Experimental evaluation of an antimicrobial protein from *Bacillus amyloliquefaciens* MBL27 for wound healing potential in rats

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Abstract
This study was aimed at assessing the ability of the antimicrobial protein (AMP) produced by *Bacillus amyloliquefaciens* MBL27 as a potent wound healant. Rat models were used to study the efficacy of AMP and AMP-incorporated chitosan sheet along with control groups. AMP and AMP-incorporated chitosan sheet significantly improved wound contraction when compared to controls. Rate of wound contraction (97.23%), decreased period of epithelialization (14 days), and the levels of biochemical markers such as hydroxyproline (collagen), total protein, uronic acid, and hexosamine in the granulation tissue on different days of wound healing revealed the wound healing efficacy of the AMP. The histological examinations also correlated well with the biochemical findings, confirming the wound healing efficacy of the AMP. The results indicate the beneficial effects of AMP from *B. amyloliquefaciens* MBL27 and it is prospective to be developed into novel therapeutic agent for dermal wound healing.

Keywords Animal models · Antimicrobial protein · *Bacillus amyloliquefaciens* · Chitosan · Collagen · Wound healing

Introduction
Wound healing is a tissue remodeling process wherein the injured tissue is removed and switched with normal tissue. This complex process is regulated by a pattern of events, in which a large number of resident and infiltrating cells are involved including keratinocytes, macrophages, fibroblasts, lymphocytes, neutrophils, and mast cells (Artue et al. 1999; Noli and Miolo 2001). Wound care and maintenance involve numerous factors, viz., dressing and administration of painkillers, healing promoting drugs, and anti-inflammatory agents. It also involves phases such as coagulation, inflammation, granulation, fibroplasia, collagenation, wound contraction, and epithelialization (Forest 1982).

The process of wound healing is reported to be accelerated by exogenous application of biological macromolecules such as collagen or chitosan, or the bioactive peptide such as growth factors on the wound surface. A biomaterial should be non-irritant, non-carcinogenic, sterilizable, non-toxic, non-antigenic, and should not cause any inflammatory reaction to the neighboring tissues. Chitin and chitosan are well known for wound healing activity (Minagawa et al. 2007; Deters et al. 2008). Chitosan excites the migration of polymorphonuclear (PMN) and mononuclear cells and speeds up the reepithelialization and regeneration of normal skin and healing occurs without excessive granulation tissue and scar formation (Kojima et al. 1998; William and Herbert 1985).

Antimicrobial peptides have been produced from bacteria, fungi, plants, and animals. Bioactive peptides have been isolated from amphibian skins (Bevins and Zasloff 1990) which exhibit microbe-killing, wound healing, and possess oxidant scavenging activities (Clarke 1997; Li et al. 2007; Conlon et al. 2004). The antimicrobial components found in the skin of *Rana dybowskii* contribute to the wound healing efficacy largely and have been used extensively in traditional Chinese medicine to heal open and burn wounds (Li–Li et al. 2009). Ranalexin, an antimicrobial peptide (AMP), in combination with lysozyme, an antistaphylococcal endopeptidase, inhibits the growth of MRSA. It could be used in wound dressings for the prevention and treatment of topical *Staphylococcus aureus* infections (Desboisa et al. 2010).
Multiple drug-resistant (MDR) bacteria have emerged in response to selective pressure created by the widespread use of antibiotics. Antibiotic resistance has become a global public-health problem; thus, it is imperative that new alternative antibacterial therapies continue to be developed. Antimicrobial peptides (AMPs) are universal multifunctional molecules and their functions extend far beyond simple antibiotics.

Many growth factors possessing angiogenic properties are commercially available in recombinant form but their widespread clinical use is hindered by their prohibitive costs. In addition, their half-life in the blood stream is likely to be short and unpredictable due to the presence of a variety of binding proteins. Therefore, the identification and characterization of alternative compounds actively promoting wound healing constitute an important part of development of new therapeutic strategies.

The by-products of Lactobacilli have inhibitory effects against pathogens both in vitro and in vivo during trials with urinary and genital infections in mice and humans (Ashahara et al. 2001; Reid 2001). Extracts from Lactobacilli cultures have medicinal effects, which includes immune system stimulating and wound healing activity (Halper et al. 2003). Lactobacillus plantarum and/or its by-products are potential therapeutic agents for the local treatment of burn infections (Valdez et al. 2005). Selection of proper healant and the method of application also determine the rate of healing. The effect of dressing on dermal repair has received great attention in recent years.

The antimicrobial protein produced by B. amyloliquefaciens MBL27 due to its broad inhibitory spectrum towards wide range of pathogens (including wound pathogens) (Vijayalakshmi et al. 2011) was used potentially in wound healing studies. The process of wound healing is reported to be accelerated by exogenous application of biological macromolecules such as collagen or chitosan or bioactive peptides (Minagawa et al. 2007; Deters et al. 2008; Clarke 1997; Li et al. 2007; Conlon et al. 2004; Mathew-Steiner et al. 2021). However, the combined efficacy of these molecules in tissue repair and regeneration is least reported and therefore, the present work focuses on the possibilities of using AMP from B. amyloliquefaciens MBL27 for wound healing studies using rat model system alone and in combination with sheets prepared using chitosan as a carrier system.

The objective in wound management is to heal the wound in the shortest time possible, minimal discomfort, pain, and scaring.

Materials and methods

Animals

Female albino Wistar rats weighing approximately 120–150 g were used for this study. The rats were purchased from King Institute of Preventive Medicine, Chennai. They were maintained in individual metabolic cages and in hygienic conditions and fed with commercial balanced diet and water ad libitum. The Institutional Animal Ethics Committee (IAEC) constituted by Central Leather Research Institute, Chennai, India approved all the protocols of animal experiments of this study (IAEC Reg. No. 06/002/08).

Preparation of AMP

A total of 1000 ml of the production medium was inoculated with 1.0% (v/v) inoculum of B. amyloliquefaciens MBL27 containing 2.2×10⁶ cells/ml and incubated at 30 °C for 36 h at 200 rpm (Vijayalakshmi and Suseela Rajakumar 2010). Antimicrobial protein (AMP) was recovered from culture supernatant by precipitation using 40% ammonium sulfate followed by centrifugation at 10,000 rpm using a refrigerated centrifuge (SIGMA, Model 3K30) at 4 °C for 15 min. It was partially purified by dialysis, filter sterilized using a sterile 0.22-μm syringe filter (Millipore, Bedford, MA, USA), and lyophilized. This preparation was used for the wound healing studies.

Preparation of chitosan sheets

Chitosan (1% w/v) was dissolved in 0.05 M acetic acid by stirring in a magnetic stirrer for 2–3 h. It was then filtered in a sintered glass filter and poured as a uniform layer onto a polypropylene plate carefully without any air bubbles. It was then allowed to dry in a drying oven at 37 °C for 24 h (Viney and Harison 2001). After drying, the sheets were peeled from the plate and washed with distilled water to remove the acetic acid and allowed to dry. It was then stored in a desiccator at room temperature and relative humidity of 60–65% for its characterization studies and wound healing studies.

Thickness of the sheet

The thickness of the sheet was measured using a micrometer (Digimatic Micrometer, Mitutoyo, Tokyo, Japan) at five locations and the mean thickness calculated.

Moisture content of the sheet

Moisture content (MC%) of the membrane was determined by drying 3 cm² pieces of samples in an oven at 105 °C for 24 h.

Water uptake study (or) swelling index

Water absorption was calculated by taking the chitosan sheet of 3×3 cm² dimension. The dry weight was taken before soaking the material in 0.1 M phosphate-buffered saline (PBS) pH 7.4. The sheet was taken out from the buffer and blotted in a filter paper and weighed. This was done at
regular intervals. The following formula was used to calculate the sample mass change resulting from water uptake:

\[
\% \Delta m = \left( \frac{m_t - m_0}{m_0} \right) \times 100
\]

where \( m_0 \) and \( m_t \) are the masses of dry and wet samples, respectively.

**Water vapor transmission rate**

The moisture permeability of the bilayer wound dressing was measured by its water vapor transmission rate (WVTR) across the material following the modified ASTM standard method E 96 at 80% relative humidity. The following formula was used to calculate the transmission rate of water vapor:

\[
\text{WVTR} = \frac{m}{\Delta t \cdot A} \text{ (g/m}^2\text{/day)}
\]

where \( m \) is the mass loss over time interval (g), \( \Delta t \) is the time interval (h), and \( A \) is the effective transfer area (10\(^{-2}\) m\(^2\)).

**Wound creation and treatment**

Female albino rats weighing approximately 120–150 g were used for the study. Hairs on the back of the rats were shaved and open excision wounds of standard size (2 × 2 cm) were made using a template. Six groups were studied, one being positive control, one being negative control, and the other four groups experimental. The animals were grouped and treated as follows and observed for healing.

- **Group I**–Wounds were topically applied with sterile water, once a day (negative control).
- **Group II**–Wounds were topically applied with cipladine ointment (povidone iodine), once a day (positive control).
- **Group III**–Wounds were topically applied with aqueous solution of the AMP at a concentration of 1 mg/cm\(^2\) wound area, once a day.
- **Group IV**–Wounds were topically applied with aqueous solution of the AMP at a concentration of 2 mg/cm\(^2\) wound area, once a day.
- **Group V**–Wounds were treated with chitosan sheets, prepared for this study using 1% (w/v) chitosan in 0.05 M acetic acid, once a day.
- **Group VI**–Wounds were treated with AMP-incorporated chitosan sheet (AMP at lower concentration 1 mg/cm\(^2\)), once a day.

**Wound assessment by planimetry**

The wound size of control and experimental animals was measured using a transparent graph sheet. The healing rate was calculated and expressed as percentage contraction (Morgen et al. 1994).

The following formula was used to calculate the percentage of wound contraction.

\[
\text{Wound contraction (\%) } = \frac{\text{Wound area day '0' - Wound area day 'n'}}{\text{Wound area day n}} \times 100
\]

(Please refer to the manuscript sent for review)

**Antimicrobial activity**

The antimicrobial efficacy of AMP-incorporated dressing was tested by using 1 cm\(^2\) of the sheet. It was placed at the center of the agar plates inoculated with a mixed culture constituting of *S. aureus, Escherichia coli*, and *Pseudomonas aeruginosa* by Kirby-Bauer disk diffusion test (Loke et al. 2000). AMP activity was measured as zone of inhibition (in mm) using zone measuring scale (HIMEDIA).

**Biochemical parameters**

Protein in granulation tissues was extracted in 5% TCA (trichloro acetic acid) by Porat et al. method (1956) and the estimation was done by Lowry et al. (1951) method. For estimation of collagen and hexosamine, the tissue samples were collected at different days of wound healing and were defatted in chloroform: methanol (2:1), dried in acetone, before use. Weighed tissues were first hydrolyzed in 6 N HCl for 18 h at 110 °C, evaporated to dryness, and then made up with a known volume of water. Collagen was estimated by Woessner (Woessner and Arch 1961) and hexosamine by Elson and Morgan method (Elson and Morgan 1933). For uronic acid estimation, digestion of the wound tissue was done with crude papain in 0.5 M acetate buffer, pH 5.5, containing 0.005 M cysteine and 0.005 M disodium salt of EDTA at 65 °C for 24 h and the estimation was done by Schiller et al. method (1961). In addition, the microbial count in terms of CFU/ml at the wound site was also determined.

**Histopathology**

Histological sectioning of the samples was done by separately fixing the samples in 10% formalin, dehydrated through graded series of alcohol, cleared in xylene, and embedded in paraffin wax. Thin sections were cut and hematoxylin and eosin staining was done. The sections were examined under a light microscope and photomicrographs were taken. Masson’s trichrome staining was also done to determine the amount of collagen deposition.

**Statistical analysis**

Data are expressed as mean ± S.E. Analysis of variance (ANOVA) followed by the student-unpaired t test was used.
to determine the significant differences among the groups. \( p \) values less than 0.05 were significant. All statistical analyses were performed using the SPSS statistical software version 11.0.

**Results**

Chitosan can be extensively used in various forms for wound healing studies due to its hemostatic and inhibitory effect against microorganisms. The results of the physical properties of the chitosan sheet prepared are given in Table 1. All used chitosan sheets were transparent and slightly yellowish in color. The SEM images show the uniform thickness of the sheet and it also shows the uniform binding of the drug throughout the sheet. The thickness of the chitosan sheet was 40–45 μm.

**Moisture content (%) and swelling index**

The moisture content of the chitosan sheet was observed to be 16.2±0.9. The swelling index of the chitosan sheet was 469.63±15. This shows the high efficiency of dressing to absorb wound exudates.

**Water vapor transmission rate**

Controlled water vapor transmission rate promotes wound healing and at the same time, there should not be accumulation of exudates in the wound area. The rate of water vapor permeability of chitosan sheets was measured at different levels and it shows a transmission rate of 180±10 done at 80% RH and at 35 °C.

**Agar diffusion test**

The AMP-incorporated chitosan sheet showed clear zone of inhibition (22 mm) against microbial consortia of gram-positive and gram-negative pathogens mainly *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. The inhibitory zone was found to be stable for more than 10 days in AMP-incorporated chitosan sheet which is shown in Fig. 1.

**Table 1** Physical properties of chitosan sheet

| Property                  | Value               |
|---------------------------|---------------------|
| Thickness (μm)            | 40–45               |
| Moisture content (MC%)    | 16.2±0.9            |
| WVTR (g/m²/day)           | 180±10              |
| Swelling index (SI)       | 469.63±15           |
| Tensile strength (N/mm²)  | 89.67±10.04         |
| % elongation              | 6.42±0.12           |

**FT-IR**

FT-IR spectrum of chitosan sheets shows the characteristic peaks of chitosan at 899.20 and 1158 cm\(^{-1}\) corresponding to saccharide structure. Strong absorption peaks at 1640.69 and 1324.02 cm\(^{-1}\) are characteristics of amide I and III peaks, respectively. The peak at 1425.18 cm\(^{-1}\) is assigned to the CH\(_3\) symmetrical deformation mode. The peak at 1040.30 cm\(^{-1}\) indicates the C-O stretching vibration in chitosan. Another broad peak at 3447 cm\(^{-1}\) is caused by amine N–H symmetrical vibration. Peak at 2950 cm\(^{-1}\) is the typical C-H stretch vibrations (Fig. 2).
Thermal studies of sheets

Thermogravimetric analyses (TGA) (Fig. 3a) revealed that the chitosan sheet showed stability up to 200 °C. The second stage of weight loss started at 200 °C and continued up to 781 °C (with 31.85% remaining).

DSC thermogram (Fig. 3b) of chitosan showed an initial broad peak at around 98 °C which is due to loss of moisture on heating. This was followed by further decomposition of chitosan as a broad exotherm in sheet at around 279 °C.

XRD studies

The wide angle X-ray diffraction (WAXD) pattern of pure chitosan powder and chitosan sheet in Fig. 4 showed the main diffraction peaks around $2\theta = 20^\circ$. But the peak intensity ratio of chitosan sheet was decreased when compared to peak intensity of pure chitosan powder, although the crystalline peaks still remain. This indicated the crystallinity of the chitosan has been decreased.

Rate of wound contraction

Visual inspection of the wound showed that all the animals had well-formed granulation tissues by day 4. A visual proof of the healing pattern of the wound was photographed on 0, 4, 8, 12, and 16 days after wound creation and depicted in Fig. 5A–F.

Wounds of group I rats (negative control) were reduced to 27.42% of the original wound area on day 4 of wound creation. The experimental groups (G II–G VI) showed significant ($p < 0.05$) wound reduction (40%, 36.3%, 40%, 32%, and 42.25%, respectively) compared to the control animals (G I) on day 4 of wound creation with more prominent reduction in animals treated with AMP-incorporated chitosan sheet. The same trend continued after 4, 12, and 16th day, indicating wound contraction was significant throughout the healing period. Animals treated with AMP-incorporated chitosan showed faster rate of wound contraction (97.23%) on day 14, followed by AMP at higher concentration (96.71%) on day 15, then by AMP at lower concentration (94.0%) on day 16, and animals treated only with chitosan showed 92.98% healing on day 16 (Fig. 6). The period of epithelialization of control and experimental groups is presented in Table 2. In our study, the wound contraction rate in AMP-treated rats was significantly higher. Furthermore, the period of epithelialization was shorter for the treated wounds and these results support the effectiveness of AMP produced by *B. amyloliquefaciens* MBL27 for wound healing.

Microbial count

Swab was taken on the wounded site on the 3rd, 5th, 7th, 14th, and 21st day taking care that the entire site of the wound was covered, from the day of wound creation and the microbial count was estimated as colony-forming units (CFU/ml). There was a significant ($p < 0.001$) reduction in bacterial population from $10^4$ to $10^1$ cells on the 3rd and 8th day, respectively for AMP-incorporated chitosan sheet. But for animals treated with chitosan sheet, the reduction is from $10^7$ to $10^2$ cells only (Fig. 7). Similarly, significant ($p < 0.05$) reduction in microbial count was noticed for other groups also when compared to control animals. The antimicrobial property of the AMP is responsible for the reduction of the microbial count.
**Biochemical analysis**

**Protein content**

Figure 8 shows the total protein content in the granulation tissues of control (G I) and experimental wounds (G II–G VI). The protein content had a statistically significant ($p < 0.05$) increase in experimental groups (G II–G VI) and was maintained up to day 12 of wound creation indicating the synthesis of other extracellular matrix proteins other than collagen in the granulation tissues by the infiltrating cells. Group III showed a significant ($p < 0.05$) increase in protein level in the granulation tissues on the 4th day by 43.19% compared to control animals (G I). Similar trend was observed on the 12th day. Group IV showed a significant ($p < 0.05$) increase in protein level in the granulation tissues on the 4th day by 59.69% compared to control animals (G I). Similar trend was observed on the 12th day by 62%. Group V showed a significant ($p < 0.05$) increase from the 4th day (30.89%) to the 12th day (47.58%), respectively, whereas group VI showed a significant ($p < 0.001$) increase from the 4th day (69.63%) to the 12th day (77.79%), respectively compared to controls (G I). The increase in the total protein content is an indication of active synthesis and deposition of matrix proteins in the granulation tissues.

**Collagen content**

The total collagen content of granulation tissues on various days is presented in Fig. 9. A significant ($p < 0.05$) increase in collagen content was observed in experimental rats (G II–G VI) compared to controls (G I), throughout the course of healing, which is an important constituent of extracellular matrix for healing. Group II animals showed a significant ($p < 0.05$) increase in collagen content on days 4, 8, 12, and 16th day when compared to controls (G I). Similar observations were found in the cases of G III, G IV, G V, and G VI animals. A significant ($p < 0.001$) increase in collagen content from days 4 (143.45%) to 8 (106.09%) respectively was observed in G VI animals compared to control animals.

Increased fibroblast proliferation and dense collagen deposition are observed during later stages of healing particularly in the remodeling phase. As a result, there is an increase in the total collagen content in all the experimental groups undergoing proper healing.

**Hexosamine content**

The results of hexosamine content in granulation tissues of control and experimental wounds are shown in Fig. 10. Hexosamine content was significantly increased in all experimental groups (G II–G VI) compared to controls (G I) with...
Fig. 5  A Photographs showing the wound healing pattern of control (G I) wounds taken on the a 0th day, b 4th day, c 8th day, d 12th day, and e 16th day after wound creation. B Photographs showing the wound healing pattern of cipladine (povidone iodine)-treated (G II) wounds taken on the a 0th day, b 4th day, c 8th day, d 10th day, and e 15th day after wound creation. C Photographs showing the wound healing pattern of wounds treated with AMP (1 mg/cm² wound area) (G III) taken on the a 0th day, b 4th day, c 8th day, d 12th day, and e 16th day after wound creation. D Photographs showing the wound healing pattern of wounds treated with AMP (2 mg/cm² wound area) (G IV) taken on the a 0th day, b 4th day, c 8th day, d 12th day, and e 15th day after wound creation. E Photographs showing the wound healing pattern of wounds treated with chitosan sheet (G V) taken on the a 0th day, b 4th day, c 8th day, d 12th day, and e 16th day after wound creation. F Photographs showing the wound healing pattern of wounds treated with AMP-incorporated chitosan sheet (G VI) taken on the a 0th day, b 4th day, c 8th day, d 12th day, and e 14th day after wound creation.
Fig 5B

Fig. 5 (continued)
Fig. 5C

Fig. 5 (continued)
Fig 5D

Fig. 5 (continued)
Fig. 5E
G VI showing increased content than all other groups. G III animals showed a significant \((p < 0.05)\) increase in hexosamine content when compared to controls (G I) and it was 48.75%, 32.26%, 13.27%, and 58.4% on 4, 8, 12, and 16th day, respectively. A significant \((p < 0.05)\) increase in hexosamine content was found in G IV animals when compared to controls (G I) and it was 90.82%, 70.92%, 31.41%, and 78.73% on 4, 8, 12, and 16th day, respectively. A significant \((p < 0.001)\) increase was observed in G VI animals also when compared to controls (G I). In addition, among the groups, G III and G VI had significantly higher values \((p < 0.05)\) than the other experimental groups, followed by G V and G VI and then by G III and G IV.

### Uronic acid content

The uronic acid levels in the granulation tissues of the control and experimental wounds are presented in Table 3. The synthesis of ground substance uronic acid was increased up to day 12 post-wounding in the treated groups (G II–G VI); thereafter, the levels decreased.

The increase in hexosamine and uronic acid contents in the granulation tissues could be attributed to the formation of glycosaminoglycans (one of the ECM proteins) for which these two ingredients form the backbone. They are the first components of ECM to be synthesized during wound healing and act as template for collagen and elastin deposition. There was a significant \((p < 0.05)\) increase in the uronic acid levels in all experimental groups (G II–G VI) compared to controls (G I), with G VI showing higher values compared to other treatment groups. Among the groups, G IV and G VI showed a statistically significant \((p < 0.001)\) increase on 8, 12, and 16th day.

### Histopathology

The first phase of normally proceeding wound healing is marked by the influx of inflammatory cellular
infiltrate consisting of polymorphonuclear leukocytes (PMNs) and macrophages under a fibrin plug. Both the control wounds and wounds treated with AMP showed good influx of inflammatory cells, which consisted first mostly of PMNs. As expected, the number of PMNs peaked in the control wounds during initial days. At day 5 and later, experimental wounds (G II–G VI) showed gradual decrease in inflammatory cellular infiltrate forming a less-dense band below the fibrin plug and newly formed granulation tissue was observed to start filling the underlying wound bed. At day 12 after wounding, the AMP-treated wounds were largely closed and covered with newly formed epidermis, and young connective tissue containing collagen and very few inflammatory cells. In contrast, control wounds showed only little microscopic changes and epidermis formation was also comparatively slower.

In G I (negative control), epidermal layer is still not formed well. But infiltration was reduced and macrophages were also observed below the epidermal layer. In G II (positive control), histological features of normal skin were clearly observed with beginning of remodeling of skin. A well-formed epidermis was noticed along with hair follicles emerging from the epidermal layer. In G III (AMP at lower concentration), well-formed epithelia were seen with dermal layer containing mature fibroblasts and collagen deposition. In G IV (AMP at higher concentration), epithelial proliferation with well-formed collagen bundles was observed. Hair follicles were also found. In G V (chitosan sheet), wounded area was covered with epithelium. Collagen deposition was also observed. In G VI (AMP-incorporated chitosan sheet), complete epithelialization was seen. Mature fibroblastic cells were seen in the dermal region with collagen deposition. Healing was complete with surface being covered by epithelial cells (Fig. 11).

Masson’s trichrome staining was used to examine the extent of collagen deposition in the wounds. Figure 12
Fig. 8  Protein content in granulation tissue during different days of wound healing on various treatment groups

shows the histological sections of both control (G I) and experimental groups (G II–G VI) taken on the 16th day. Masson’s trichrome staining stains collagen and yields a blue color. The pattern of staining intensity corresponds to the relative quantity of collagen-fiber deposit, which reflects the process of synthesis and degradation and remodeling as well as the timing of the lesion. Experimental groups (G II–G VI) showed well-formed epithelial layer with intense collagen deposition when compared to controls (G I). Well-formed hair follicles along with sebaceous gland were also observed. Experimental groups (G II–G VI) also depict the compact and well-aligned arrangement of collagen layers except for controls (G I) where only very slight collagen was observed. The bundles of collagen were also thicker in AMP-incorporated chitosan sheet (G VI)-treated group than controls (G I).

Discussion
This study describes the beneficial effects of AMP produced by *B. amyloliquefaciens* MBL27 when used alone and in combination with chitosan on rat dermal wound for healing of the wound. The positive influence of supernatants from *Lactobacillus* cultures on wound healing and angiogenic properties (Halper et al. 2003) has prompted us to investigate the AMP produced by *B. amyloliquefaciens* MBL27 which had very good antimicrobial effects on wound pathogens.

Topically applied drugs were effective in quick wound contraction because of larger availability at the wound site. More than 90% wound contraction observed in 14 days for AMP-treated groups showed effective healing process and similar findings were reported by Saratha et al. (2009).
and Sumitra et al. (2009) during the excision wound model studies.

Hexosamine and uronic acid are matrix molecules which act as ground substances for the synthesis of extracellular matrix. The levels of hexosamine and uronic acid were increased during the early stages of wound healing, following which normal levels are restored (Saratha et al. 2009; Nithya et al. 2003). Talekar et al. (2017) observed similar findings in the wound healing studies where the level of hexosamine was found to be $28.4 \pm 2.2$ mg/gm of dry tissue. The early increase in hexosamine and uronic acid revealed that the fibroblasts synthesize the base substratum on which collagen is placed. By providing more fluid uronic acid in the wound attracts fibroblasts and stimulates collagen synthesis. This facilitates greater cell mobility and early remodeling, and assists faster healing of wounds with no scar formation.

Both the control wounds and wounds treated with AMP showed good influx of inflammatory cells, initially which consisted first mostly of PMNs. Later the inflammatory cell count decreased in the experimental group particularly in AMP-incorporated chitosan-treated group. The well-formed epithelial layer in the regenerated wounds in AMP-treated group may be related to the chemotactic nature of collagen, which attracts and helps in the proliferation of cells that provided a moist environment and enhanced epithelialization. The surrounding extracellular matrix and the connective tissue in the wound gel play a key role in the contractile process. These matrices make available the anchoring points and connecting cables to which contractile cells (Majno et al. 1971) bind and reduce the wound volume through an active contraction process (Gabbiani and Majno 1972).

The role of skin protein collagen in wound repair is significant. It participates directly or indirectly in every
stage of wound healing and its metabolism throughout the process and ultimately contributes to the quality of wound repair (McPherson and Piez 1988). Scar tissue formation made of collagenous fibers is the ultimate result of wound healing and it reveals the importance of collagen. Collagen synthesis is directly proportional to hexosamine content. Similar pattern was also observed by Saratha et al. (2009), Talekar et al. (2017), and Suguna et al. (2002) during the evaluation of wound healing potential of excision wound models.

Histological examination of the tissues which showed well-formed epithelial layer in the regenerated wounds in AMP-treated group may be related to the chemotactic nature of collagen, which attracts and helps in the proliferation of cells that provided a moist environment and enhanced epithelialization. Similar patterns were observed by Talekar et al. (2017) and Karodi et al. (2009) in the excision wound studies. The biochemical aspects that accompany wound healing are governed by the metabolism of the cells infiltrating the wound (Falcone and Caldwell 1990). The rate of wound repair is influenced positively by presence on non-damaged connective tissue cells and factors, which controls proliferation and movement.

**Table 3** Uronic acid content (mg/100 mg dry tissue) in granulation tissue of control and experimental groups (mean ± S.E)

| Groups | 4th day | 8th day | 12th day | 16th day |
|--------|---------|---------|----------|----------|
| G I    | 0.47 ± 2.49 | 1.58 ± 0.97 | 1.23 ± 0.33 | 1.20 ± 1.2 |
| G II   | 2.11 ± 1.2* | 4.23 ± 0.81* | 4.68 ± 0.74* | 3.95 ± 1.05* |
| G III  | 2.82 ± 1.33* | 3.88 ± 0.66* | 4.05 ± 1.0* | 2.87 ± 0.66* |
| G IV   | 4.11 ± 1.05* | 5.95 ± 2.36** | 5.23 ± 1.35** | 4.12 ± 0.33** |
| G V    | 3.4 ± 0.66* | 4.86 ± 0.78* | 4.23 ± 0.43* | 4.03 ± 0.81* |
| G VI   | 5.4 ± 0.84** | 5.99 ± 0.83** | 5.06 ± 1.05** | 4.87 ± 1.33* |

* *p<0.05; **p<0.001
Fig. 11 Photomicrographs (100×) of healing wound tissue taken on the 16th day (a) negative control (G I), (b) positive control (G II), (e) AMP at lower concentration (G III), (d) AMP at higher concentration (G IV), (e) chitosan sheet (G V), and (f) AMP-incorporated chitosan sheet (G VI) Please include: E - Epidermis, H - Hair follicle, S - Sebaceous glands
Fig. 12 Photomicrographs (100×) of healing wound tissue taken on the 16th day (a) negative control (G I), (b) positive control (G II), (c) AMP at lower concentration (G III), (d) AMP at higher concentration (G IV), (e) chitosan sheet (G V), and (f) AMP-incorporated chitosan sheet (G VI). Please include: E - Epidermis and H - Hair follicles.
Conclusion

This study showed that the use of AMP produced by *B. amyloliquefaciens* MBL27 seemed to have promoting effect on wound healing according to the collagen content and arrangement. To the best of our knowledge, this was the first detailed study on the wound healing efficacy and antimicrobial potential of the bioactive antimicrobial protein from bacterial source done using rat animal model studies captivating the various levels of biochemical parameters such as total protein, hexosamine, uronic acid, and collagen prevailing during the period of wound healing, as markers of good wound healing.

The results of the study indicate that AMP produced by *B. amyloliquefaciens* MBL27 may be a prospective candidate for dermal wound healing in view of its antimicrobial properties and also its positive influence on different phases of the healing process.

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Author contribution Dr. K. Vijayalakshmi - methodology, conceptualization, practical investigation, formal analysis, writing - original draft. Dr. G. Suseela Rajakumar - editing and review of the draft, supervision, validation.

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Declarations

Ethics approval The Institutional Animal Ethics Committee (IAEC) constituted by Central Leather Research Institute, Chennai, India approved all the protocols of animal experiments of this study (IAEC Reg. No. 06/002/08).

Consent to participate N/A.

Consent for publication Consent is given.

Competing interests The authors declare no competing interests.

References

Artue M, Hermes B, Steclings UM, Grutzkau A, Henz BM (1999) Mast cells and their mediators in cutaneous wound healing-active participants or innocent by staders. Exp Dermatol 8:1-16. https://doi.org/10.1111/j.1600-0625.1999.tb00342.x

Ashahara T, Nomoto K, Watanuki M, Yokokura T (2001) Antimicrobial activity of intraperieuthronally administered probiotic *Lactobacillus casei* in a murine model of *Escherichia coli* urinary tract infection. Antimicrob Agents Chemother 45:1751–1760. https://doi.org/10.1128/AAC.45.6.1751-1760.2001

Bevins CL, Zasloff M (1990) Peptides from frog skin. Annu Rev Biochem 59:395–414. https://doi.org/10.1146/annurev.bi.59.070190.002143

Clarke BT (1997) The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol Rev Camb Philos Soc 72:365–379. https://doi.org/10.1017/s0006323197005045

Conlon JM, Kolodziejek J, Nowotny N (2004) Antimicrobial peptides from rain frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochem Biophys Acta 1696:1–14. https://doi.org/10.1016/j.bbapap.2003.09.004

Desboisa AP, Gemmell CG, Cootea PJ (2010) In vivo efficacy of the antimicrobial peptide ranalexin in combination with the endopeptidase lysostaphin against wound and systemic meticillin-resistant *Staphylococcus aureus* (MRSA) infections. Int J Antimicrob Agents 35:59–565. https://doi.org/10.1016/j.ijantimicag.2010.01.016

Deters A, Petereit F, Schmidgall J, Hensel A (2008) N-Acetyl-D-glucosamine oligosaccharides induce mucin secretion from colonic tissue and induce differentiation of human keratinocytes. J Pharm Pharmacol 60:1–8. https://doi.org/10.1016/j.jpharma.2006.07.007

Elson LA, Morgan W TG (1933) A colorimetric method for the determination of glucose and chondrosamine. J Biochem 27:1824–1828. https://doi.org/10.1042/bj0271824

Falcone P, Caldwell M (1990) Wound metabolism. Clin Plast Surg 17:443–456

Forest RD (1982) Early history of wound treatment. J R Soc Med 75:198–205

Gabbiani G, Majno G (1972) Dupuytren’s contracture: fibroblast contraction? An ultrastructural study. Am J Pathol 66:131–146

Halper J, Leshin LS, Lewis SJ, Wi Li (2003) Wound healing and angiogenic properties of supernatants from *Lactobacillus* cultures. Exp Biol Med 228:1329–1337. https://doi.org/10.1177/15353702032280111

Jin LL, Li Q, Song SS, Feng K, Zhang DB, Wang QY, Chen YH (2009) Characterization of antimicrobial peptides isolated from the skin of the Chinese frog, *Rana dybowskii*. Comp Biochem Physiol B Biochem Mol Biol 154:174–178. https://doi.org/10.1016/j.cbpb.2009.05.015

Karodi R, Jadhav M, Rub R, Bafna A (2009) Evaluation of the wound healing activity of crude extract of *Rhabia cordifolia L.* (Indian madder) in mice. Int J Appl Res 2:12–18

Kojima K, Okamoto Y, Miyatake K, Kitamura Y, Minami S (1998) Collagen typing of granulation tissue induced by chitin and chitosan. Carbohydr Polym 37:109–113. https://doi.org/10.1016/s0008-6215(97)00055-1

Li J, Xu X, Xu C, Zhou W, Zhang K, Yu H, Zhang Y, Zheng Y, Rees HH, Lai R, Yang D (2007) Anti-infection peptidomics of amphibian skin. Mol Cell Proteomics 6:882–894. https://doi.org/10.1074.mcp.M600334-MCP200

Loke WK, Lau SK, Yong LL, Khor E, Sum CK (2000) Wound dressing with sustained anti-microbial capability. J Biomed Mater Res 53:8–17. https://doi.org/10.1002/1097-4636(2000)53:1%3C:aid-jbm2%3E;2.3

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Majno G, Gabbian G, Hirschel BJ, Ryan GB, Statkov PR (1971) Contraction of granulation tissue in vitro: similarity to smooth muscle. Science 173:548–550. https://doi.org/10.1126/science.173.3996.548

Mathew-Steiner SS, Roy S, Sen CK (2012) Collagen in Wound Healing Bioengineering (basel) 8(5):63. https://doi.org/10.3390/bbapap.8050063

McPherson JM, Piez KA (1988) The molecular and cellular biology of wound repair. Plenum, New York, pp 471–496

Minagawa T, Okamura Y, Shigemasa Y, Minami S, Okamoto Y (2007) Effects of molecular weight and deacetylation degree of chitin/
chitosan on wound healing. Carbohyd Polym 67:640–644. https://doi.org/10.1016/j.carbpol.2006.07.007

Morgen PW, Binnington AG, Miller CW, Smith DA, Valliant A, Presscott JF (1994) The effect of occlusive and semiocclusive dressings on the healing of acute full-thickness skin wounds on the forelimbs of dog. Vet Surg 23:494–502. https://doi.org/10.1111/j.1532-950x.1994.tb00511.x

Nithya M, Suguna L, Rose C (2003) The effect of nerve growth factor on the early responses during the process of wound healing. Biochem Biophys Acta 1620:25–31. https://doi.org/10.1016/s0304-4165(02)00501-9

Noli C, Miolo A (2001) The mast cell in wound healing. Vet Dermatol 2:303–313. https://doi.org/10.1046/j.0959-4493.2001.00272.x

Porat S, Roussa M, Shosan S (1956) Improvement of the gliding functions of flexor tendons by topically applied enriched collagen solution. J Bone Joint Surg 62:315–323. https://doi.org/10.1012/0301-620X.62B2.6245095

Reid G (2001) Probiotic agents to protect the urogenital tract against infection. Am J Clin Nutr 73:437S-443S. https://doi.org/10.1093/ajcn/73.2.437s

Saratha V, Subramanian S, Sivakumar S (2009) Evaluation of wound healing potential of Calotropis gigantea latex studied on excision wounds in experimental rats. Med Chem Res 19:936–947. https://doi.org/10.1007/s00044-009-9240-6

Schiller S, Slover GA, Dorfman A (1961) A method for the separation of acid mucopolysaccharides: its application to the isolation of heparin from the skin of rats. J Biol Chem 236:983–988

Suguna L, Singh S, Sivakumar P, Sampath P, Chandrakasan G (2002) Influence of Terminalia chebula on dermal wound healing in rats. Phytoter Res 16:227–231. https://doi.org/10.1002/ptr.827

Sumitra M, Manikandan P, Gayathri VS, Suguna L (2009) Influence of honey on energy metabolism during wound healing in rats. Sch Res Exch. https://doi.org/10.3814/2009/715320

Talekar YP, Apte KG, Paygude SV, Tondare PR, Parab PB (2017) Studies on wound healing potential of polyherbal formulation using in vitro and in vivo assays. J Ayurveda Integr Med 8:73–81

Valdez JC, Peral MC, Rachid M, Santana M, Perdigon G (2005) Interference of Lactobacillus plantarum with Pseudomonas aeruginosa in vitro and in infected burns: the potential probiotics in wound treatment. Clin Microbiol Infect 11:472–479. https://doi.org/10.1111/j.1469-0691.2005.01142.x

Vijayalakshmi K, Premalatha A, Rajakumar S (2011) Production and antimicrobial potential of a broad spectrum antimicrobial protein from a new strain of Bacillus amyloliquefaciens MBL27. Int J Pharm Pharm Sci 3(4):243–249

Vijayalakshmi K, Suseela Rajakumar G (2010) Antimicrobial protein production by Bacillus amyloliquefaciens MBL27: an application of statistical optimization technique. Afr J Microbiol Res 4:2388–2396

Viney C, Harison D (2001) Chitosan-preparation and properties. Indian Drugs 39:191–194

William GM, Herbert JQ (1985) Method of achieving hemostasis inhibiting fibroplasia and promoting tissue regeneration in a tissue wound. US patent No. 4532134

Woessner JF, Arch. (1961) The determination of hydroxyproline in tissue and protein samples containing small portions of this imino acid. Arch Biochem Biophys 93:440–447. https://doi.org/10.1016/0003-9861(61)90291-0

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