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Potentiation of Bioactivity in Keratin and Probiotic Wound Healing Composite Hydrogels

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Abstract:
Challenges of antimicrobial resistance by pathogens, cellular toxicity, and allergic reactions to conventional wound healing therapies, constitute major drawbacks in wound management. It has, therefore, become imperative to expand the search for biomaterials that possess promising wound healing properties. Among the therapeutic attributes of probiotics are antimicrobial and tissue regeneration potentials. On the other hand, keratin, a biopolymer, is also known to support tissue regeneration, thereby facilitating wound healing. It has been shown to possess a measurable degree of antimicrobial properties. The combination of keratin and a probiotic in a wound-healing formulation should expectedly produce mutual potentiation in their antimicrobial and tissue regeneration properties. In the present study, keratin in chicken feather was extracted by alkaline hydrolysis. Hydrogels were prepared with combinations of the keratin and a probiotic (Lactobacillus acidophilus) at two levels of bio-load (1 x 108 CFU/ml and 2 x 108 CFU/ml). The probiotic-loaded keratin-based hydrogels were subjected to in vivo tests for wound-healing efficacy and control of multi-drug resistant wound pathogens. Fourier transform infra-red (FTIR) spectroscopic characterization of keratin showed that the protein was structurally intact at the end of extraction process and in the formulation. The formulated hydrogels retained its homogeneity throughout the period of study. The probiotic-enriched hydrogel produced comparatively better re-epithelialization of skin wounds, and control of microbial infection within the period of 16 days, when analysed statistically ($p < 0.05$). This study has demonstrated the potentials of enhanced therapeutic efficacy when L. acidophilus, as a probiotic, is combined with keratin for the management of wounds infected by multi-drug resistant bacteria.

Keywords: Keratin, Lactobacillus acidophilus, multi-drug resistant pathogens, probiotics, skin wound healing.

I. INTRODUCTION

Chronic wounds are inherently associated with disruptions in the skin microflora as a result of polymicrobial infections [1]. Generally, treatment of wounds, especially chronic wounds, is expensive, labour-intensive and relies on a wide range of approaches. Advancements in diagnosis, antimicrobial combination therapies, application of high-efficacy antiseptics and surgeries are among the treatment modalities adopted in managing non-healing wounds [2]. An ideal wound healing formulation should be biocompatible, non-antigenic, protective to damaged tissues, and able to provide moist environment for re-epithelialization. Additionally, the therapy should efficiently facilitate all the phases of wound healing [3, 4]. In spite of these requirements, antimicrobial resistance, cellular toxicity and allergic reactions are still major drawbacks in wound management. Some therapies need to be administered frequently, which may result in cellular toxicity, antimicrobial resistance and allergenicity, ultimately affecting patient compliance and clinical response [5]. There is constant necessity to conduct evidence-based studies on biocompatibility of therapies and the upgrading of existing wound care interventions.

Long before the discovery of microbes and antibiotics, fermented products made with specific species of bacteria were consumed orally as probiotics [6]. These organisms were often used as treatment in cases of diarrhoea and other gastrointestinal disorders as well as a number of other disease conditions. They were also applied topically to wound sites where they played the role of antiseptics and prevented infections, thereby, improving healing [6]. Probiotics, which are generally considered to be beneficial to human health, have been used therapeutically in various disease conditions. Several earlier studies have demonstrated the antimicrobial, anti-inflammatory and tissue regeneration potentials of probiotics [7 – 9].

On the other hand, keratin, a biopolymer, seems to have similar properties as probiotics. Keratin is intrinsically biocompatible and has antibacterial as well as wound healing potentials [10]. A study was done to test the ability of topically administered keratin-based hydrogel loaded with ciprofloxacin to inhibit wound infection from Pseudomonas aeruginosa [11]. The treated wounds were assessed on days 3, 7 and 11 in comparison with untreated wounds. It was observed that ciprofloxacin-loaded keratin hydrogels eradicated up to 99.9% of Pseudomonas aeruginosa in the treated wounds. Also, from the drug release study, 60 % of the loaded drug was released in 10 days, thereby consistently preventing bacterial growth. The intrinsic ability of keratin to facilitate cell proliferation, retain adequate wound moisture as well as its antimicrobial properties has led to diverse applications of the protein in surgical and non-surgical wound treatments [12]. Loan et al. [13] conducted a study on Keragel

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and Keramatrix. These keratin-based products were evaluated, in comparison with some standard products, to determine their efficacy on superficial and partial thickness burn injuries. The products were found to facilitate healing with minimal scarring. It was also reported that the products were capable of repairing injuries at the dermal and epidermal layers. They were well tolerated as a result of very minimal pain and allergic reactions. A case study was reported by Paulsen and Bygum [14] where keratin-rich gel was topically applied as an adjuvant in the treatment of pyoderma gangrenosum, an ulcerative skin disease. Treatment with the keratin gel was chosen as a better alternative to long term anti-inflammatory therapy. As a last resort, the keratin gel was applied concurrently with a TNF-α antagonist which was administered systemically. This resulted in accelerated healing, thereby, making the gel a safe and effective topical adjuvant in the healing process.

It has been established that keratinous proteins serve as scaffolds, which support tissue regeneration and formation of extracellular matrices [15]. There is sufficient evidence on the stabilizing effect of keratin for mammalian skin, thus promoting the healing of skin wounds. But there are no reports, in literature, showing the complementary effects of combinations of L. acidophilus and keratin in the care of wounds, especially those infected by methicillin-resistant Staphylococcus aureus (MRSA) or multi-drug resistant Pseudomonas aeruginosa (MDR-PA). Consequently, in this study, we seek to determine the effects of combining a probiotic and keratin on multidrug-resistant wound pathogens, and their potentials in enhancing the process of wound healing.

II. Materials and Methods

Materials

Chicken feathers were obtained from a local chicken processing plant. Keratin was extracted from the chicken feathers in our laboratories. The following analytical grade reagents were used in this study: petroleum ether (ACS Chemicals, India), sodium sulphide (Loba Chemie PVT, India), sodium hydroxide (Loba Chemie PVT, India), hydrochloric acid [GFS Chemicals, Inc. Columbus, OH]), sodium carbonate (Merck, KGaA, Germany), anhydrous copper sulphate (Qualikems Lifesciences Pvt. Ltd, India), anhydrous sodium tartrate (Qualikems Lifesciences Pvt. Ltd, India), polyvinyl alcohol (Sigma-Aldrich, USA), triethanolamine (Lab Alley Essential Chemicals, USA). L. acidophilus (Mason Natural, USA) was obtained from a commercial source. MRSA and MDR-PA were obtained as clinical isolates, and characterised in our laboratories. The microbiological media were: mannitol salt agar (TM Media, India), cetrimide agar (TM Media, India), and De Man, Rogosa and Sharpe (MRS) agar (HiMedia Laboratories Pvt. Ltd, India).

Extraction of Keratin from Chicken Feathers

Chicken feathers (obtained from a local chicken processing plant) were washed under running water to get rid of dirt. The feathers were sun-dried, pulverized, and de-fatted with petroleum ether. In an alkaline hydrolysis process, 100 g of chopped feather was added to two litres of mixture consisting of 0.2 M sodium sulphide in 5 % sodium hydroxide solution. The mixture was stirred at reaction temperature of 40 °C until the feather was completely dissolved (approximately 6 hours). The resulting solution was centrifuged at 10,000 rpm for 10 minutes. The supernatant liquid was maintained at a temperature of 40 °C with the aid of a magnetic hot plate/stirrer (Bioevopeak Inc., USA). Equal proportions (500 ml of feather solution and 500 ml of 2 M hydrochloric acid) were brought to the same temperature of 40 °C. The hydrochloric acid solution was gradually added to the feather solution with constant stirring. The mixture was left to stand for 60 minutes to allow for complete precipitation of the protein. The precipitated protein was collected by filtration using Whatman filter paper of 0.25 µm pore size, and dried in a desiccator chamber. The percentage yield of keratin was calculated using Equation 1.

$$\text{Percentage yield} = \frac{(\text{Mass of protein})}{(\text{Mass of the feathers})} \times 100 \qquad (1)$$

Lowry Assay of Keratin

The assay of keratin in the precipitate was determined using the method of Swati and Arun [16]. The standard Lowry solutions were prepared as follows [17]:

Solution A (alkaline solution): A 4.3 g quantity of sodium carbonate and 2.8 g of sodium hydroxide were dissolved in 500 ml of double distilled water.

Solution B: A 1.4 g quantity of anhydrous copper sulphate was dissolved in 100 ml double distilled water.

Solution C: A 2.8 g quantity of anhydrous sodium tartrate was dissolved in 100 ml double distilled water.

Lowry solution mixture (freshly prepared prior to use): Solution A + Solution B + Solution C in a ratio of 100:1:1 by volume.

An accurate amount of 0.5 g of the extracted keratin was dissolved in 10 ml of 5 % sodium hydroxide solution. The keratin solution was centrifuged at 3000 rpm for 10 minutes. An aliquot (0.1 ml) of the supernatant was diluted to 1 ml with distilled water. To four replicate test tubes was added 5 ml of the Lowry solution mixture and incubated for 10 minutes at room temperature in the dark. At the end of incubation time, 0.1 ml Folin reagent (earlier prepared by adding 5 ml of 2 N Folin and Ciocalteu's phenol reagent into 5 ml of double distilled water, and stored in an amber container) was added to the test tubes and votexed. The tubes were incubated further in the dark for 30 minutes at room temperature. The absorbance readings for keratin were taken at 750 nm with a UV/Vis spectrophotometer (Macylab Instruments Inc., Shanghai), and quantitatively estimated using bovine serum albumin as standard protein.

Preparation of Keratin-Based Hydrogel

A solution consisting of 2 g polyvinyl alcohol, 3 ml glycerin and 40 ml of distilled water was prepared by continuous stirring at 10,000 rpm and 80 oC for 1 hour using a magnetic stirrer (ThermoFisher Scientific, USA). A separate solution

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consisting of 0.5 g of keratin powder in 2 ml of triethanolamine was also prepared. The two solutions were mixed at 40 oC, and stirred continuously for 2 hours. The resulting hydrogel was left to equilibrate at room temperature. The test probiotic, L. acidophilus, was incorporated into the hydrogel at two varying inoculum concentrations.

Fourier transform infra-red (FT-IR) spectroscopy for keratin or hydrogel. The FT-IR spectroscopic analysis was conducted at the Central Science Laboratory, University of Nigeria, Nsukka, using a Shimadzu FT-IR 8300 spectrometer (Shimadzu, Tokyo, Japan) in the wavelength region of 4000 to 400cm-1. A smart attenuated total reflection (SATR) accessory was used for data recording. The potassium bromate (KBr) plate used for the study was cleaned with a tri-solvent (acetone–toluene–methanol in 3:1:1 ratio) mixture for baseline scanning. Exactly, 0.1 g of keratin or hydrogel was mixed with 0.1 ml nujul diluent. The solution was introduced into the potassium bromate (KBr) plate and compressed into discs by applying a pressure of 5 tonnes for 5 minutes in a hydraulic press. The pellets were placed in the light path and spectra were recorded in 60 seconds using Gram A1 spectroscopy software. Afterwards, the chemometrics was performed using TQ Analyzer1.

In vivo wound healing tests on the keratin-based probiotic-loaded hydrogel. Albino male rats (n = 40) with weight range of 25 – 35 g were used for this study. The rats were kept under pathogen free conditions. Feeds and water were provided ad libitum while the animals were acclimatizing to the laboratory environment. Prior to infliction of skin wounds, the rats were anesthetized by injection of Ketamin® (Hameln Pharma Ltd, UK), 80 mg/kg b. w., intramuscularly at the dorsal part. When the animals were fully sedated, a clean shaving device was used to remove hair from the dorsal part of each rat and the exposed skin was disinfected with 70 % (v/v) ethanol. With the aid of sterile surgical blades, circular excision wounds of 2.54 mm in diameter were inflicted on each rat. The wounds were left open for the duration of study. The animals were kept singly in separate cages, but randomized in groups of 5 animals. After 24 hours, animals in each group were inoculated with 0.1 ml fresh culture of MRSA (1.8 x 108 CFU/ml) or MDR-PA (1.8 x 108 CFU/ml). Subsequently, the animals received wound healing treatments thus:

(a) Gentamicin sulphate skin ointment once daily for 16 days.
(b) Keratin-only hydrogel once daily for 16 days.
(c) Keratin + probiotic (1 x 108 CFU/ml) hydrogel once daily for 16 days.
(d) Keratin + probiotic (2 x 108 CFU/ml) hydrogel once daily for 16 days.

Assessment of Wound Size and Microbial Load

Clear photographs of the healing process were taken on days 0, 4, 8, 12 and 16. A digital vernier caliper (Shaghai Trisun, China) was used to measure the wound diameter (mm) prior to treatments and during course of the study. The percentage wound closure was calculated using Equation 2.

$$\%\ \text{Wound closure} = \frac{(D_o - D_t)}{D_o} \times 100 \quad (2)$$

where D_o is the original wound diameter and D_t is the diameter of wound at the time of measurement. Bacterial load at the wound site was determined prior to treatments and on subsequent days. Swab samples were collected from the wounds, transferred separately into 5 ml of sterile normal saline and homogenized. Ten-fold serial dilutions of the microbial suspensions were performed. An aliquot of 0.1 ml of dilution was aseptically spread on three replicate plates of sterile mannitol salt agar and cetrimide agar respectively for S. aureus and P. aeruginosa. All plates were incubated at 37 °C for 48 hours. The mean of bacterial colonies (n) per group was determined, and mean colony forming units were derived using Equation 3.

$$\text{Mean CFU/group} = \frac{5n \times \text{Level of dilution}}{0.1} \quad (3)$$

Over the course of treatment, viability of L. acidophilus was evaluated. Using a sterile loop, the probiotic loaded gel was collected and streaked on the surface of De Man, Rogosa and Sharpe (MRS) agar. The plates were incubated at 37 °C for 48 hours. The presence of off-white coloured colonies was indicative of viable L. acidophilus cells.

Statistical Analysis

Data from the in vivo studies were expressed as mean and standard deviation. Student's T-test was used for pair-wise comparison of effects of formulated gels and commercial gentamicin ointment on rates of wound closure and bio-load reduction. The statistical significance was determined at 5 % level of significance (p = 0.05).

III. Overall Format Specifications

The title and author data are in one-column format, while the rest of the paper is in two-column format. To accomplish this, *Word* has section break commands that will separate the one and two-column format. There are two ways to setup this format: 1) Use this template as a guide, 2) make your own formatted template.

To make your own template, open a new document and begin by inserting the title and author information in the standard one-column format. After you type in your title and your author information, double space. Click the Insert menu, select Break, then select Section Break—Continuous. This will set your paper up in sections so you can now proceed to a two-column section for the body of your paper.

IV. Results

Upon stirring the chicken feathers in a solution of the reducing agents, complete dissolution was observed within 30 minutes. And at the end of the extraction process, 86 g of keratin was obtained from 500 g of chicken feather, giving a percentage yield of 17.2 %. Further characterization of the protein revealed that the extraction process did not cause any chemical alteration or damage to the protein. The Lowry assay confirmed the integrity of extracted keratin. Quantitative assay

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was determined using standard calibration of bovine serum albumin (BSA) as shown in Figure 1.

The Fourier transform infra-red (FT-IR) spectrum of the keratin (Figure 2) demonstrated a sharp peak observed at 1117 cm-1 corresponding to -C-N- group stretching vibration. The observed peak at 1237 cm-1 corresponds to -CN-H functional group comprising -C-N- and -C-C- stretching vibrations as well as -N-H bending vibration. The spectrum showed characteristic peaks between 1600 – 1700 cm-1 which represents α and β confirmation of amide I (Table 1). The carbonyl group of keratin amides can be found within this region as well. Peaks in the range of 1500 – 1560 cm-1 confirmed the presence of amide II. Within the region of 2900 – 3100 cm-1, stretching vibrations like -C-H, CH3 and -N-H were observed. From the spectrum, amide A absorption band was seen at 3286 cm-1 and amide B was seen around the region of 3062 cm-1 to 3074 cm-1. The sharp peak at 3349 cm-1 corresponds to hydrogen-bonding in the -N-H and -O-H stretching of amide groups [18]. These results particularly showed the presence of functional groups like -N-H, -C=O, -C-N- and -CN-H which make up the building blocks of amino acids of keratin protein.

Some of the peaks observed at 1900 cm-1, 1345 cm-1 and within the range of 1228 – 1251 cm-1 were suggestive of the following amino acids: cysteine, glutamic acid and tryptophan respectively. From the above analysis, it can be seen that the extracted protein showed characteristics of secondary structure of keratin and matched typical spectra of keratinous protein samples from chicken feathers [19].

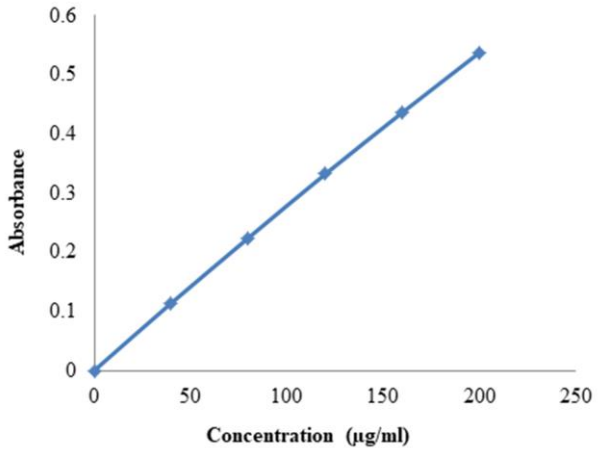

Figure 1: Calibration plot for assay of keratinous protein (standard protein = BSA).

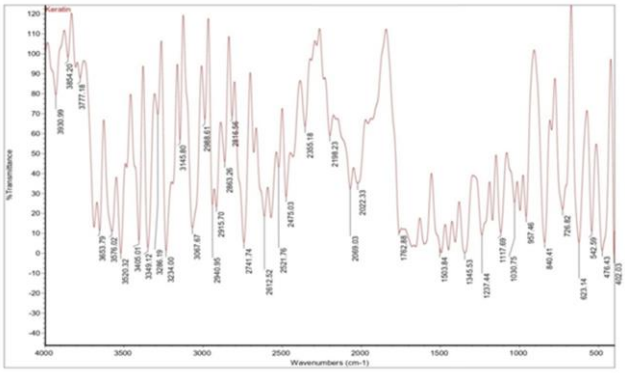

Figure 2: FT-IR spectrum of keratin.

TABLE 1: ANALYSIS OF FT-IR SPECTRUM FOR KERATIN.

Frequency (cm^{-1})	Frequency (cm^{-1}) from FT-IR	Functional groups
3417	3405.01	Amide
3300	3349	-N-H and -O-H
3287	3286.19	Amide A
3062 – 3074	3067.67	Amide B
2900 – 3100	2940.95	-N-H
1951	1900	C=O
1600 – 1700	1600	Carbonyl
1556 – 1587		Tryptophan
1500 – 1560	1503.84	Amide II
1311	1345.53	Glutamic acid
1230	1237.44	Amide III
1228 – 1251	1237.44	Amide III
1100	1117.69	-C-N- stretch

The FT-IR spectroscopy of the hydrogel was conducted to ascertain possible compatibility between keratin and formulation excipients. The spectrum is shown in Figure 3, and the analysis is presented in Table 2. Characteristic bands were analyzed in order to confirm the protein nature of the extracted keratin via the functional groups. As expected, there were characteristic peaks corresponding to important functional groups like -N-H-, -NH2, -C-N-, -C-H, and C=O. The peaks at 559 and 922 cm-1 represent C-S and S-S bonds of cystine amino acids. The amide I peak of keratin was seen to be around 1600 cm-1. For the hydrogel, this peak shifted to 1644.85 cm-1. This could be as a result of breaking of hydrogen bonds between the chains of keratin molecules and formation of covalent bonds with water during the processing of hydrogel formation [19]. The peaks observed at 2558.52 which fell within the region of 2500 – 3000 cm-1 showed the presence of -N-H stretch functional group. The peak at 3364.19 cm-1 showed -O-H stretch/bend functional group. For the keratin extract, this was observed at a close range of 3349.12 cm-1. From the above comparison, it would appear that the keratin and other hydrogel excipients were

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appreciably compatible, with only minimal physical or chemical reactions.

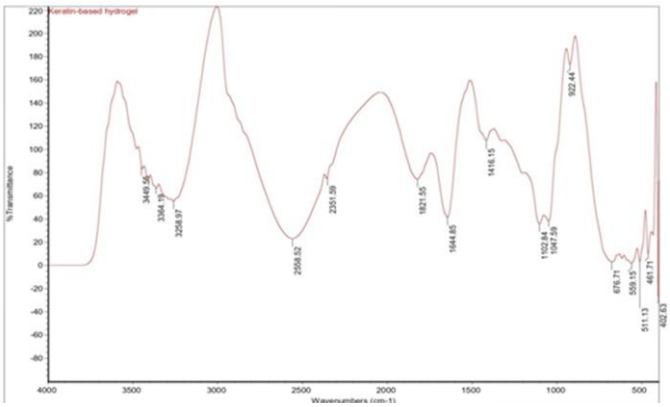

Figure 3: FT-IR spectrum for keratin-based hydrogel.

TABLE 2: ANALYSIS OF FT-IR SPECTRUM FOR KERATIN-BASED HYDROGEL.

FT-IR Frequency (cm^{-1}) range	Frequencies (cm^{-1}) of keratin transmission	Functional groups
3417	3449.95	Amide group
3300	3364.19	-OH alcohol stretch
3287		
3062 – 3074		
2900 – 3100		
2500 – 3000	2558.52	-NH amines bending
1600 – 1700	1644.85	Carbonyl, Amide I
1556 – 1587		
1400 – 1500	1416.15	
1100	1102.84	-CN- stretch
920 – 950	922.44	C-S and S-S bonds of
568	559.15	cysteine
500 – 400	461.71	C-S and S-S bonds of
		cysteine

As illustrated in Figures 4 – 7, a typical pattern of healing was observed in all groups. On day 0, the wound sizes were 2.54 mm for all experimental groups. On day 1, the wound surfaces were observed to be physically stable with no exudates. The fresh wounds were then inoculated with MRSA and MDA-PA. Subsequently, wound healing efficacy and antimicrobial potential of the formulations vis-á-vis commercial gentamicin skin ointment were determined under four experimental conditions:

(i) Infected wounds treated with gentamicin skin ointment
(ii) Infected wounds treated with keratin-only hydrogel
(iii) Infected wounds treated with keratin + probiotic (1 x 10^8 CFU/ml) hydrogel
(iv) Infected wounds treated with keratin + probiotic (2 x 10^8 CFU /ml) hydrogel

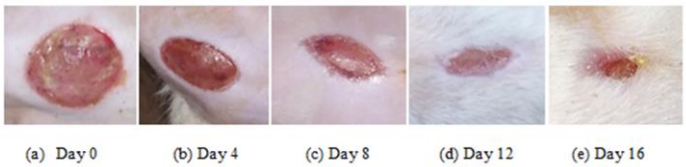

Figure 4: Group receiving gentamicin sulphate skin ointment.

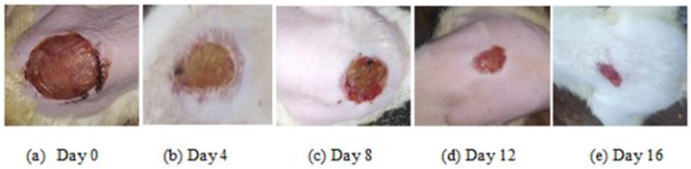

Figure 5: Group receiving gel base treatment.

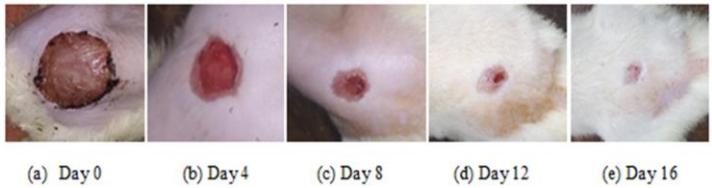

Figure 6: Group receiving hydrogel containing *Lactobacillus acidophilus* (1.0 x 10^8 CFU/ml).

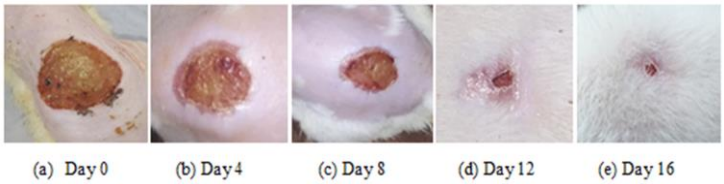

Figure 7: Group receiving hydrogel containing *Lactobacillus acidophilus* (2.0 x 10^8 CFU/ml).

The mean wound diameter and percentage reduction in wound diameter over the duration of treatment are shown in Tables 3 and 4 respectively. A slight decrease in wound diameter was observed across all experimental groups on the 4th day. However, groups receiving keratin-only or probiotic-loaded hydrogels had higher percentage reduction in wound area (12, 16, and 12 %) compared to the gentamicin treated group (8 %). The results show that, in all cases, healing was progressive. On the 12th day, notable wound contraction was observed in the groups treated with L. acidophilus (1 x 108 CFU/ml) and L. acidophilus (2 x 108 CFU/ml) (80 % and 72 % respectively). Correspondingly, colony counts showed a progressive decrease in microbial load. This was not the case in the gentamicin-treated group as microbial load did not decrease significantly over the duration of treatment (Tables 5 and 6).

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TABLE 3: MEAN WOUND DIAMETER, MM (± SD) OVER 16 DAYS

Formulation	Day 0	Day 4	Day 8	Day 12	Day 16
Gentamicin skin ointment	2.54	2.33 ± 0.06	2.03 ± 0.12	1.73 ± 0.17	1.42 ± 0.12
Keratin-only gel	2.54	2.24 ± 0.07	1.93 ± 0.11	1.22 ± 0.12	0.72 ± 0.13
L. acidophillus (1.0×10^8 CFU/ml) gel	2.54	2.13 ± 0.10	1.42 ± 0.14	0.51 ± 0.14	0.10 ± 0.04
L. acidophillus (2.0×10^8 CFU/ml) gel	2.54	2.24 ± 0.06	1.52 ± 0.14	0.72 ± 0.10	0.30 ± 0.11

TABLE 4: PERCENTAGE (%) REDUCTION IN MEAN WOUND DIAMETER OVER 16 DAYS.

Formulation	Day 0	Day 4	Day 8	Day 12	Day 16
Gentamicin skin ointment	0	8	20	32	44
Keratin-only gel	0	12	24	52	72
L. acidophillus (1.0×10^8 CFU/ml) gel	0	16	44	80	96
L. acidophillus (2.0×10^8 CFU/ml) gel	0	12	40	72	88

TABLE 5: ASSESSMENT OF REDUCTION IN MICROBIAL LOAD OF MRSA

	Day 0	Day 4	Day 8	Day 12	Day 16
Gentamicin skin ointment	1.5×10^8	1.2×10^8	1.1×10^8	9.6×10^7	7.8×10^7
Keratin gel base	1.5×10^8	9.2×10^7	5.9×10^7	1.2×10^7	5.0×10^6
L. acidophillus (1.0×10^8 CFU/ml) gel	1.5×10^8	6.2×10^7	2.2×10^7	8.0×10^6	0
L. acidophillus (2.0×10^8 CFU/ml) gel	1.5×10^8	7.0×10^7	3.8×10^7	1.1×10^7	0

*Unit of average colony forming units were recorded in CFU/ml

TABLE 6: ASSESSMENT OF REDUCTION IN MICROBIAL LOAD OF MDR-PA

	Day 0	Day 4	Day 8	Day 12	Day 16
Gentamicin skin ointment	1.8×10^8	1.5×10^8	1.4×10^8	9.2×10^7	8.8×10^7
Keratin gel base	1.8×10^8	1.2×10^8	7.5×10^7	2.2×10^7	3.0×10^6
L. acidophillus (1.0×10^8 CFU/ml) gel	1.8×10^8	8.2×10^7	3.1×10^7	7.0×10^6	0
L. acidophillus (2.0×10^8 CFU/ml) gel	1.8×10^8	9.7×10^7	4.6×10^7	1.0×10^7	0

*Unit of average colony forming units were recorded in CFU/ml

V. DISCUSSION

Keratin-based biomaterials have been variously applied in therapeutic formulations due to their intrinsic biocompatibility, biodegradability, mechanical stability and natural abundance [14]. The structural rigidity of keratin promotes formation of extracellular matrices, facilitates cell to cell interactions, and may account for its significance in wound healing [20, 21]. In formulating the therapeutic hydrogel, extracted keratin served both as a bioactive polymer and gelling agent. Upon successful formulation, the keratin-based hydrogel showed good physical qualities appearing in cream colour with homogenous consistency. Keratin-based hydrogel was selected as the topical delivery system for this study due to its capacity to sustain the moist environment needed for autolytic debridement of necrotic tissue and skin regeneration [22]. Other major advantages of hydrogels in wound healing are: enabling exchange of gases, absorption of excess exudates, provision of protection and acting as barrier to prevent invasion of pathogenic microorganisms, decrease of surface necrosis in wounds, ease of application and removal, soothing effect on wound surface and reduction of pains [23]. On the contrary, wound dressings that involve the use of cotton and gauze bandages tend to absorb a lot of the moisture, thereby, leaving the wound surface dry. This can result in increased discomfort and delayed healing [23].

Hydrogels can be used on burns, surgical wounds, skin tears, and pressure ulcers. The hydrogels used in this study consisted of keratin, polyvinyl alcohol, glycerol and at least 70 % of water. Keratin served as a crosslinking agent in the hydrogels. Its biocompatible nature was also considered to be of great value in wound care. The hydrogels were prepared in pH range of 5.5 – 6.5. It has been established that dressings with slightly acidic pH are more ideal for topical application as the low pH creates unfavourable environment for bacterial proliferation [24, 25]. Other factors that were put into consideration when formulating the keratin-based hydrogels included: depth and location of the wound, as well as type of infection.

In the attempt to combat the challenges of wound infections, various wound dressings have been impregnated with antimicrobial agents, which act by either killing the pathogens or inhibiting their growth. Therapeutic agents, such as vitamins, minerals, growth factors that play vital roles in tissue restoration, have also been incorporated into wound dressings [26]. Over the years, antibiotic-loaded gauze, hydrocolloids, hydrogels, alginates, patches and films have been used to deliver therapeutic agents in wound management [27]. The present study, however, adopts the concept of "using bacteria to fight bacteria", and keratin-based hydrogel is considered as an efficient vehicle for delivery of the probiotic to wound sites. L. acidophilus was chosen as a model for eliminating infection and facilitating rapid epithelialisation. It is a lactic acid producing probiotic that plays beneficial roles in the biological system. It does not release virulent factors when administered in adequate amounts [28]. From the study, it could be seen that L. acidophilus was able to protect the wounds from pathogenic effects of MRSA and MDR-PA. Probiotic bacteria can act against a range of pathogens simultaneously, including drug-resistant bacteria and fungi [29]. It has been established that lactic acid producing bacteria secrete substances like hydrogen peroxide (H2O2), lactic acid

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and bacteriocin, which inhibit microbial proliferation. This class of probiotic bacteria inhibits the pathogenic action of microorganisms by competing with them for nutritional requirements. Lactobacillus species has been shown to interfere specifically with the ability of pathogenic bacteria to form biofilms and produce quorum-sensing molecules [29]. The presence of L. acidophilus on the skin could also trigger immune cells which secondarily enable the host to fight any super-infecting pathogens. Acidic pH has been reported to contribute to the activation of cells involved in immune response and tissue repair [30]. The acidic nature of L. acidophilus may have played an important role in interfering with pathogenicity of MRSA and MDR-PA on the wound sites.

Skin contraction is a very significant phase in the healing of excision wounds. This process which involves movement of the wound edges towards the centre leads to a decrease in wound diameter over time. Generally, contraction takes about 14 – 18 days during which period healing is expected to have been completed. In this study, the excision wounds of 2.54 mm were subjected to various treatment conditions and the rate of epithelization was monitored over time. From analysis of the data obtained, it was seen that the groups treated with probiotic-loaded formulations had more significant reduction in wound diameter over the duration of this study ($p < 0.05$). Essentially, the presence of multi-drug resistant isolates did not impair wound healing efficacy of the formulation. The re-epithelialization was almost complete (96 %) in the group receiving single strength of L. acidophilus compared to other treatment groups ($p < 0.05$). This observation suggests that keratin-based hydrogels enriched with L. acidophilus (1 x 108 CFU/ml), in addition to enhancing the eradication of bacterial colonization also accelerates tissue regeneration. Lactic acid producing species of probiotic bacteria possess the intrinsic ability to modulate release of growth factors, trigger the proliferative phase of wound healing, and remodel epidermal and dermal cells [31]. The physical appearances of wounds treated with probiotic-loaded hydrogels were observed to be similar to those of a normal skin, demonstrating complete restoration of the epidermal layer and rejuvenation of hair follicles. There were no allergenic reactions observed on the skin. It was observed that treatment of the infected wounds with L. acidophilus (2 x 108 CFU/ml)-loaded gel did not produce a higher rate of healing than the groups receiving single strength probiotic. There was no significant difference in the mean percentage decrease in wound diameter ($p > 0.05$). This would indicate that efficacy of probiotic in wound healing is not dose-dependent.

In the gentamicin treated group, progression in wound healing remained slow and largely incomplete throughout the duration of treatment compared to groups receiving keratin and probiotic-loaded hydrogels. This could be attributed to possible resistance of the pathogens to gentamicin. It is, therefore, obvious that the keratin-probiotic formulations yielded better antibacterial and tissue regeneration effects than commercial gentamicin ointment.

VI. Conclusion

Keratin was successfully extracted from chicken feather using the alkaline hydrolysis and acid precipitation method. By regulating certain variables, such as extraction time, temperature and solvent, good quality and reasonable yield of keratin were obtained. Keratin was the biopolymer of choice for this study because of its properties, notably biocompatibility, tissue regeneration and antimicrobial potentials. FT-IR spectroscopy of extracted keratin showed the presence of characteristic functional groups present in keratinous proteins while the FT-IR spectra of the hydrogels indicated that there were no strong chemical interactions between the keratin and formulation excipients. It can be concluded that under appropriate thermo-chemical conditions, structure and quality of extracted keratin can be preserved. Keratin-based hydrogel was successfully formulated as a vehicle for localized administration of the probiotic to wound sites. Progressive wound contraction was observed in groups treated with probiotic-enriched, keratin-based hydrogels. Moreover, pathogenic effects of multi-drug resistant bacteria on the wound sites were hindered by the combined antimicrobial activities of keratin and L. acidophilus. Suppression of pathogenic infections and the re-epithelialization effect of this therapeutic combination were found not to be dose-dependent. This study has unequivocally demonstrated the usefulness of integrating probiotic-loaded, keratin-based hydrogels in the treatment regimens for wounds infected by multi-drug resistant bacteria, especially MRSA and MDR-PA. The concept adopted in this work is also in line with a long-held cliché of “fighting bacteria with bacteria”.

VII. Recommendations

This work does not claim to have exhausted the thoughts expounded herein. The ideas are open to further investigation. It is recommended that more work should focus on determining whether the antibacterial and wound healing actions of the combination of L. acidophilus and keratin are limited to mutual potentiation or involve classical synergism. In addition, the present study can be expanded to include other skin wound pathogens, such as Escherichia coli, Streptococcus pyogenes and fungi. Formation of bacterial biofilms is known to complicate the process of skin wound management. With this in mind, it would be an interesting area of study to ascertain the potentials of probiotics, or their combinations with keratin, in inhibiting bacterial biofilm formation in order to promote rapid wound healing. These and other innovative ideas should form the basis of further studies on this subject.

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IX. Conflict of Interest

No conflicts of interest were declared by the authors.

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X. REFERENCES

1. Nahid M. A, Griffin J. M, Lustik M. B, Hayes J. J, Fong K., Horseman T. S., Menguito M, Snesrud E. C, Barnhill J. C, and Washington M. A. (2021) A longitudinal evaluation of the bacterial pathogens colonizing chronic non-healing wound sites at a United States military treatment facility in the pacific region. Infection and Drug Resistance, 14: 1–10. https://doi.org/10.2147/IDR.S260708.
2. Mahmoudi M, and Gould L. (2022) Opportunities and challenges of the management of chronic wounds: A multidisciplinary viewpoint. Chronic Wound Care Management and Research, 7: 27– 36. https://doi.org/10.2147/CWCMR.S260136.
3. Gurtner G. C., and Chapman M. A. (2016) Regenerative medicine: Charting a new course in wound healing. Advances in Wound Care, 5(7): 314–328. https://doi.org/10.1089/wound.2015.0663.
4. Boateng J, and Catanzano O. (2015) Advanced therapeutic dressings for effective wound healing--A review. Journal of Pharmaceutical Sciences, 104(11): 3653–3680. https://doi.org/10.1002/jps.24610.
5. Frykberg R. G, and Banks J. (2015) Challenges in the treatment of chronic wounds. Advances in Wound Care, 4(9): 560–582. https://doi.org/10.1089/wound.2015.0635.
6. Brandi J, Cheri S, and Manfredi M. (2020) Exploring the wound healing, anti-inflammatory, anti-pathogenic and proteomic effects of lactic acid bacteria on keratinocytes. Scientific Reports, 10 (1), 1–14.
7. Tan-Lim C, Esteban-Ipac N, Mantaring J, Chan SYE, Recto M, Sison OT, and Alejandria MM. (2021) Comparative effectiveness of probiotic strains for the treatment of pediatric atopic dermatitis: A systematic review and network meta-analysis. *Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology*, 32(1): 124–136. https://doi.org/10.1111/pai.13305.
8. McFarland LV, Ship N, Auclair J, and Millette M. (2018) Primary prevention of Clostridium difficile infections with a specific probiotic combining Lactobacillus acidophilus, L. casei, and L. rhamnosus strains: assessing the evidence. Journal of Hospital Infection, 99(4): 443–452. doi: 10.1016/j.jhin.2018.04.017.
9. Surmeli M, Macin S, Akyon Y, and Kayikcioglu AU. (2019) The protective effect of *Lactobacillus plantarum* against meticillin-resistant *Staphylococcus aureus* infections: an experimental animal model. *Journal of Wound Care*, 28: 29 – 34. doi: 10.12968/jowc.2019.28.sup3b.s29.
10. Raina N, Rani R, Pahwa R, and Gupta M. (2022) Biopolymers and Treatment Strategies for Wound Healing: An Insight View. International Journal of Polymeric Materials and Polymeric Biomaterials, 71: 359–375. https://doi.org/10.1080/00914037.2020.1838518.
11. Roy D. C, Tomblyn S, Burmeister D. M, Wrice N. L, Becerra S. C, Burnett L. R, Saul J. M. and Christy R. J. (2015) Ciprofloxacin-loaded keratin hydrogels prevent Pseudomonas aeruginosa infection and support healing in a porcine full-thickness excisional wound. Advances in Wound Care, 4(8), 457–468. https://doi.org/10.1089/wound.2014.0576.
12. Patrucco A, Visai L, Fassina L, Magenes G. and Tonin C. (2019) Keratin-based matrices from wool fibers and human hair. Materials for Biomedical Engineering, Elsevier, 375–403. http://dx.doi.org/10.1016/B978-0-12-816872-1.00013-3.
13. Loan F, Cassidy S, Marsh C. and Simcock J. (2016) Keratin-based products for effective wound care management in superficial and partial thickness burns injuries. Burns, 42, 541–547.
14. Paulsen E. and Bygum A. (2019) Keratin Gel as an adjuvant in the treatment of recalcitrant pyoderma gangrenosum ulcers: A Case Report. Acta Dermato-Venereologica, 99(2), 234–235. https://doi.org/10.2340/00015555-3081.
15. Singaravelu S, Ramanathan G, Muthukumar T, Raja M. D, Nagiah N, Thyagarajan S, Aravinthan A. P. G, Natarajan T. S, Geetha S, Kim JH, and Sivagnanam U. T. (2016) Durable keratin-based bilayered electrospun mats for wound closure. Journal of Materials Chemistry, 4(22): 3982–3997. https://doi.org/10.1039/c6tb00720a.
16. Swati S, and Arun G. (2016) Sustainable Management of Keratin Waste Biomass: Applications and Future Perspectives. Brazilian Archives of Biology and Technology, 59: 1-14. https://doi.org/10.1590/1678-4324-2016150684.
17. Lowry O. H, Rosebrough N. J, Farr A. L, and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry, 193(1): 265–275.
18. Mckittrick J, Chen P. Y, Bodde S. G, Yang W, and Novitskaya E. (2012) The structure, functions, and mechanical properties of keratin. Journal of the Minerals, Metals & Materials Society, 64: 449-468. https://doi.org/10.1007/s11837-012-0302-8.
19. Gindaba G, Filate S, and Belay B. (2019) Extraction and characterization of natural protein (keratin) from waste chicken feather. International Journal of Modern Science and Technology, 4(7): 174-179.
20. Chandini D, Charulatha M, Radhakrishnan L, and Sundaram M. (2017) In vitro evaluation of natural keratin-based hydrogel from chicken feather waste for controlled

drug release. International Journal of Current Microbiology and Applied Sciences, 6: 3488-3495. 10.20546/ijcmas.2017.610.411.

21. Chandini D, Charulatha M, Radhakrishnan L, and Sundaram M. (2017) In vitro evaluation of natural keratin-based hydrogel from chicken feather waste for controlled drug release. International Journal of Current Microbiology and Applied Sciences, 6: 3488-3495. 10.20546/ijcmas.2017.610.411.
22. Feroz S, Muhammad N., Ranayake J., and Dias G.. (2020) Keratin-based materials for biomedical applications. Bioactive Materials, 5(3): 496–509. https://doi.org/10.1016/j.bioactmat.2020.04.007.
23. Priyaah K., Arun G., and Swati S. (2017) Synthesis of wound-healing keratin hydrogels using chicken feathers proteins and its properties. International Journal of Pharmacy and Pharmaceutical Sciences, 9(2): 171-178. https://doi.org/10.22159/ijpps.2017v9i2.15620.
24. Jain S., Domb A. J., and Kumar N. (2014) In Focal Controlled Drug Delivery. 2nd edn. Boston, US, Springer, p. 585.
25. Saarai A., Kasparkova V., Sedlacek T., and Saha P.. (2013) On the development and characterisation of crosslinked sodium alginate/gelatine hydrogels. Journal of the Mechanical Behavior of Biomedical Materials, 18: 152–166. https://doi.org/10.1016/j.jmbbm.2012.11.010.
26. Hui-Jeong G., Youn-Mook L., Sung-Jun A, Min-Ho Y., Seol-Hee H., Ho-Nam C., and Young-Chang N.. (2009) Characterization of PVA/glycerin hydrogels using γ-irradiation for advanced wound dressings. Korean Journal of Chemical Engineering, 26(6): 1686-1688. DOI: 10.1007/s11814-009-0246-z.
27. Saghazadeh S., Rinoldi C., Schot M., Kashaf S. S., Sharifi F., Jalilian E., Nuutila K., Giatsidis G., Mostafalu P., Derakhshandeh H, Yue K, Swieszkowski W, Memic A, Tamayol A., and Khademhosseini A.. (2018) Drug delivery systems and materials for wound healing applications. Advanced drug delivery reviews, 127, 138–166. https://doi.org/10.1016/j.addr.2018.04.008.
28. Gudadappanavar A. M., Hombal P. R., Timashetti S. S., and Javali S. B. (2017) Influence of Lactobacillus acidophilus and Lactobacillus plantarum on wound healing in male Wistar rats - an experimental study. International Journal of Applied & Basic Medical Research, 7(4): 233–238. https://doi.org/10.4103/ijabmr.IJABMR_329_16.
29. Satish L, Gallo P. H., Johnson S, Yates C. C., and Kathju S. (2017) Local Probiotic Therapy with Lactobacillus plantarum Mitigates Scar Formation in Rabbits after Burn Injury and Infection. Surgical Infections, 18(2): 119–127. https://doi.org/10.1089/sur.2016.090.
30. Peral M. C., Martinez MA, and Valdez JC. (2009) Bacteriotherapy with Lactobacillus plantarum in burns. International Wound Journal, 6(1): 73–81. https://doi.org/10.1111/j.1742-481X.2008.00577.x.
31. Lukic J, Chen V, Strahinic I, Begovic J, Lev-Tov H, Davis SC, Tomic-Canic M, and Pastar I. (2017) Probiotics or pro-healers: the role of beneficial bacteria in tissue repair. Wound Repair and Regeneration, 25(6): 912–922. https://doi.org/10.1111/wrr.12607.