Original Research Article

Wound Healing Effects of Bacteriocin extracted from Lactobacillus rhamnosus WE1-30 in *P. aeruginosa* infected mice model.

ABSTRACT

Background: Probiotic are beneficial microorganisms that are able to compete with and kill pathogenic bacteria without doing any harm to the host. Lactobacillus rhamnosus is a well characterized probiotic and has been shown to have many health benefits.
Objectives: this study evaluates the wound healing potentials of probiotic strain *L. rhamnosus WE1-30* (LrWE1-30) using albino mice model.
Materials and methods: Probiotic Lactobacillus rhamnosus WE1-30 (LrWE1-30) was isolated from fermented nono milk using MRS agar media. The antimicrobial assay was carried out against *P. aeruginosa, S. aureus and E. coli* isolated from infected wound and the wound healing properties were assessed on albino mice. Wound was induced on the mice and crude bacteriocin from probiotic strain LrWE1-30 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.
Result: crude bacteriocin of probiotic strain LrWE1-30 showed excellent inhibitory effects on both gram-positive *S. aureus* and gram negative *P. aeruginosa* and *E. coli* isolated from wound infection in the agar well diffusion assay. Topical application of LrWE1-30 bacteriocin showed a faster and better wound healing when compared to penicillin treated groups in *P. aeruginosa* infected wound model. Histology examination revealed an increase wound healing time in LrWE1-30 bacteriocin treated mice.
Conclusion: The extracted LrWE1-30 bacteriocin had various beneficial actions on wounds and could be used as alternatives to traditional antibiotics for wound healing.

Keywords: crude bacteriocin, probiotics, wound healing, probiotic bacteria, Lactobacillus rhamnosus.

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1. INTRODUCTION

Treatment and management of chronic wound infection present a significant clinical healthcare burden as they leave patients with serious pain and open to microbial infection [1]. Chronic wounds, unlike acute wounds can take long to heal and leave a person at greater risk of developing infection, so early recognition along with effective treatment are more important than ever in reducing its economic and healthcare challenges [2]. Treatment of wound infection includes 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 leads to problems such as drug-specific adverse effects and selection of multidrug-resistant bacteria [3].

The antimicrobial resistance has increased recently as a result of irrational use of antibiotics [4]. The world health organization has declared antimicrobial drug resistance one of the greatest

healthcare problems and the situation is common in the case of bacterial infection associated with non-healing wounds [5,6], therefore, alternatives to the antibiotics are of great interest and antimicrobial peptides such as bacteriocin produced by beneficial microorganisms *Lactobacillus spp* is 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]. Some examples of *lactobacillus spp* such as *L. rhamnosus* has been designated as 'generally recognized as safe' by the US FDA [8]. *Lactobacillus spp* has been reported to be a rich source of bacteriocin and the other antimicrobial substances [9]. Recent studies have demonstrated several antimicrobial mechanisms of Lactobacillus such as production of inhibitory substances, nutrient competition, immune stimulation, and competition for binding sites. In addition, Lactobacillus can secret certain antimicrobial substances such as bacteriocin [9].

Bacteriocins are small ribosomally synthesized antimicrobial peptides which are produced and secreted by bacteria for self defense against the growth of closely related bacteria species [10]. 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]. Many studies have shown the inhibitory effect of different bacteriocin against methicillin resistant *Staphylococcus aureus* [12], *Clostridiun defficile* [13], and *Pseudomonas aeruginosa* [14]. 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 [15]. The present study aimed to evaluate wound healing activity of probiotic strain L. rhamnosus WE1-30 bacteriocin using *P. aeruginosa* infected albino 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 Hospital in Anambra State. The samples were processed and analyzed by standard Microbiological analysis. Morphological and biochemical identification was done based on Bergy's Manual of Systematic Bacteriology [16]

2.2. Isolation and Identification of Probiotic *Lactobacillus sp.*

The bacteria *Lactobacillus sp*. was isolated from nono milk sample by pour plate method using deMan Rogosa and Sharpe (MRS) medium. Ten-fold serial dilution was performed with saline solution. One ml aliquote of the 10^{-5} of these dilutions were aseptically spread on sterile plates. Man Rogosa and Sharpe agar was poured onto it and allowed to set. Plates were incubated at 37^0C under anaerobic conditions for 48 hours. After incubation, the single colony of *Lactobacillus* was isolated by observing their colony morphology, physiological and biochemical characteristics as described in the Bergey's Manual of Systematic Bacteriology [16]

2.3. Production of Crude Bacteriocin by the Isolated *Lactobacillus Strains*

For Bacteriocin Extraction**:** Probiotic *Lactobacillus* strains were grown anaerobically in 1000ml MRS broth for 48 hours at 37^0C. After incubation, the broth was centrifuged at 5000 rpm for 10 minutes to obtain a cell-free culture supernatant. Ammonium sulphate was used to directly precipitate the crude bacteriocin. The cell-free supernatants were saturated with 10% ammonium sulphate. After stirring on a magnetic stirrer, it was kept undisturbed at 4^oC overnight. The cell free supernatant (CFS) was discarded on a sterile tube while precipitates formed (crude bacteriocin) were collected by centrifugation at 10,000 rpm for 10 minutes and re-dissolved in 20 ml of sodium phosphate buffer with pH adjusted to 6.0. The crude purified bacteriocins were collected in sterile containers and stored at -20^oC [17].

2.4. 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 [18] against three wound pathogenic bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli.* 2ml aliquot of crude bacteriocin of LrWE1-30 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). Plates were incubated at 37^oC for 24 h. After 24 hours, diameters of the growth inhibition zones were measured. A well containing only distilled water was considered as the negative control and penicillin served as the positive control.

2.5. *In vivo* study

2.4.1. Animal selection

Eighteen healthy albino mice weighing 300g of both sexes were used in the evaluation of the wound healing potential of crude bacteriocin of LrWE1-30. 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 during the period of acclimatization and throughout the experimental period. The mice were divided into three groups (each consisting of six mice) receiving different treatment according to the experimental protocol. Group I: Infected with *P. aeruginosa* without treatment (negative control), Group II: Infected with *P. aeruginosa* and treated with penicillin (positive control) and Group III: Infected with *P. aeruginosa* and treated with crude bacteriocin of LrWE1-30. The study was performed in accordance with ethical norms approved by Nnamdi Azikiwe University Animal Ethical Committee.

2.4.2. Induction of Wound and Inoculation of Test Microorganisms

The healthy albino mice were anaesthetized with 100 mg/ml diethyl ether and the hair on their skin dorsal area were surgically clipped and 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. The tracing paper was placed on a graph and traced out, the squares were counted and the area recorded. After the skin excision, all the wounds 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 and disease establishment Afterwards, group III were topically inoculated with 1.5 ml of 1.1 x 10^7 cfu/ml of crude bacteriocin from LrWE1-30 in order to initiate competitive inhibition. Group II were treated with penicillin while group I was left without treatment as stated already in the group arrangements.

2.4.3. Evaluation of probiotic wound healing potential

The crude bacteriocin of LrWE1-30 as well as penicillin ointment were applied topically once daily for 14 days. The wound area of each animal was aseptically measured on the 1st, 3rd, 5th, 9th, and 14th day post treatment. Percentage wound healing on these days was determined. Digital photograph of the wounds was taken on days 1, 9, and 14. 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

2.5. 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. Blood samples were collected from the wounded mice and transferred to an EDTA bottle. 0.02 ml of the blood samples were mixed with 0.038 of Tursk 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 (0.1)) × Dilution factor

2.6. Histopathological Studies

At the end of the experiment, mice were sacrificed and skin tissue was excised. 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

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2.7. Data Analysis:

The collected data were analysed using Microsoft Excel 2007 and SPSS version 17. Graph and tables were extracted from the data and the results were expressed as the means ± standard deviations of three independent replicates using one-way analysis of variance (ANOVA). P value < 0.05 was considered as significant.

3.0. RESULT

3.1 Isolation and identification of wound pathogens

Most of the wound swabs processed showed single bacterial growth. The organisms were identified as *Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli*

3.2 Isolation and identification of *Lactobacillus spp*. from Nono

Table 1 show the morphological and biochemical characteristics of the isolated *Lactobacillus sp.* 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 anaerobic bacteria

Table 1: Morphological and Biochemical Characteristics of test Isolates *L. rhamnosus* WE1-30

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	+
Fructose	+

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

3.3. Antibacterial activity of Crude Bacteriocins of LrWE1-30

The results of antimicrobial activity showed that crude bacteriocin LrWE1-30 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 of LrWE1-30 displayed highest inhibitory effect against *P. aeruginosa* (31±2 mm), followed by *S. aureus* (28±2 mm) and *E. coli* (27±1mm). Table 2 show the inhibitory effects of LrWE1-30 bacteriocin.

Table 2: Antimicrobial activity of Crude Bacteriocin of Lr *WE1-30*

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 LrWE1-30 bacteriocin

The LrWE1-30 bacteriocin was applied topically on the mice twice daily. Healing wounds were compared between non-treated wound in mice as negative control, mice treated with penicillin ointment as positive control group and LrWE1-30 bacteriocin treated mice as experimental group during the wound healing process. 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 (Figure 1). The wound treated with LrWE1-30 resulted in a faster reduction of the wound size than the negative control and positive control group (Figure 2). By day 14, there were no sign of inflammation and wound reached complete closure in LWE1-30 bacteriocin treated mice. However, in both the positive control group and negative control groups, the wound remains until 14 days

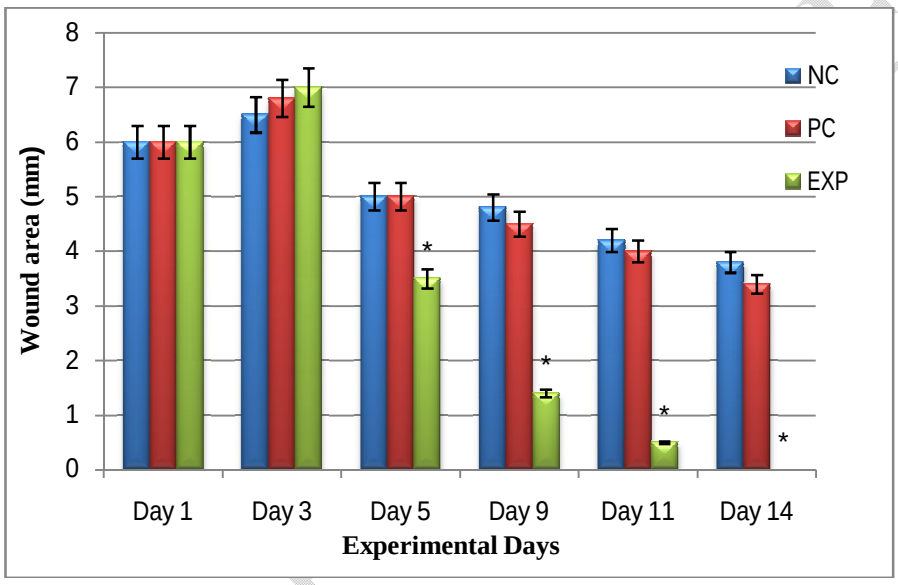


Figure 1: Effect of topical treatment of LrWE1-30 bacteriocin on wound area. data are expressed as the mean ±SD, (* $p < 0.05$ vs control). **NC:**Negative control (no treatment), **PC**:Positive (penicillin) control, EXP: Experimental group (LrWE1-30 bacteriocin treatment

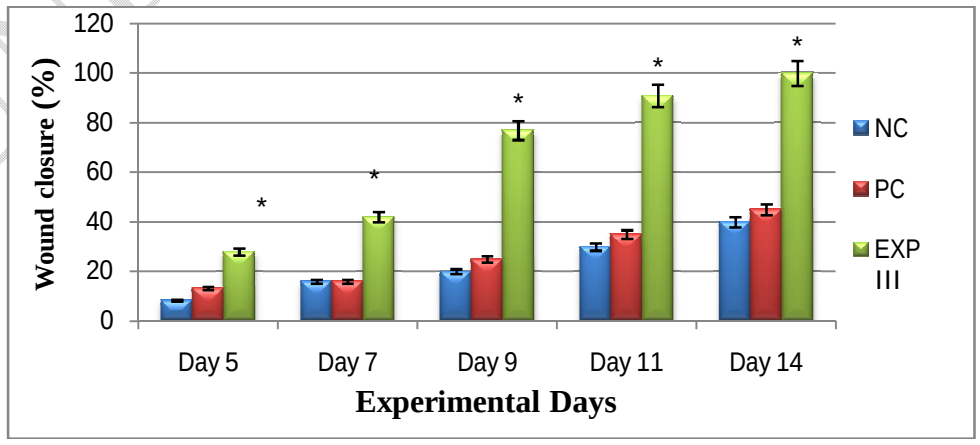


Figure 2: Effect of topical treatment of LrWE1-30 bacteriocin on Wound Closure (%) during treatment duration in mice. Data are expressed as the mean ±SD, (* $p < 0.05$ vs control). **NC:**Negative control (no treatment), **PC**:Positive (penicillin) control, EXP: Experimental group (LrWE1-30 bacteriocin treatment)

3.3. Total White Blood Cell Count

White blood cell was measured using haemocytometer on days 1, 3, 5, 9, and 14 for all the groups (Figure 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.

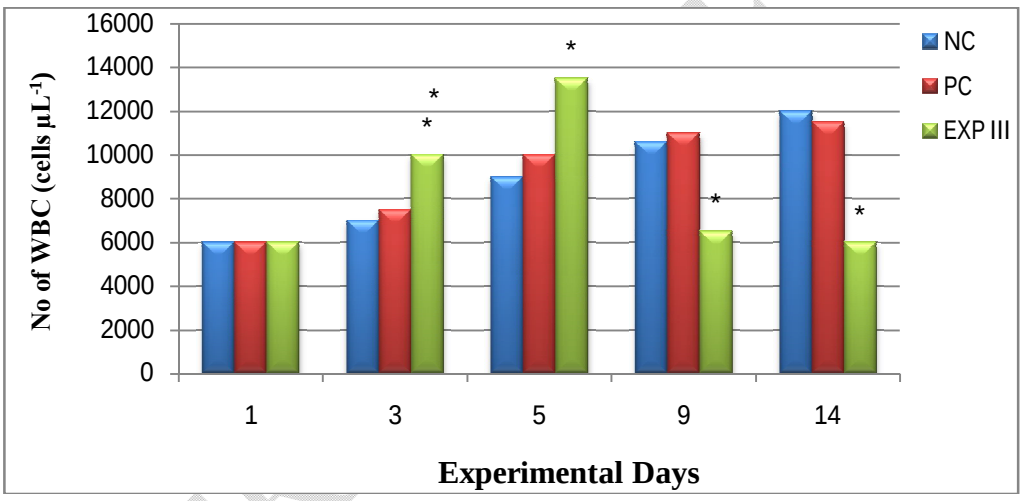
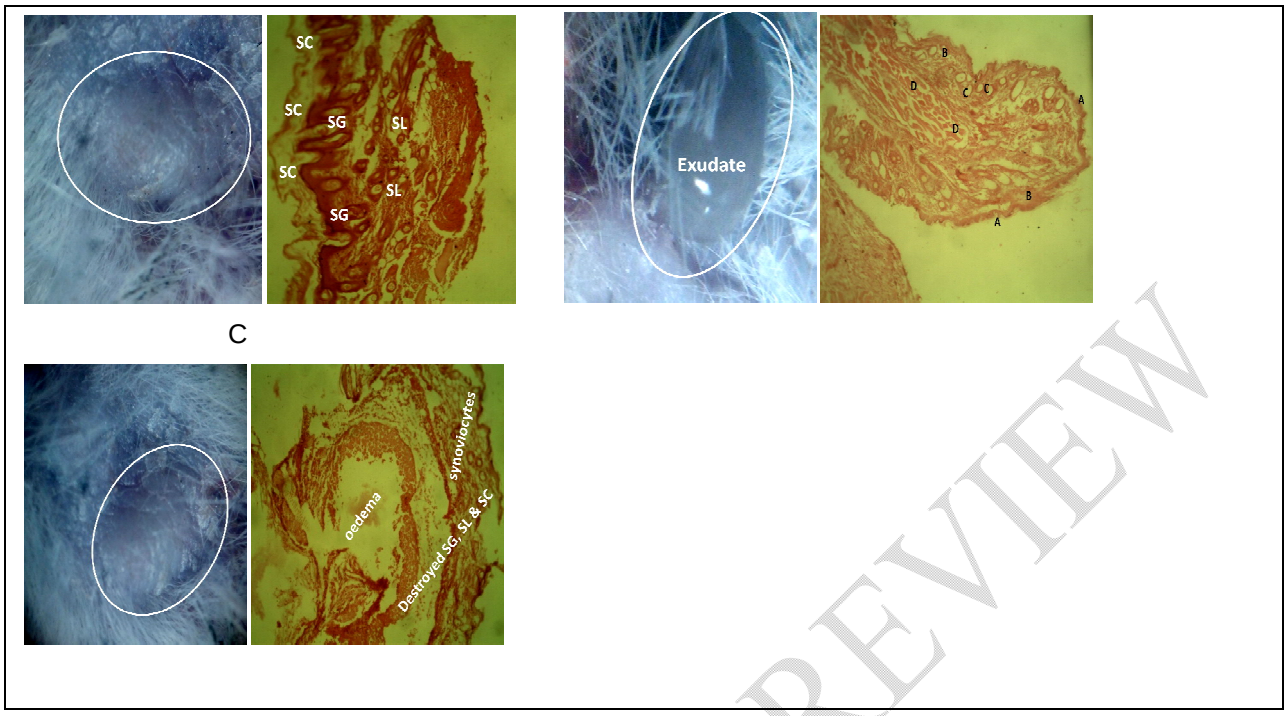


Figure 3: White Blood Cell Count (Cells in μL^{-1})
Graph showing the total number of WBC in negative control, Positive control and experimental mice on various days on the wound healing process.NC: Negative control (no treatment}, PC: Positive control (positive treatment). EXP III: Experimental control A (LrWE1-30 bacteriocin treatment.

3.4.Histopathological Studies

Skin sections of the different treatment groups are represented in (Figure 4). The negative control group showed intense lymphocytic infiltrations associated with foreign body and exudates while in positive control group, intense inflammation with fibroblastic reaction was detected. On the contrary, in the group treated with LrWE1-*30* bacteriocin, there was presence of scattered lymphocytes and destroyed inflammatory elements.

A	B



Figer 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, indicating sign of infection and incomplete wound healing. **C:** showing scattered lymphocytes and destroyed inflammatory elements, indicating complete wound healing.

DISCUSSION

In the present study, we investigated the antimicrobial and wound healing activities of crude bacteriocin of LrWE1-30 isolated from nono milk. Our result showed that crude purified bacteriocin LrWE1-30 has significant inhibitory effect on growth of both gram positive *S. aureus and* gram negative *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* [19-22]. The obtained result was consistent with the study conducted by [23] that reported antibacterial effect of *Lactobacillus fermentum* HFY02-from fermented soy milk on D-galactose-induced aging mouse model. Another study by [24] also reported that bacteriocin isolated from *L. rhamnosus* GP1 has antibacterial effects on pathogenic bacteria.

In order to investigate the in-vivo LrWE1-30 bacteriocin wound healing activity, mice model of *P. aeruginosa* infection was treated with LrWE1-30 bacteriocin. An open skin excision wound was made on the dorsal surface of the mice and the LrWE1-30 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 [25]. Another study done on *Lactiplantibacillus* by [26] 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 LrWE1-30 bacteriocin treated groups did not show any healing. This finding is similar to previous studies by [27,28]. 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 LrWE1-30 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 [29] 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 LrWE1-30 bacteriocin treated group was significantly higher ($p<0.001$) than control groups. This was similar to study done by [29]. From the 9th day after wound induction and onwards, the healing activity of mice treated with LrWE1-30 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 LrWE1-30 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 LrWE1-30 bacteriocin on the reduction of time needed to achieve wound closure. Our results are in agreement with recent work done by [30] 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 LrWE1-30 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 LrWE1-30 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 [31] 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 LrWE1-30 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 LrWE1-30 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 LrWE1-30 bacteriocin treated wound, 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 LrWE1-30 bacteriocin treated groups. The results showed that crude bacteriocin from *L. rhamnosus* WE1-30 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 LrWE1-30 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 LrWE1-30 bacteriocin. The findings of our wound healing study provide evidence that the topical application of crude bacteriocins from *L. rhamnosus* WE1-30 to P. aeruginosa infected wound demonstrated rapid healing inhibition of pathogenic bacteria and re-epithelization. This findings, therefore suggests that LrWE1-30 bacteriocin have potential for treatment of wound infection.

CONCLUSION

LrWE1-30 bacteriocin used in the above studies had various beneficial actions on wounds. LrWE1-30 inhibited the growth of wound pathogens and increase wound healing time. Crude bacteriocin of LrWE1-30 could be used as alternatives to traditional antibiotics for wound healing.

ETHICAL CONSIDERATION

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

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