ammonium sulphate was used to directly precipitate the crude bacteriocin. The cell-free supernatants were saturated with 10% ammonium sulphate at 4°C to precipitate the proteins. After stirring on a magnetic stirrer, it was kept undisturbed at 4^{o}C overnight. The cell free supernatant (CFS) was discarded on a sterile tube while pellets formed (crude bacteriocin) was collected by centrifugation at 10,000 rpm for 10 minutes and re-dissolved in 20 ml of sodium phosphate buffer with pH adjusted to 6.5. The crude purified bacteriocins were collected in sterile containers and stored at -20^{o}C.

2.5 Assay for Antibacterial Activity of Crude Bacteriocin of *Lactobacillus* sp.

Antibacterial activity of crude bacteriocin was determined by the agar well diffusion assay as described by Zhou [19] against three wound pathogenic bacteria *S. aureus*, *P. aeruginosa* and *E. coli.* 2 mL aliquot of LrWE1-30 bacteriocin were placed in wells cut on Mueller-Hinton agar plates previously seeded with the wound isolates (which were incubated in nutrient broth for 24 h, diluted to 0.06 at 600nm, which is equivalent to the McFarland standard 0.5). A well containing only distilled water was considered as the negative control and penicillin served as the positive control. The cultured Plates were incubated at 37^{o}C for 48 h. After 48 hours, diameters of the growth inhibition zones were measured using a ruler calibrated in millimeter. Each experiment was replicated three times and the results were expressed as average values.

2.6 *In vivo* Study

2.6.1 Animals selection

Twenty healthy albino mice weighing 300g of both males and females were used in the evaluation of the wound healing potential of *L. rhamnosus* bacteriocin. The animals were housed and maintained under controlled conditions and were provided with a diet food pellet supplied from animal house and clean drinking water throughout the experimental period. The mice were divided into four groups (each consisting of five mice) receiving different treatment according to the experimental protocol. Negative control group (Infected with *P. aeruginosa* without treatment), positive control group (Infected with *P. aeruginosa* and treated with penicillin ointment), test group (Infected with *P. aeruginosa* and treated with *L. rhamnosus* bacteriocin) and healthy group (non-infected mice). This study was performed in accordance with ethical norms approved by Nnamdi Azikiwe University Animal Ethical Committee.

2.6.2 Induction of wound and drug administration

The healthy albino mice were anaesthetized with 100 mg/mL diethyl ether and the hair on their skin dorsal area were surgically clipped and 2cm^{2} skin-excision wound was made on the disinfected dorsal area of the skin using a sterilized scalpel. The wound areas were measured immediately by placing a transparent

tracing paper over the wound. After the skin excision, all the wounds except healthy control group were inoculated with 1.5 ml of 10^5 CFU/ml of *Pseudomonas aeruginosa* with the aid of a sterile pipette and left for a period of 5 days to give room for proper pathogen incubation. Afterwards, experimental group were topically inoculated with 1.5 ml of 1.1 x 10^7 cfu/ml of *L. rhamnosus* bacteriocin in order to initiate competitive inhibition once daily for 14 days. Positive control groups was treated with 1% penicillin ointment, while negative control group was left without treatment as stated already in the group arrangements [20].

2.6.3 Wound contraction determination

The maximum length and width of each wound was measured with calipers in tertian. The wound area of each animal was calculated from these measurements as a function of time that had passed during the treatment. Digital photographs of the wounds were taken on days 1, 9, and 14 [21].The percentage of wound closure was determined from the difference between the initial and final areas of the wounds and the result expressed in cm^2 using the formula below.

> Percentage of wound contraction = ((initial wound area –specific day wound area) / Initial wound area) × 100

2.7 Total White Blood Count

This was done in order to monitor leucocytes roles in the experiment owing to the fact that they play role in body defense as described by Cheesbrough [17]. Blood samples were collected from the wounded mice and transferred to an EDTA bottle. 0.5 mL of the blood samples were mixed with 0.038 of Turk's diluents in a test tube. A small amount was used to fill the counting chamber of the already charged Neubauer chamber. This set up was charged again for 5-10 minutes by placing the counting chamber on a damp towel. Thereafter, the underside of the chamber was cleaned and placed under the microscope where it was viewed using x10 objective lens.

> Cells/(µL)=(Number of cells in 1 large square)/(volume factor ($0.1mm^3$)) × Dilution factor

2.8 Histopathological Studies

At the end of the experiment, mice were sacrificed and skin tissue was excised as described by Weinheimer-Haus [22]. Skin sections of specimens from all groups were fixed in 10% formalin and then preserved in 1 mL of phosphate buffer solution and processed for routine histology. The section was stained with hematoxylin –eosin and photographed with a bright-field Olympus microscope.

2.9 Data Analysis

The collected data were analyzed using Microsoft Excel 2007 and SPSS version 20. All results were expressed as the means ± standard deviations of three independent replicates. One-way analyses of variance (ANOVA) were applied to test statistically significant differences among groups. P value ≤ 0.05 was considered as significant.

3. RESULTS

3.1 Isolation and Identification of Wound Pathogens

Most of the wound swabs processed showed single bacterial growth. The organisms were identified as *S. aureus, P. aeruginosa* and *E. coli.*

3.2 Isolation and Identification of *Lactobacillus rhamnosus*

Table 1 show the morphological and biochemical characteristics of the isolated *Lactobacillus rhamnosus.* After 48 hours culturing on selective MRS agar, one lactic acid bacteria was isolated as forming creamy mucoid colony on selective MRS. The result of the biochemical test revealed it to be Gram-positive, rod shaped, catalase negative, oxidase negative and citrate negative anaerobic bacteria.

3.3 Antibacterial Activity of Crude Bacteriocins of LrWE1-30

The results of antibacterial activity showed that *L. rhamnosus* bacteriocin displayed antagonistic effect in the agar well diffusion test against all three wound pathogenic bacteria chosen as an indicator strains (*S. aureus, P. aeruginosa* and *E. coli*). The strength of the inhibition was variable among the different wound pathogens as shown by wide diameter of inhibition zones. The crude bacteriocin displayed highest inhibitory effect against *P. aeruginosa* (31±2 mm), followed by *S. aureus* (28±2 mm) and *E. coli* (27±1mm). Antibacterial activity of the crude bacteriocin from K. rhamnosus is shown in Table 2.

Table 1. Morphological and biochemical characteristics of *L. rhamnosus*

Parameters	Observation
General characteristics	
MRS broth	Turbidity
MRS agar	Creamy, mucoid short rods
Colour	Creamy
Pigmentation	No
Gram staining	Gram negative, rod
Biochemeical Characteristics	
Catalase test	Negative
Oxidase	Negative
Citrate utilization test	-
Carbohydrate fermentation	
Glucose	+
Lactose	+
Sucrose	+
Fructose	+

'+' indicates a positive result and '−' indicates a negative result; MRS, Man Rogosa Sharpe

Table 2. Antimicrobial activity of *L. rhamnosus* Bacteriocin

Wound Pathogens	Bacteriocin activity	Diameter of zone of Inhibition (mm)	P- Value
P. aeruginosa	+++	31±2	
S. aureus	+++	28±2	0.005
E. coli	+++	27±1	
Distilled water (control)	-	0	

Zone of inhibition was measured in mm, and the results are expressed as the means ± standard deviations of three independent replicates, bacteriocin activity was expressed as strong susceptible +++ (≥ 21mm), moderate ++(≥16 -20mm) Resistance – (≤15 mm)

3.4 Wound Healing Potential of *L. rhamnosus* bacteriocin

Wound healing were compared between negative control group that is non-treated mice, positive control (mice treated with penicillin ointment), and experimental group (mice treated with *L. rhamnosus* bacteriocin) during the wound healing process. The wound of mice in different groups differed in macroscopic appearance. From the measurement of wound area, the result showed that the wound sizes of negative control and positive control groups were significantly larger ($P< 0.05$) than that of the experimental group at all days after wounding process (Fig. 1). Experimental group showed fewer purulent exudates than positive control and negative control groups. There was a delay in wound healing in positive control and negative control groups (>14 days) compared with experimental group (14 days). There were no sign of inflammation and wound reached complete closure in *L. rhamnosus* bacteriocin treated mice. However, in both the positive control group and negative control groups, the wound remains after 14 days.

3.5 Total White Blood Cell Count

White blood cell was measured using haemocytometer on days 1, 3, 5, 9, and 14 for all the groups (Fig. 3). From the result obtained, it was observed that *LrWE1-30* bacteriocin treated group showed a marked difference in leukocyte levels as compared to the positive and negative groups. At day 3, the leukocyte count of LrWE1-30 bacteriocin group was much higher (100,000 uL^{-1}), as compared to the negative control groups (70,000 uL^{-1}) and positive control group (75,000 uL^{-1}) $P< 0.001$. By the 5^{th} day, the leukocyte count reached its highest peak (135,000 uL^{-1}) in the LrWE1-30 treated groups, as compared to the control groups ($P< 0.05$). After which the leukocyte count decreased indicating the end of inflammation stage.

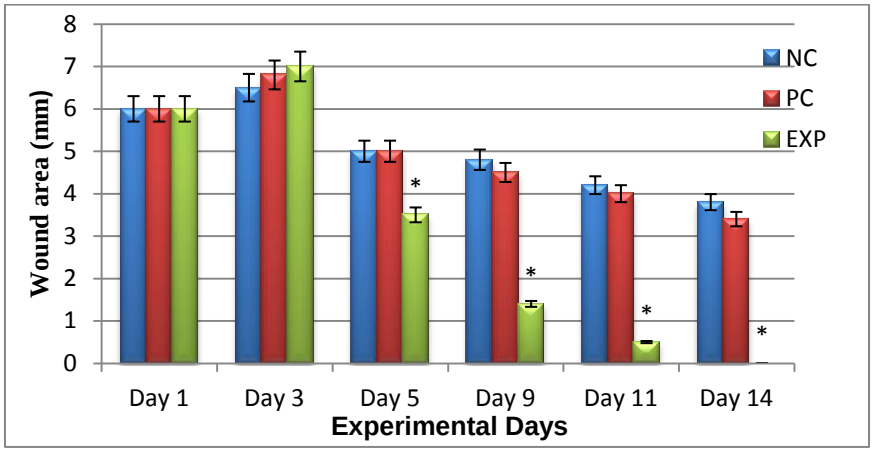

Fig. 1. Effect of topical treatment of *L. rhamnosus* bacteriocin on wound area

Data are expressed as the mean ±SD, (p < 0.05 vs control). **NC:**Negative control (no treatment), **PC**:Positive (penicillin) control, EXP: Experimental group (LrWE1-30 bacteriocin treatment*

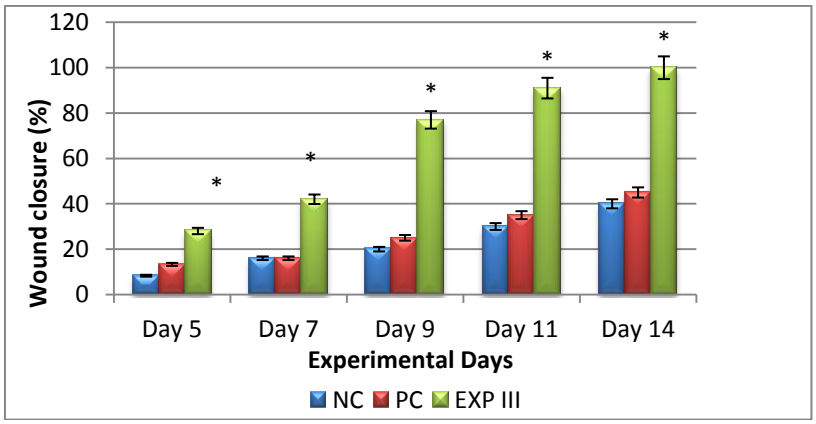

Fig. 2. Effect of topical treatment of *L. rhamnosus* bacteriocin on Wound Closure (%) during treatment duration in mice

Data are expressed as the mean ±SD, (p < 0.05 vs control).*
NC: Negative control, PC: Positive control, EXP 111: Experimental group (bacteriocin treatment)

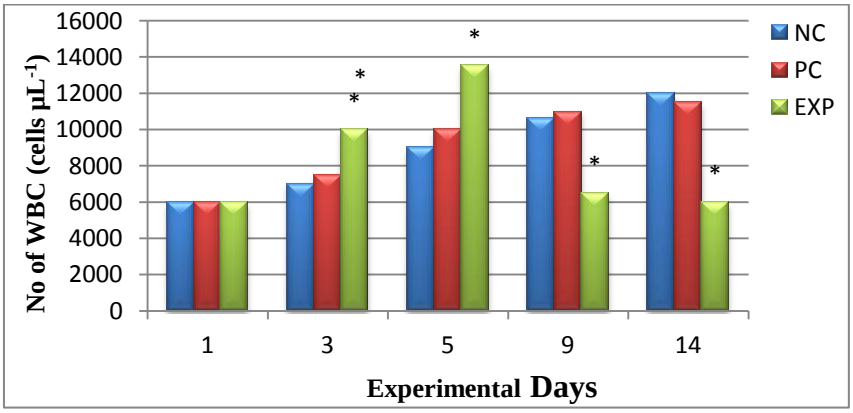

Fig. 3. White blood cell count (Cells in μL^{-1})

Graph showing the total number of WBC in negative control, Positive control and experimental mice on various days on the wound healing process.NC: Negative control (no treatment}, PC: Positive control (positive treatment). EXP III: Experimental control A (LrWE1-30 bacteriocin treatment.

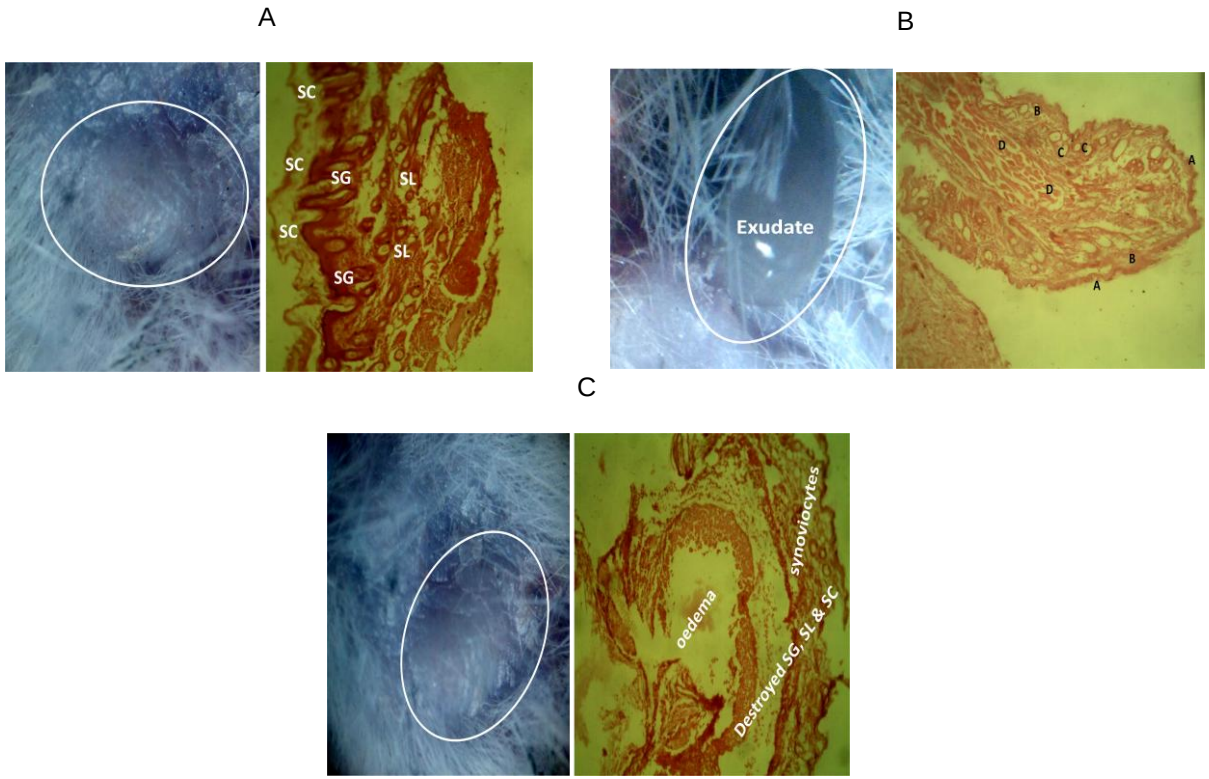

Fig. 4. Photographic representative of skin tissue of positive control mice taken on day 14 of wound healing study, stained with haematoxylin-eosin and photographed with Olympus microscope at resolution of 40x. A: negative control (mice without treatment) Showing persistence inflammatory cells, greater macrophage aggregation, edema, indicating incomplete wound healing. B: positive control group (antibiotic treatment) Showing intense inflammation, edema, exudates, and incomplete wound healing. C: showing scattered lymphocytes and destroyed inflammatory elements, indicating complete wound healing

3.6 Histopathological Studies

Skin sections of the different treatment groups are represented in (Fig. 4). Histological studies on day 14 showed oedema, intense inflammatory infiltrations associated with foreign body and exudates, with neutrophils evenly distributed throughout the entire surrounding tissues in both negative and positive control groups. On the contrary, in the group treated with *L. rhamnosus* bacteriocin, there was presence of scattered lymphocytes and destroyed inflammatory elements.

4. DISCUSSION

In the present study, we investigated the antimicrobial and wound healing activities of crude bacteriocin from *L. rhamnosus* isolated from nono milk. Our result showed that *L. rhamnosus* bacteriocin has significant inhibitory effect on growth of S*. aureus, P. aeruginosa* and *E. coli* isolated from wound infection in agar medium. Many studies have characterized the antimicrobial activity of various bacteriocin produced by *Lacticaseibacillus rhamnosus, L. fermentum, L. plantarum and L. garvieae* [23-26]. The obtained result was consistent with the study conducted by Hu [27] that reported antibacterial effect of *Lactobacillus fermentum* HFY02-from fermented soy milk on D-galactose-induced aging mouse model. Another study by Sarika [28] also reported that bacteriocin isolated from *L. rhamnosus* GP1 has antibacterial effects on pathogenic bacteria.

In order to investigate the in-vivo effect of *L. rhamnosus* bacteriocin on wound healing, mice model of *P. aeruginosa* infection was treated with crude bacteriocin. An open skin excision wound was made on the dorsal surface of the mice and the *L. rhamnosus* bacteriocin was administered to the infected mice on day 1 after wound induction till the wound was healed completely. Previous studies have reported the wound healing activity of various *Lactobacillus* species and their metabolites in animal study [29]. Previous study done on *Lactiplantibacillus* by Dubey [30] demonstrated that the topical application of Lp2621 in wound may promote wound healing.

On day 3 of the wound healing experiments, there was an increase in wound area as compared to the day 1. We discovered that the inflammatory response in all the groups was very high; therefore, the positive control groups, negative control groups and the *L. rhamnosus* bacteriocin treated groups did not show any healing. This finding is similar to previous studies by Liu and Sinha [31,32]. The increase in the wound area within the first three days of the study was a result of the wound being in the inflammatory phase of wound healing. On the fifth day of this study, there was a reduced inflammation in *L. rhamnosus* bacteriocin treated groups than the other control groups. The wounded area was found to be healing and reducing in size as compared to the other two control groups. Our findings are consistent with the recent work by Ong [33] where the wound healing activity was considerably promoted by the administration of *L. plantarum* extract by day 5 post wounding.

The percentage of the wound healing on the *L. rhamnosus* bacteriocin treated group was significantly higher ($p<0.001$) than control groups. This was similar to study done by Barzegari [34]. From the 9th day after wound induction and onwards, the healing activity of mice treated with *L. rhamnosus* bacteriocin was significantly greater than that observed for the negative control and positive control groups. On the 14^{th} day, significant healing was observed in *L. rhamnosus* bacteriocin treated mice compared to both negative and positive control mice. The increase in wound healing observed up to day 14 in comparison to positive control and negative control group represent a significant positive wound healing effect of bacteriocin on the reduction of time needed to achieve wound closure. Our results are in agreement with recent work done by Ashoori [35] who observed that the rate of wound healing was faster in the groups treated with both *L. fermentum* and *L. reuteri* supernatant loaded chitosan nanogel.

The whole WBC count was measured over a period of 14 days in the wounded mice. According to the result the *L. rhamnosus* bacteriocin treated groups showed a marked significant difference ($p<0.005$) in leukocyte levels compared to the positive control and the negative control. Total white blood cell count in blood from *L. rhamnosus* bacteriocin treated mice reached peak value on day 5, followed by subsequently decreasing to day 14, indicating an improved wound healing process from day 5 onwards. Our finding is similar to the study done by Ashoori [35] which demonstrated that bacteriocin formulation promotes inflammatory response during tissue repair in mice when applied into the wounded area and thereby,

accelerating process of wound healing. The results of this study also showed that in the groups treated with *L. rhamnosus* bacteriocin, the total number of white blood cells started decreasing from day 9. It was significantly lower than the negative control group and the positive control group indicating wound healing process in the *L. rhamnosus* bacteriocin treated groups by reduction in wound area and reducing the time required for complete wound recovery.

The wounded sections were also analyzed with Haematoxylin- Eosin staining. In the experimental group, a higher level of neutrophils and macrophage migration was observed at day 5, indicating faster wound-healing from day 5. The treated wounds showed nearly full regeneration of skin tissue. On a contrary, the controls showed lower levels of leukocytes migration and persistence inflammatory elements. No sign of infection was found in the *L. rhamnosus* bacteriocin treated groups. The results showed that crude bacteriocin from *L. rhamnosus* prevent infection in wounds by inhibiting wound pathogenic bacteria. Measurement at the end of the experiment (day 14) revealed significant differences in the mean values for bacteriocin treated groups during the healing process as compared to the positive control and negative control groups, indicating improved wound healing activity for the group treated with *L. rhamnosus* bacteriocin. The findings of our wound healing study provide evidence that the topical application of crude bacteriocins from *L. rhamnosus* to *P. aeruginosa* infected wound demonstrated rapid healing inhibition of pathogenic bacteria and re-epithelization. This findings, therefore suggests that *rhamnosus* bacteriocin have potential for treatment of wound infection.

5. CONCLUSION

The present study revealed promising wound healing activity of crude bacteriocin from *L. rhamnosus.* The *L. rhamnosus* bacteriocin used in the above studies had various beneficial actions on wounds. It inhibited the growth of wound pathogens and increase wound healing time compared to antibiotic used. Crude bacteriocin *L. rhamnosus* can be used as topical treatment therapy in treatment of wound infection.

ETHICAL APPROVAL

Ethical approval was obtained from Nnamdi Azikiwe University Animal Research ethics committee. P.M.B. 5025 Awka, Anambra State, with ethical approval Ref: NAU/AREC/2023/ 00060.

ACKNOWLEDGEMENT

The authors are grateful to management of General Hospital Onitsha for sample provision and Nnamdi Azikiwe University Animal Research ethics Committee (AREC) for their support on use of laboratory animals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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