Physiology and Pharmacology 29 (2025) 408-423
Experimental Research Article

Evaluation of the effect of biodegradable dressing prepared from chitosan and external microvesicles of *Bifidobacterium bifidum* on burn wound healing in male Wistar rats

Seyed Amin Mousavinezhad[1], Naser Harzandi[1*], Azam Marjani[2,3], Parvaneh Jafari[4]

1. Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran
2. Department of Chemistry, Arak Branch, Islamic Azad University, Arak, Iran
3. Halal Reserch Center of IRI, Food and Drug Administration, Ministry of Health and Medical Education, Tehran, Iran
4. Department of Microbiology, Arak Branch, Islamic Azad University, Arak, Iran

ABSTRACT

Introduction: The burn wound healing process is multi-variable, and various factors are involved. Chitosan-based dressings are acceptable materials for wound healing. PProbiotics and postbiotics can in-crease the effectiveness of these dressings.

This study aimed to investigate the effect of a biodegradable dressing prepared from chitosan and external microvesicles of *Bifidobacterium bifidum* on burn wound healing in male Wistar rats.

Methods: *Bifidobacterium bifidum* was cultured, the activated crude supernatant (ACS) was separated, and the microvesicles were isolated with the help of Ultra-centrifugation at 150,000×g at 4°C for 30 minutes. Chitosan films containing and without microvesicles were prepared using a heater stirrer at 29°C. The effectiveness of the membranes was evaluated. For this purpose, 60 male rats with second-degree burns were randomly divided into 5 groups (n=12) and were treated for 21 days. The process of wound healing was examined macroscopically and microscopically (wound histology and evaluation of gene expression of cytokines interleukin-8, interleukin-10, and VEGF) on the days 3, 7, 14 and 21. The obtained data were analyzed by IBM SPSS.21 software and using Kolmogorov-Smirnov, Kruskal-Wallis, and Mann-Whitney tests with $p \leq 0.05$.

Results: The average diameter of microvesicles was 174 ± 52nm. The thickness of the layers was 2mm. The wounds of the group post-biotic and positive control were closed on the 14th day. Collagen production and epithelialization, as well as inflammation control in the treatment groups, were higher than negative control.

Conclusion: The use of chitosan membranes, particularly those enhanced with ACS and microvesicles, represents a significant advancement in wound care by inflammation control, offering a promising strategy for improving the healing of burn wounds.

Keywords:
Wound healing
Microvesicle
Chitosan
Bifidobacterium bifidum

* Corresponding author: Naser Harzandi, naser.harzandi@kiau.ac.ir

Received 7 September 2024; Revised from 29 November 2024; Accepted 31 December 2024

Citation: Mousavinezhad S.A., Harzandi N, Marjani A, Jafari P. Evaluation of the effect of biodegradable dressing prepared from chitosan and external microvesicles of *Bifidobacterium bifidum* on burn wound healing in male Wistar rats. Physiology and Pharmacology 2025; 29: 408-423. http://dx.doi.org/10.61186/phypha.29.4.408

Introduction

The complex biological process of wound healing includes hemostasis, inflammation, proliferation, and regeneration (Guo and DiPietro 2010). Inflammation is one of the necessary stages of wound healing, which begins with the presence of neutrophils, macrophages, and the production of several pro-inflammatory factors (Falanga 2005). The effect of cytokines and chemokines on wound healing has been proven in many studies (Sorg et al., 2017). Interleukin-8 (IL-8), interleukin-10 (IL-10), and vascular endothelial growth factor (VEGF) are among the cytokines effective in wound healing (Barrientos et al., 2008; Ghaneialvar et al., 2017). Skin wounds are closed by epithelial regeneration and wound contraction. For example, rodents recover primarily by contraction (Sorg et al., 2017; Volk and Bohling 2013). Epithelialization of the skin wound depends on the location, depth, size, microbial contamination, health conditions related to the patient, genetics, and epigenetics, etc (Sorg et al., 2017). Angiogenesis is an important step in the wound healing process, although studies on increasing or decreasing angiogenesis have provided conflicting results(DiPietro 2016).

Biological dressings are the most suitable materials for healing chronic wounds and severe burns. By protecting the wound, maintaining microbial control, and accelerating the maturation of the wound, these dressings create a suitable mechanical and physiological effect on healing the wound (Wang et al., 2020). Among the biological dressings are chitosan compounds. Chitosan is a non-toxic, biocompatible, and biodegradable polymer with many medical applications, including wound dressing, tissue engineering, implant coating, and drug delivery systems (McFadyen and Kaplan 2015; Yin et al., 2021). Studies have shown that chitosan increases wound granulation, so it is a stimulating agent for healing open and deep wounds. Due to coagulation and antimicrobial properties, chitosan-based dressing improves wound healing (Dong et al., 2020). Probiotics are live non-pathogenic microorganisms that can positively affect the host (Mousavi Nezhad et al., 2015). The possibility of inflammatory reactions increases with excessive consumption of probiotics (Sanders 2009). Probiotics prevent the growth of pathogenic bacteria by producing inhibitory compounds such as organic acids, fatty acids, propionate, butyrate, hydrogen peroxide, and bacteriocin compounds (Savadogo et al., 2006). The compounds are primarily released into the environment through vesicles (Wang et al., 2021). Attempts have been made to replace probiotics with prebiotics and postbiotics (probiotic metabolites) (Sabahi et al., 2023). Active crude supernatant of *Bifidobacterium bifidum* has the most significant inhibition in the growth of *Pseudomonas* species (Choi and Shin 2021; Mousavinezhad et al., 2023). As an opportunistic pathogen that causes secondary infections in certain conditions, such as cancer or burn patients (Kambouris et al., 2023). In 2021, Jamaran et al. published a study demonstrating the effectiveness of a new wound dressing that uses postbiotic/chitosan film. The study found that a chitosan membrane containing bacterial supernatant accelerates the healing of skin wounds (Jamaran et al., 2021).

Vesicles are a form of intracellular and extracellular communication used by archaebacteria, bacteria, and eukaryotes (Al-Nedawi et al., 2015). Extracellular vesicles, as bilayer spherical sacs, are produced by many cell types, including Gram-positive bacteria; the sizes of these bags are reported to be 10-400 nm (Brown et al., 2014; Domínguez Rubio et al., 2017).

The present study was conducted with the aim of investigating the effect of chitosan-based wound dressing containing bacterial active crude supernatant and microvesicles extracted from the probiotic *Bifidobacterium bifidum* on burn wound healing in male Wistar rats.

Materials and Methods

The present study was an experimental intervention study that had two phases. In the in vitro phase, a Biodegradable dressing containing *Bifidobacterium bifidum* microvesicles was made; Then, in the in vivo phase, its effect on the healing of burn wounds in male Wistar rats was investigated. This research has been approved by the Research Ethics Committee of the Islamic Azad University of Karaj with ID number IR.IAU.K.REC.1399.020.

Preparation of microvesicles

To isolate microvesicles from active crude supernatant (ACS), probiotic *Bifidobacterium bifidum* strain DSM22892 was taken from PoratebGostar Company. Then, it was inoculated in 1000 ml of sterile MRS broth culture medium containing 0.25 g of L-cysteine at 37°Cfor 48h (Ceylan and Atasoy 2022). After the bacterial growth phase, the optical density (OD600), pH, and bacterial count were measured using the pour plate

method.

Following bacterial growth, the bacterial culture was centrifuged at 7000×g at 4°C. Subsequently, the supernatant was filtered successively through 0.45 and 0.2 µm filters to prepare ACS .Ultra-centrifugation (Beckman Co.) was performed at 150,000 ×g at 4°C for 30 minutes to pellet microvesicles and separate them from the soluble components in the ACS. This step aimed to enrich the sample in microvesicles, which are membrane-bound vesicles shed by cells. The microvesicles separated from the sample were examined using scanning electron microscopy (SEM) to determine their morphology and size.

Preparation of chitosan films

Medium molecular weight chitosan (CAS number: 9012-76-4) manufactured by Sigma-Aldrich was purchased. A 10% (w/v) chitosan solution was prepared in 30 ml of distilled water and placed on a stirring heater for 30 minutes; the temperature of the heater did not exceed 30°C. While 50 mL of distilled water (DW) was added to the mixture, the pH of the solution was adjusted to about 4 by adding glacial acetic acid. The resulting solution was placed on a heater stirrer at 29°C for 120 minutes and then for 90 minutes in an ultrasonic bath at 3000 rpm. Then, 2/3 of the petri dish was filled with the solution and incubated for 48 hours at 25°C (Mousavinezhad et al., 2022). To prepare the ACS-containing film, instead of the distilled water (described in the chitosan film preparation section), 50 mL of ACS was added to the solution (Jamaran et al., 2021). To prepare the microvesicles-containing films, the microvesicles isolated from 50 ml of ACS were diluted in 50 mL of DW and added to chitosan as before. Finally, wound dressing films were examined for contamination; The structural morphology and surface structure of the films were investigated with a scanning electron microscope (SEM) VEGA model (TESCAN company).

The swelling index of the films was determined by the formula:

$$SI\% = \frac{Wwet - Wdry}{Wdry} \times 10$$ (Touré et al., 2003).

In vivo phase of the study

Mail wistar rats aged 8-10 weeks (weight 180-220 g) were procured from the Royan Research Institute. The rats were housed under standard conditions with a 12h light-dark cycle (24 ± 3°C, 45 to 50%humidity) in the Islamic Azad University of Karaj laboratory animal storage facility. Throughout the experimental period, the rats had ad libitum access to standard rodent chow and water. These conditions were carefully maintained to ensure the health and well-being of the rats and to minimize environmental stress that could potentially affect experimental outcomes. After 2 weeks, the rats were divided into 5 experimental groups:

- Negative Control (NG): Treated with sterile gauze.
- Positive Control (PG): Treated with sterile gauze containing 1% sulfadiazine.
- Experimental Group 1 (CG): Treated with chitosan film.
- Experimental Group 2 (SG): Treated with chitosan film containing ACS
- Experimental Group 3 (MG): Treated with chitosan film containing microvesicles.

Wound formation and treatment

The rats were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) (Jara et al., 2021). The hairs of rats were shaved, and the skin was disinfected with Povidone-iodine. A metal plate weighing approximately 50 g was heated to 300°C using direct flame exposure. Subsequently, the heated plate was placed in direct contact with the animal skin for precisely one second. This method ensured rapid and controlled thermal transfer to the skin, leading to the formation of a standardized burn injury. Immediately from day zero after inducing the burn injury, the wound was washed with normal saline serum. Following the initial care, wound management commenced with the application of appropriate wound dressings tailored to each experimental group.

$$\text{Wound healing}\% = \frac{\text{The size of the wound on the first day} - \text{The size of the wound on the examined day}}{\text{The size of the wound on the first day}} \times 100$$

TABLE 1: The primer sequence of genes evaluated in the study

Gene	Primer sequence	Amp size (pb)	efficiency	reference
GAPDH	Forward: 5'- GTTACCAGGGCTGCCTTCTC-3' Revers: 5'- GGGTTTCCCGTTGATGACC-3'	120	1.07	(Yoshikawa et al., 2009)
IL-10	Forward:5'- CGGGAAGACAATAACTGCACCC-3¢ Revers: 5'- CGGTTAGCAGTATGTTGTCCAGC-3'	130	0.98	(Xu et al., 2019)
IL-8	Forward: 5'- CTCTCAAGGGCGGTCAAAAAGTT-3' Reverse: 5'- TCAGACAGCGAGGCACATCAGGTA-3'	208	0.90	(Zhao 2016)
VEGF	Forward: 5'- CACTGGACCCTGGCTTTACT-3' Reverse: 5'- GACGTCCATGAACTTCACCA-3'	111	0.92	(Miyagi et al., 2018)

Wound examination

On the days 3, 7, 14, and 21 post-burn injury, 3 rats were randomly selected from each group and euthanized under anesthesia while adhering to ethical principles. The area of the burn wound was measured using ImageJ software. Subsequently, tissue from the wound site, along with surrounding tissue, was surgically excised. The excised tissue was divided into two equal parts. One part was fixed in formaldehyde (10%) for histological examinations. The other part was placed in DNase and RNase-free Eppendorf tubes for gene expression analysis.

Histological analysis

Paraffin blocks were prepared from formalin-fixed tissue samples. The tissue samples were dehydrated through a series of alcohol solutions and then cleared with alcohol and toluene mixtures. Subsequently, the samples were embedded in paraffin wax. Sections of 5 micrometers were obtained from the paraffin blocks using a microtome. These sections were mounted onto glass slides. The tissue sections were stained using the hematoxylin and eosin (H&E) staining method.

Histological studies were conducted to assess the progression of wound healing, including the quantification of inflammatory cells, collagen deposition, extracellular matrix formation, and re-epithelialization.

Gene expression analysis

RNA was extracted using the RNX-plus kit (Sinacolon, Iran). A total of 10 µL of RNA was used in the cDNA synthesis reaction, along with 2 µL of 10X buffer, 1 µL of dNTPs, 0.5 µL of Reverse Transcriptase (RT) enzyme, 1 µL of primer (1.5µM), and nuclease-free water to achieve a final volume of 20 µL. The reaction conditions in an A100 PCR thermocycler were as follows: initial priming at 25°C for 3 minutes, followed by cDNA synthesis at 42°C for 1 hour, and then inactivation of the RT enzyme at 70°C for 5 minutes.

Gene expression levels were assessed via RT-qPCR using a reaction mixture composed of 5 µL of 4x Q-PCR Green Master Mix-Biotechrabbit, 1.5 µL of primer 1.5(µM10.5 ,) µL of ddH_2O, and 3 µL of cDNA. PCR conditions involved initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 10 seconds, annealing at 52°C for 20 seconds, and extension at 72°C for 20 seconds per kilobase, concluding with a final extension at 72°C for 300 seconds. The primers used are detailed in Table 1, with *GAPDH* selected as the internal reference gene for normalization. Gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method (Yoshikawa et al., 2009):

Statistical Analysis

The resulting data were entered into IBM SPSS.21 software for statistical analysis; the normal distribution of the data was tested with the Kolmogorov-Smirnov method, and finally, by using the Kruskal-Wallis and Mann-Whitney tests, the data were analyzed with the criterion of $p \leq 0.05$. They were reported as mean ± standard deviation.

Results

Microvesicle isolation

Microvesicles were isolated from the culture medium of *Bifidobacterium bifidum* using an ultracentrifuge. The

TABLE 2: The Swelling Index (SI) values for the different groups over the time range of 60 to 300 minutes.

Time/Groups	Chitosan	Chitosan-ACS	Chitosan-Microvesicles
60	121.66±10.41a	121.66±7.64a	112.00±5.29a
90	303.33±20.82a	303.33±32.14a	280.00±10.00a
150	756.66±40.41a	780.00±26.45a	776.66±25.17a
200	938.33±53.93a	953.33±55.07a	883.33±20.82a
300	966.00±42.75a	960.00±55.68a	910.00±65.57a

Similar letters mean no significant difference among groups.

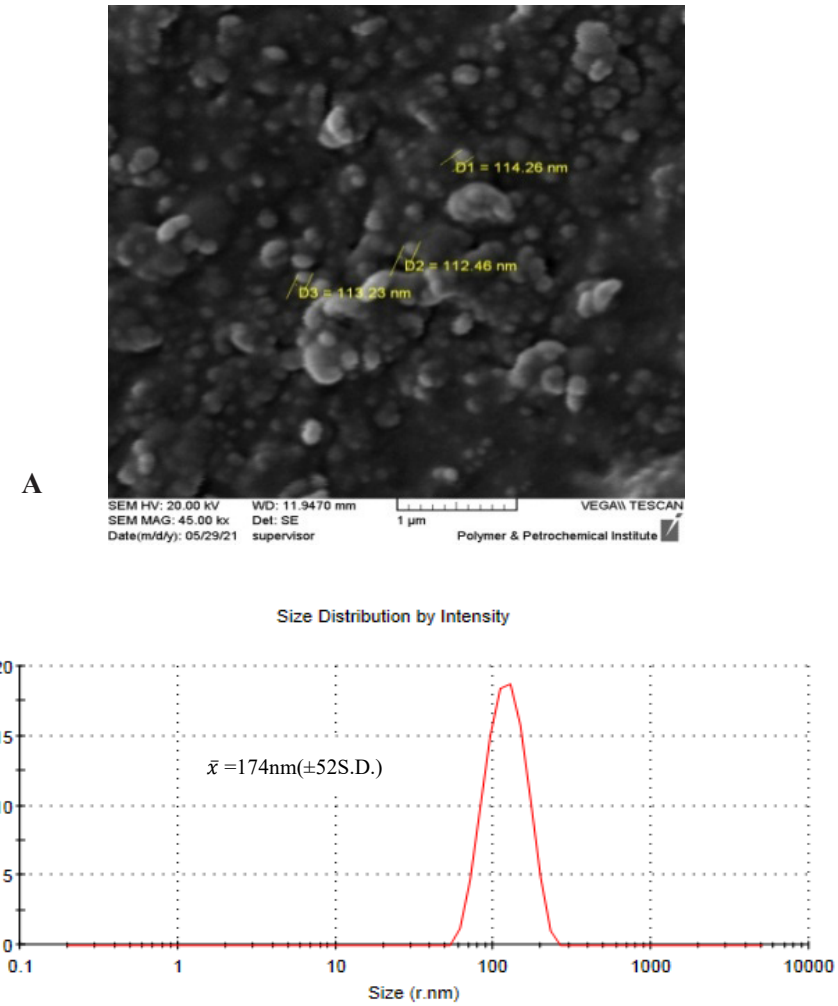

FIGURE 1. A: the image of microvesicles isolated from the supernatant, B: Determining the size of microvesicles with the help of the Dynamic Light Scattering technique

characterization of these microvesicles was performed via electron microscopy and dynamic light scattering (DLS). Our analysis revealed a high degree of uniformity and stability in the solution. The microvesicles exhibited an average diameter of 174 ± 52 nm (Figure 1), highlighting their consistent size distribution and potential suitability for subsequent experimental applications.

Wound dressing properties

The thickness of the films after drying, measured with a digital caliper from five different areas, was consistently 2 mm (Figure 2). Notably, the film containing microvesicles displayed a clear yellowish color, while the film containing ACS exhibited an opaque brown color.

The results showed (Table 2) that the Swelling Index (SI) of all films increased over time and eventually sta-

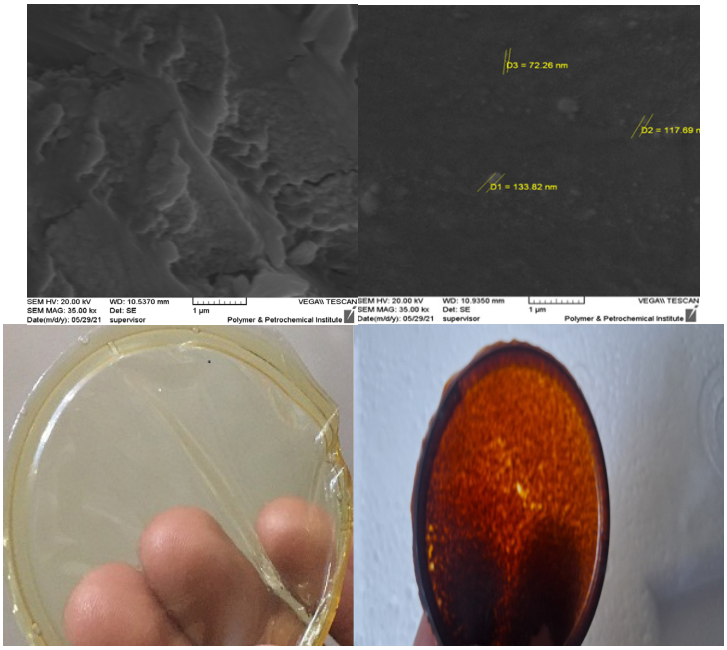

FIGURE 2. A: The image of microvesicles scattered in the background of chitosan after preparing the membrane. B: The image of ACS scattered in the background of chitosan after preparing the membrane. C: Image of chitosan transparent film containing microvesicles. D: Image of chitosan film containing ACS

bilized after 150 minutes. Interestingly, there was no significant difference in the SI among the different films. After 5 hours, the SI varied between 910% and 966% for all films. This indicates that these films are particularly suitable for wounds with high exudate levels, as they can absorb excess fluids and prevent wound maceration.

Wound healing and histological analysis

In this study, burn wound healing was evaluated in five different groups of rats (NG, PG, CG, SG, MG) on the days 3, 7, 14, and 21. The results showed that on day 3, there were no significant differences among the groups. The percentage of wound healing was approximately similar in all groups, indicating that in the early stages of healing, the treatments did not significantly affect the rate of wound healing. On the day 7, the PG, CG, SG and MG groups showed significant improvement compared to the negative control group (NG). PG had the highest percentage of wound healing, but there were no significant differences among the PG, CG, SG, and MG groups. On the day 14, the PG, SG, and MG groups had significantly higher percentages of wound healing compared to the negative control group (NG), with their healing rates nearing 100%. The CG group also showed good improvement, but it was not as effective as the other groups. On the day 21, all groups showed almost complete healing. The SG and MG groups demonstrated complete healing (100%), while the other groups also had healing rates very close to 100%.

Overall, the results indicate that using chitosan films containing ACS and microvesicles (SG and MG groups) is highly effective in promoting faster and more complete healing of burn wounds compared to the other groups. These films are particularly suitable for wounds with high exudate levels, as they can absorb excess fluids and prevent maceration. In summary, the films containing ACS and microvesicles exhibited the best performance in accelerating the healing of burn wounds (Table 3, Fig. 3).

The regeneration status of the epithelial tissue in various groups was systematically assessed. Figure 4 vividly depicts the progression of epithelial regeneration over different time points. The results showed that over time, wound contraction and closure were observed in all groups. Statistical analysis indicated no significant differences among the groups across the evaluated days.

Based on our results that on the day 3 of the study, no statistical difference was observed among the groups regarding the organization and structure of the matrix and collagen formation. However, by the seventh day,

TABLE 3: Percentage of wound closure

Group name	Third day	Seventh day	Fourteenth day	Twenty-first day
NG	9.24±0.68[a]	20.55±2.81[b]	49.56±6.49[c]	96.84±3.57[a]
PG	8.87±1.86[a]	59.52±5.12[a]	91.00±1.30[a]	99.67±0.58[a]
CG	10.01±1.02[a]	56.56±3.17[a]	72.67±4.00[b]	96.93±2.72[a]
SG	10/90±1/65[a]	54/11±2/85[a]	90/49±6/73[a]	100±0/00[a]
MG	8.97±1.77[a]	55.30±1.61[a]	95.77±4.02[a]	100±0.00[a]

Similar letters mean no significant difference among groups.

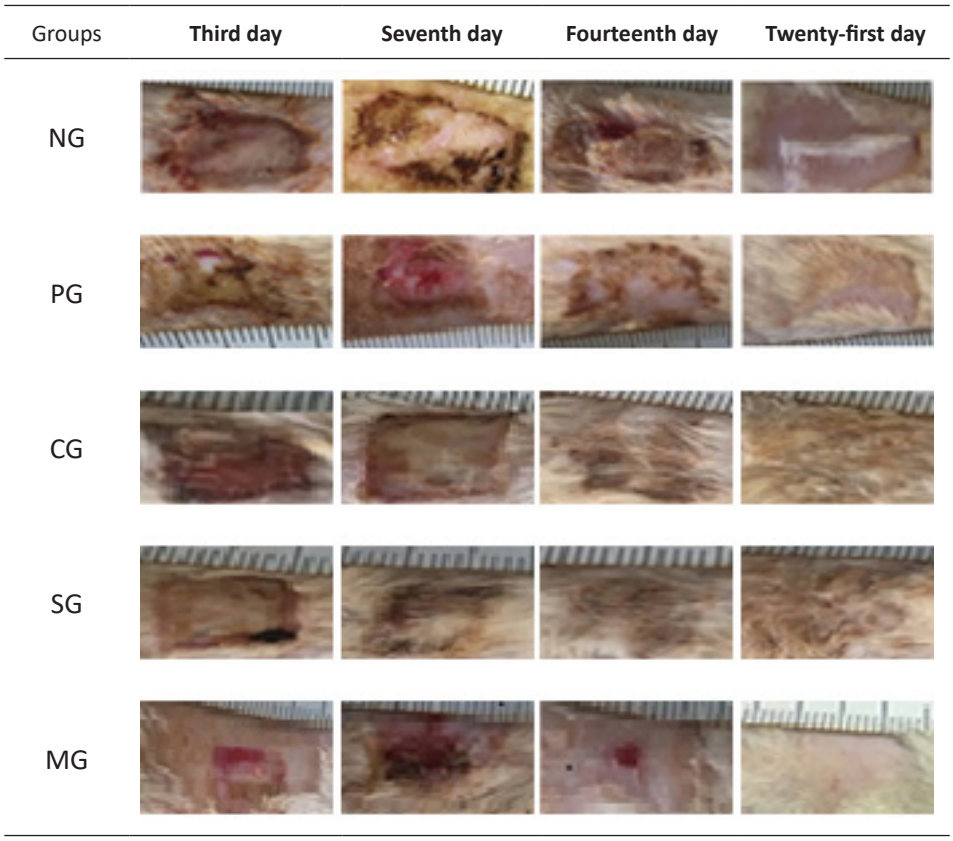

FIGURE 3. Wound images on different days. NG: Negative control group, PG: Positive control group treated with sulfadiazine, GC: the group treated with chitosan film, SG: the group treated with chitosan containing ACS, and MG: the group treated with chitosan and microvesicles.

a significant difference was noted between the PG and MG groups compared to the NG group. In contrast, no significant difference was observed between the NG and CG groups. These observations were assessed across four stages of tissue organization: immature, thin, medium, and thick matrix structures, which were evaluated on different days (Table 4, Fig. 5)

Figure 6 shows the samples' average number of inflammatory cells on specific days. The statistical analysis showed no significant difference between the average number of inflammatory cells on the 3rd and 7th days. However, a significant difference was observed among the groups on the day 14. On the day 21, the groups were similar. It is essential to consider these findings when interpreting the study results.

TABLE 4: State of organization and structure of matrix and collagen formation

Group name	Third day				Seventh day				Fourteenth day				Twenty-first day			
Score	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
NG	+++				++	+					+++					+++
PG	++	+				++	+					+++				+++
CG	++	+			+	++					+	++				+++
SG	++	+				++	+				+	++				+++
MG	++	+				++	+					+++				+++

The stage of immature organization and inflammatory tissue (Score: 1), structure and organization of thin and inflammatory matrix (Score: 2), matrix organization with moderate regeneration (Score: 3), and good and thick collagen structure (Score: 4)

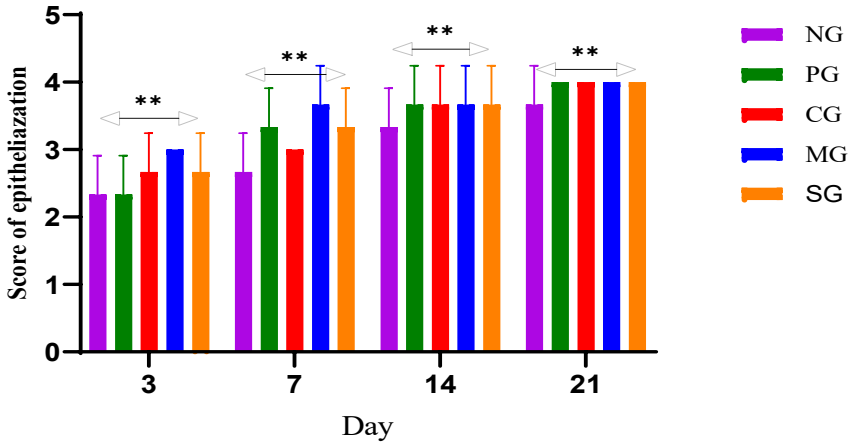

FIGURE 4. The trend of changes in epithelization of the groups. NG: Negative control group, PG: Positive control group treated with sulfadiazine, GC: the group treated with chitosan film, SG: the group treated with chitosan containing ACS, and MG: the group treated with chitosan and microvesicles. Pvalues were regarded as significantly different at $^{*}P < 0.05$, not significantly different $^{**}P > 0.05$

Gene expression assay

On the day 3, IL-8 expression is relatively similar across all groups, with no significant differences. This suggests that the inflammatory response, as marked by IL-8 expression, is not significantly influenced by the treatments at this early stage. By the 7th day, a significant decrease in IL-8 expression is observed in the PG, SG, and MG groups compared to NG. This suggests that sulfadiazine, ACS, and microvesicle treatments effectively reduce the inflammatory response at this stage. On the day 14, IL-8 expression remains significantly lower in the SG and MG groups compared to NG. The CG group shows increased IL-8 expression, indicating a prolonged inflammatory response, whereas PG shows a moderate increase but is not significantly different from NG. By the 21st day, the SG and MG groups continue to show significantly lower IL-8 expression compared to NG, indicating a sustained reduction in inflammation. The PG and CG groups show IL-8 levels similar to NG, suggesting the resolution of the inflammatory phase.

The treatments involving chitosan film containing ACS and microvesicles (SG and MG) are significantly more effective in reducing IL-8 expression and controlling inflammation during wound healing compared to the other groups. This highlights the potential benefits of these treatments in promoting better wound healing outcomes by mitigating prolonged inflammatory responses.

On the day 3, IL-10 expression is significantly higher in the PG, CG, and SG groups compared to the NG and MG groups. This indicates that the presence of sulfadiazine, chitosan film, and ACS enhances anti-inflammatory cytokine IL-10 production early in the healing process. By the 7th day, IL-10 expression decreases across

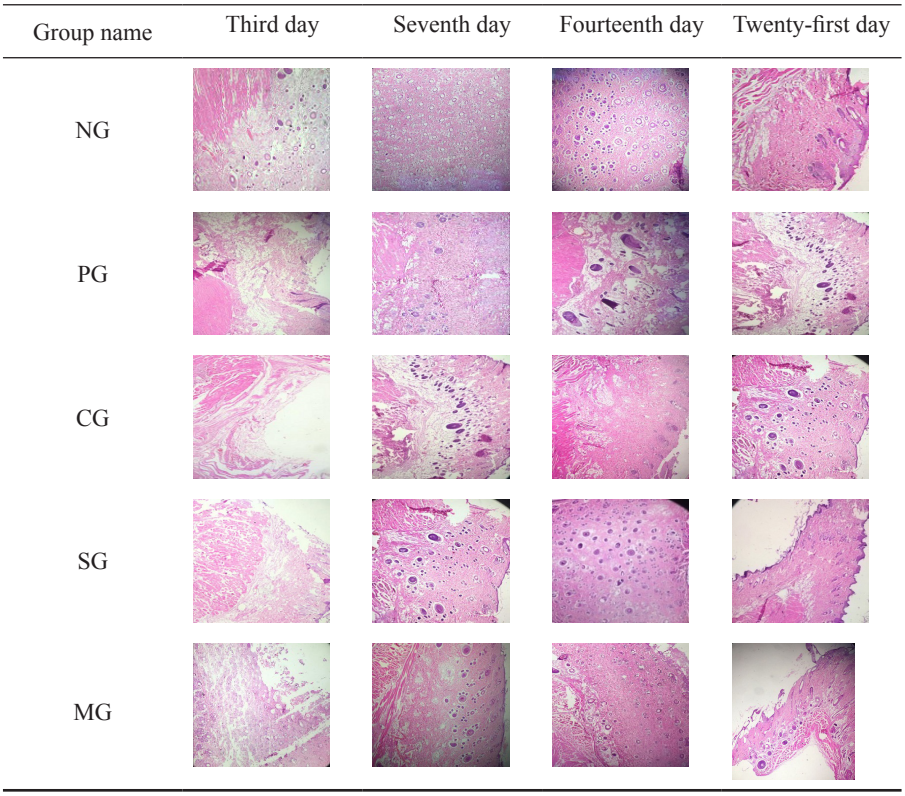

FIGURE 5. State of organization and structure of matrix and collagen formation in histological images (shown by arrows) (magnification: 4X)

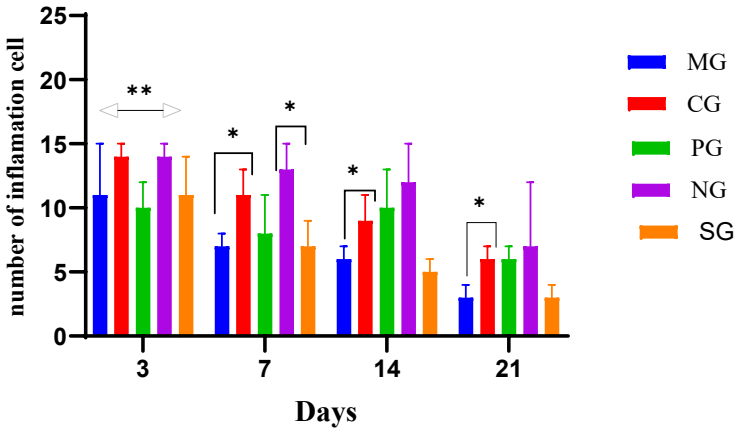

FIGURE 6. The trend of changes in inflammatory cells of the groups. NG: Negative control group, PG: Positive control group treated with sulfadiazine, GC: the group treated with chitosan film, SG: the group treated with chitosan containing ACSand MG: the group treated with chitosan and microvesicles. Pvalues were regarded as significantly different at $^{*}P < 0.05$, not significantly different $^{**}P > 0.05$

all groups, with significant reductions observed in SG and MG compared to NG, PG, and CG. This suggests that while SG and MG initially boosted IL-10 levels, they later reduced them, potentially balancing the inflammatory response. On the day 14, IL-10 levels are low across all groups, with no significant differences. This indicates that by this time point, the anti-inflammatory responses have normalized, and no group shows a pronounced anti-inflammatory effect. By the 21st day, IL-10 expression is significantly elevated in the PG, CG, SG, and MG groups compared to NG. This suggests a robust anti-inflammatory response in these treatment

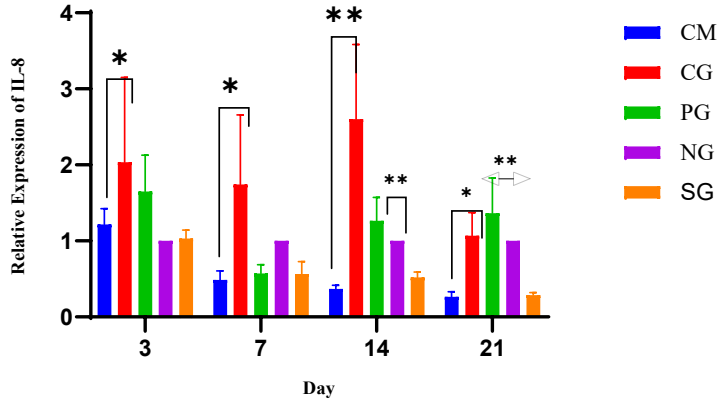

FIGURE 7. Interleukin-8 expression status compared to the NG. NG: Negative control group, PG: Positive control group treated with sulfadiazine, GC: the group treated with chitosan film, SG: the group treated with chitosan containing ACS and MG: the group treated with chitosan and microvesicles. P values were regarded as significantly different at $^{*}P < 0.05$, not significantly different $^{**}P > 0.05$

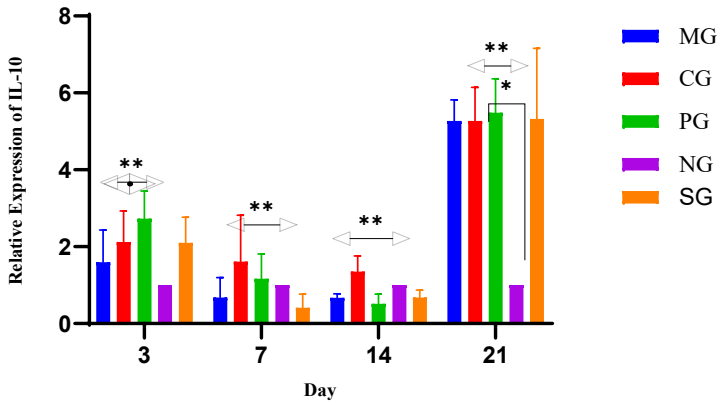

FIGURE 8. Interleukin-10 gene expression status compared to the NG. NG: Negative control group, PG: Positive control group treated with sulfadiazine, GC: the group treated with chitosan film, SG: the group treated with chitosan containing ACS, and MG: the group treated with chitosan and microvesicles. P.values were regarded as significantly different at $^{*}P < 0.05$, not significantly different $^{**}P > 0.05$

groups, promoting wound healing at the later stage of the healing process. The data show that treatments involving chitosan films, whether containing ACS or microvesicles, as well as sulfadiazine, significantly modulate IL-10 expression during different stages of wound healing. Early on, these treatments boost IL-10 levels, potentially aiding in the initial inflammatory phase. By the 7th and 14th days, IL-10 levels normalize, avoiding excessive anti-inflammatory responses. By the 21st, IL-10 levels are significantly elevated again in the treatment groups, indicating a strong anti-inflammatory response that likely aids tissue remodeling and wound closure. This modulation of IL-10 suggests that these treatments are effective in promoting a balanced inflammatory response conducive to optimal wound healing (Figure 7).

On the day 3, *Vegf* gene expression was significantly increased in PG, SG, and MG groups compared to NG group. This increase indicated the effect of the groups containing microbial postbiotics (SG, MG), such as sulfadiazine, on the increase of VEGF cytokine expression; On other days, there was no significant change in the level of gene expression among the groups (Figure 8).

Discussion

Burns and burn-related infections are an important global public health concern. Hence, addressing the matter of burn wound healing by focusing on infection control and accelerating the healing process is regarded as a crucial aspect of treatment. Conversely, researchers today prioritize minimizing antibiotic usage in light of

pathogen resistance and exploring viable alternatives to antibiotics.

Chitosan plays a crucial role as a functional compound in the production of innovative wound dressings. Chitosan-based hydrogels can control skin infections(Mohite et al., 2023). Due to its non-toxicity, good biocompatibility, biodegradability, as well as chitosan's anti-inflammatory, hemostatic, and antimicrobial properties, the compound can increase the wound-healing process (Dong et al., 2020; McFadyen and Kaplan 2015). The anti-inflammatory properties of chitosan are due to suppressing the expression of prostaglandin E2 cyclooxygenase-2 and reducing the expression of inflammatory cytokines such as IL-1, IL-6, and TNFα (Ahn et al., 2016). Based on these characteristics, chitosan has been used as the main material to make wound dressings in this research.

In our research, to be more effective in the infection control process, the properties of probiotics were used to inhibit inflammation and the growth of pathogenic microorganisms; therefore, probiotic products were used as the second component of the membrane.

The studies have demonstrated that probiotics generate compounds that can inhibit the growth of pathogens. These compounds include lactic acid, acetic acid, and fatty acids like acetate, propionate, and butyrate. Additionally, probiotics produce bacteriocin compounds, which are effective against the growth of pathogens (Kouitcheu Mabeku et al., 2020; Mirzaian et al., 2015; Sinha et al., 2018).

Many of these substances are released from bacteria through packages called vesicles. Based on the size, the vesicles obtained from the tested bacteria were of the microvesicle type.

Active crude supernatant (ACS) facilitates the wound healing process by activating the PI3K-Akt signaling pathway. PI3K-Akt is a signal transduction pathway that promotes survival and growth in response to extracellular signals. The key proteins involved are PI3K (phosphatidylinositol 3-kinase) and Akt or PKB (protein kinase B) (Ilyas 2022).

In this research, the silver sulfadiazine cream was used as a positive control (PG). This antibiotic is used in the treatment of burns. The drug hypothetically produces its bactericidal effects by increasing cell wall permeability (Naz et al., 2021). It is not known precisely which substance in ACS activates this pathway. By examining microvesicles and their effectiveness on wound healing, it can be claimed that wound healing is due to the presence of microvesicles.

Clinical investigation of wound dressing

The formation of burn wounds in all groups was identical in terms of temperature, duration of heat exposure, depth of burns, and size of the wounds. Therefore, it can be claimed that the level of burn injury on the day 0 was the same across all groups (Cai et al., 2014). The percentage of wound closure in SG and MG groups, as well as the PG group, reached completion by the fourteenth day. In simpler terms, the wounds in these three groups were fully closed within fourteen days. Namely, it can be asserted that bacterial microvesicles, similar to the ACS, had a positive impact on the speed of wound closure. Since the appearance of the wound is not definitive proof of the effectiveness of the wound dressing, tissue repair, and some cytokines involved in the wound healing process were examined on different days.

In general, wound healing has four stages, including hemostasis, inflammation, proliferation, and regeneration. The hemostasis stage is in the first hour, and the regeneration stage begins after the wound is closed which begins after the fourth week (Mercandetti and Cohen 2017). Therefore, in this study, two stages of inflammation and proliferation were considered.

Inflammatory response phase

Inflammatory cells include white blood cells. It was expected that the number of inflammatory cells would decrease in the final days of wound examination. Based on the results, this expectation was realized in chitosan-repaired groups, but in the NG group, there was no significant difference among the examined days. Therefore, it is inferred that chitosan reduces inflammatory cells.

Additionally, a significant reduction of inflammatory cells was evident in the MG group than the CG group. This means that microvesicles have also been effective in reducing inflammation. Rodrigues, et al.(2005) investigated the healing of skin wounds using kefir and noticed the anti-inflammatory and regenerative activity of the microorganisms in kefir (Rodrigues et al., 2005), which was consistent with the results of this study and indicated that probiotics can control inflammation and inflammatory cells.

Results of the MG group showed a decrease in the expression of the IL-8 genes as an inflammatory factor and an increase on the day 3 in the expression of the IL-10 genes as an anti-inflammatory factor (although to a lesser extent), indicating a moderate anti-inflammatory response due to the presence of microvesicles.

By the 7th day, the MG group showed reduced IL-10 levels like other treatment groups, consistent with the anti-inflammatory effects of microvesicles. The significant reduction in IL-10 expression on the day 14 in the SG and MG groups underscores the enhanced effectiveness of ACS and microvesicles in promoting a balanced anti-inflammatory response. By the 21st day, all treatment groups showed significantly increased IL-10 levels, indicative of tissue remodeling and repair processes that require modulation of inflammation. The NG group remained at baseline IL-10 levels, highlighting the lack of effective healing in untreated wounds. Cytokine interleukin-10 is an anti-inflammatory cytokine, and the main biological function of this cytokine is to attenuate adaptive immune responses (Babbitt et al., 2013). Th2 cells are the major source of IL-10, which inhibits the production of proinflammatory cytokines (e.g., tumor necrosis factor-alpha) by monocytes, macrophages, T cells, and NK cells (Oertelt-Prigione, 2012). Comparison of the MG and CG groups on days 7 and 14 demonstrated a significant decrease in interleukin-8 gene expression, indicating the role of microvesicles in downregulating IL-8 expression. Bazjo et al. (2022), in their study on chitosan wound dressings containing Bifidobacterium bifidum ACS, observed that ACS effectively controlled inflammation in rat burn wounds. (Bazjou et al., 2022).

Proliferation stage

This phase consists of the subphases of fibroplasia, matrix deposition, angiogenesis, and reepithelialization, which occur simultaneously (Mercandetti and Cohen 2017).

VEGF is a protein produced during tissue repair's proliferation phase. It plays a crucial role in recruiting fibroblasts from the surrounding non-damaged tissue and forming new blood vessels by promoting the migration of endothelial cells (Rodrigues et al., 2005).

On the day 3, congestion and bleeding were evident when comparing the amount of angiogenesis in the tissue of different groups. However, no statistically significant difference was observed among the groups. By the 5th day, congestion was reduced, and on average two vessels were constructed, blood was drained from the internal vessels, but some groups, such as the NG, still exhibited congestion.

The *Vegf* gene expression is an important factor in angiogenesis and wound healing. This cytokine is primarily synthesized by keratinocytes at the wound's edge, as well as platelets, neutrophils, and macrophages (Diomede et al., 2020; Grazul-Bilska et al., 2003).

According to Figure 8, the expression of *Vegf* gene increased compared to the negative control group on the third day. However, there was no significant increase in the expression of this gene in the following days compared to the negative control.

In a study conducted by Li et al. in 2009 on silver nanoparticles and wound healing, it was found that the expression of the *Vegf* gene enhances the speed of the healing process. This finding is consistent with the results of the current research (Li et al., 2009).

Upon analyzing the organization and structure of collagen, as well as the differences observed in the groups on the day 3 (P Value: 0.054), day 7 (P Value: 0.026), and day 14 (P Value: 0.08), it became apparent that there were no significant differences among the groups on the day 21 (P Value: 0.002). Further investigation revealed that the microvesicle group exhibited complete organization and matrix structure on the fourteenth day, surpassing the other groups in terms of wound healing speed. This highlights the significant role of microvesicles in the healing process.

In a study conducted by Kojima et al. in 2004, the impact of chitin and chitosan on collagen synthesis and burn repair was examined. The results indicated an increase in collagen production in the chitosan group, which aligns with the findings of our study (Kojima et al., 2004).

Furthermore, when comparing the amount of epithelium regeneration among the groups, it was observed that all groups exhibited similar levels of epithelium regeneration on the third and twenty-first days. However, the intervention groups on the days 7 and 14 showed more development of epithelial cells than the control group. On the 14thday, most of the samples from the chitosan groups had reached the intermediate and complete epithelial repair stage.

Finally, it can be concluded that the microvesicle

group, through increased angiogenesis and collagen production, effectively accelerated the wound healing process compared to the NG group.

By observing the results of wound healing in the PG group, it seems that the effectiveness of the drug decreases over time, so periodic administration of the drug on the wound is necessary, but this issue was not observed in the SG and MG groups. The presence of various compounds in ACS increases the likelihood of allergic reactions in humans, although this issue was not addressed in this study. Meanwhile, the use of bio-degradable chitosan dressings containing ACS and microvesicles is better than routine methods and the use of sulfadiazine due to the removal of antibiotics.

Conclusion

The results of this study demonstrate that bio-degradable dressings, particularly those containing ACS and microvesicles, significantly enhance the wound healing process in burn wounds. The chitosan film alone effectively modulates the initial inflammatory response, while the further addition of ACS and microvesicles enhances the anti-inflammatory effects and promotes balanced wound healing. The PG group, treated with 1% sulfadiazine, showed effective inflammation control, but the advanced chitosan formulations (SG and MG) provided superior overall results.

These findings suggest that the incorporation of ACS and microvesicles into chitosan films offers a promising approach for developing advanced wound dressings that facilitate faster and more effective healing of burn wounds. Future research should focus on optimizing these formulations and exploring their potential applications in clinical settings to improve outcomes for patients with burn injuries and other types of wounds.

In summary, the study underscores the importance of modulating the inflammatory response in the wound healing process and highlights the potential of advanced biomaterials to enhance the effectiveness of wound dressings. The use of chitosan films, particularly those enhanced with ACS and microvesicles, represents a significant advancement in wound care, offering a promising strategy for improving the healing of burn wounds and potentially other types of chronic and acute wounds.

Acknowledgements

The authors would like to thank the "Research Center for Life & Health Sciences & Biotechnology of the Police" for their collaboration and extend their appreciation to its staff.

Conflict of interest

All authors state that there is no conflict of interest

Funding

We had no financial support

Ethics approval

This study has been approved by the research ethics committee of Islamic Azad University, Karaj branch (IR.IAU.K.REC.1399.020).

The study was conducted on laboratory rats based on accepted national and international principles and norms of ethics in research and according to the instructions of the research ethics committee of Islamic Azad University, Karaj branch

Competing interests

The authors declare that they have no competing interests.

Author's Contributions

S A M is the main researcher in this study, N H is the supervisor of the study and A. M was the guide of the chemistry section of the study and P J was the guide of the microbiology section of the study. All authors read and approved the final manuscript.

References

Ahn C-B, Jung W-K, Park S-J, Kim Y-T, Kim W-S, Je J-Y. Gallic acid-g-chitosan modulates inflammatory responses in LPS-stimulated RAW264. 7 cells via NF-κB, AP-1, and MAPK pathways. Inflammation 2016; 39: 366-374. https://doi.org/10.1007/s10753-015-0258-2

Al-Nedawi K, Mian M F, Hossain N, Karimi K, Mao Y K, Forsythe P, et al. Gut commensal microvesicles reproduce parent bacterial signals to host immune and enteric nervous systems. The FASEB Journal 2015; 29: 684-695. https://doi.org/10.1096/fj.14-259721

Babbitt D M, Diaz K M, Feairheller D L, Sturgeon K M, Perkins A M, Veerabhadrappa P, et al. Endothelial activation microparticles and inflammation status improve with exercise training in African Americans. International Journal of Hypertension 2013; 2013: 538017. https://doi.org/10.1155/2013/538017

Barrientos S, Stojadinovic O, Golinko M S, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair and Regeneration 2008; 16: 585-601. https://doi.org/10.1111/j.1524-475X.2008.00410.x

Bazjou A, Jafari P, Marjani A, Akbari N. Effect of cell-free supernatant of bifidobacterium bifidum combined with chitosan biodegradable film on full thickness wound healing in rats. Physiology and Pharmacology 2022; 26: 468-479.

Brown L, Kessler A, Cabezas-Sanchez P, Luque-Garcia J L, Casadevall A. Extracellular vesicles produced by the G ram-positive bacterium B acillus subtilis are disrupted by the lipopeptide surfactin. Molecular Microbiology 2014; 93: 183-198. https://doi.org/10.1111/mmi.12650

Cai E Z, Ang C H, Raju A, Tan K B, Hing E C H, Loo Y, et al. Creation of consistent burn wounds: a rat model. Archives of Plastic Surgery 2014; 41: 317-324. https://doi.org/10.5999/aps.2014.41.4.317

Ceylan H G, Atasoy A F. Lactobacillus rhamnosus gg ve bifidobacterium bifidum bb-12 içeren yenilebilir filmlerin karakterizasyonu. Adıyaman Üniversitesi Mühendislik Bilimleri Dergisi 2022; 9: 194-203. https://doi.org/10.54365/adyumbd.1073615

Choi Y J, Shin H S. Antibacterial effect of eight probiotic strains of bifidobacterium against pathogenic Staphylococcus aureus and Pseudomonas aeruginosa. Journal of Bacteriology and Virology 2021; 51: 128-137. https://doi.org/10.4167/jbv.2021.51.3.128

Diomede F, Marconi G D, Fonticoli L, Pizzicanella J, Merciaro I, Bramanti P, et al. Functional relationship between osteogenesis and angiogenesis in tissue regeneration. International Journal of Molecular Sciences 2020; 21: 3242. https://doi.org/10.3390/ijms21093242

DiPietro L A. Angiogenesis and wound repair: when enough is enough. Journal of Leucocyte Biology 2016; 100: 979-984. https://doi.org/10.1189/jlb.4MR0316-102R

Domínguez Rubio A P, Martínez J H, Martínez Casillas D C, Coluccio Leskow F, Piuri M, Pérez O E. Lactobacillus casei BL23 produces microvesicles carrying proteins that have been associated with its probiotic effect. Frontiers in Microbiology 2017; 8: 1783. https://doi.org/10.3389/fmicb.2017.01783

Dong Y, Cui M, Qu J, Wang X, Kwon S H, Barrera J, et al. Conformable hyaluronic acid hydrogel delivers adipose-derived stem cells and promotes regeneration of burn injury. Acta Biomaterialia 2020; 108: 56-66. https://doi.org/10.1016/j.actbio.2020.03.040

Falanga V. Wound healing and its impairment in the diabetic foot. The Lancet 2005; 366: 1736-1743. https://doi.org/10.1016/S0140-6736(05)67700-8

Ghaneialvar H, Arjmand S, Sahebghadam Lotfi A, Soleimani M, Mashhadi Abbas F. Influence of adipose derived mesenchymal stem cells on the effective inflammatory factors of diabetic wound healing in animal models. Journal of Mazandaran University of Medical Sciences 2017; 27: 12-21.

Grazul-Bilska A T, Johnson M L, Bilski J J, Redmer D A, Reynolds L P, Abdullah A, et al. Wound healing: the role of growth factors. Drugs Today (Barc) 2003; 39: 787-800. https://doi.org/10.1358/dot.2003.39.10.799472

Guo S a, DiPietro L A. Factors affecting wound healing. Journal of Dental Research 2010; 89: 219-229. https://doi.org/10.1177/0022034509359125

Ilyas S. Immunohistochemistry examination to reveal the pathogenesis of Oral Squamous Cell Carcinoma. International Journal of Ecophysiology 2022; 4: 5-25. https://doi.org/10.32734/ijoep.v4i1.11150

Jamaran S, Jafari P, Marjani A, Akbari N, Feizabad M M. Novel wound dressing based on postbiotic/chitosan film accelerates cutaneous wound healing. Jundishapur Journal of Microbiology 2021; 14. https://doi.org/10.5812/jjm.120806

Jara C P, Carraro R S, Zanesco A, Andrade B, Moreira K, Nogueira G, et al. A smartphone app for individual xylazine/ketamine calculation decreased anesthesia-related mortality in mice. Frontiers in Veterinary Science 2021; 8: 651202. https://doi.org/10.3389/fvets.2021.651202

Kambouris A R, Brammer J A, Roussey H, Chen C, Cross A S. A combination of burn wound injury and Pseudomonas infection elicits unique gene expression that enhances bacterial pathogenicity. mBio2023; 14: e0245423. https://doi.org/10.1128/mbio.02454-23

Kojima K, Okamoto Y, Kojima K, Miyatake K, Fujise H, Shigemasa Y, et al. Effects of chitin and chitosan on collagen synthesis in wound healing. Journal of Veterinary Medical Science 2004; 66: 1595-1598. https://doi.org/10.1292/jvms.66.1595

Kouitcheu Mabeku L B, Ngue S, Bonsou Nguemo I, Leundji H. Potential of selected lactic acid bacteria from Theobroma cacao fermented fruit juice and cell-free supernatants from cultures as inhibitors of Helicobacter pylori and as good probiotic. BMC Research Notes 2020; 13: 1-7. https://doi.org/10.1186/s13104-020-4923-7

Li D, Zhang C, Song F, Lubenec I, Tian Y, Song Q. VEGF regulates FGF-2 and TGF-β1 expression in injury endothelial cells and mediates smooth muscle cells proliferation

and migration. Microvascular Research 2009; 77: 134-142. https://doi.org/10.1016/j.mvr.2008.09.007

McFadyen J D, Kaplan Z S. Platelets are not just for clots. Transfusion Medicine Reviews 2015; 29: 110-119. https://doi.org/10.1016/j.tmrv.2014.11.006

Mercandetti M, Cohen A. Wound healing and repair. Emedicine 2017; 14: 12-20.

Mirzaian F, Amirizadeh N, Nikougoftar Zarif M, Atarodi K, Nakhlestani M. Isolation and characterization of Placenta-MSC derived microvesicles. Scientific Journal of Iran Blood Transfus Organ 2015; 12: 135-142.

Miyagi M, Uchida K, Takano S, Fujimaki H, Aikawa J, Sekiguchi H, et al. Macrophage-derived inflammatory cytokines regulate growth factors and pain-related molecules in mice with intervertebral disc injury. Journal of Orthopaedic Research® 2018; 36: 2274-2279. https://doi.org/10.1002/jor.23888

Mohite P, Rahayu P, Munde S, Ade N, Chidrawar V R, Singh S, et al. Chitosan-based hydrogel in the management of dermal infections: a review. Gels 2023; 9: 594. https://doi.org/10.3390/gels9070594

Mousavi Nezhad S A, Tajabadi Ebrahimi M, Zilabi R. The Effect of Iran probiotic fermented milk beverage on cholesterol and liver function biomarkers of rat. Journal of Police Medicine 2015; 4: 49-56.

Mousavinezhad S A, Harzandi N, Marjani A, Jafari P. Effect of culture supernatant of three probiotic bacterial strains of Bifidobacterium on Pseudomonas aeruginosa. EBNESINA 2023; 25: 25-32.

Mousavinezhad S A, Harzandi N, Marjani A, Jafari P. The effectiveness of chitosan wound dressing in the form of a film compared to its hydrogel on burn wound healing in the male wistar rat model. Journal of Police Medicine 2022; 11: 1-17.

Naz I, Fatima A, Alhewairini S S, Rehman A. Assessment of antibacterial activity of Syzygium aromaticum extracts, antibiotics and silver sulphadiazine ointment against pathogenic bacteria isolated from the burned and unburned skin. Malaysian Journal of Microbiology 2021; 17. https://doi.org/10.21161/mjm.200895

Oertelt-Prigione S. The influence of sex and gender on the immune response. Autoimmunity Reviews 2012; 11: A479-A485. https://doi.org/10.1016/j.autrev.2011.11.022

Rodrigues KL, Caputo LRG, Carvalho JCT, Evangelista J, Schneedorf J M. Antimicrobial and healing activity of kefir and kefiran extract. International Journal of Antimicrobial Agents 2005; 25: 404-408. https://doi.org/10.1016/j.ijantimicag.2004.09.020

Sabahi S, Homayouni Rad A, Aghebati-Maleki L, Sangtarash N, Ozma M A, Karimi A, et al. Postbiotics as the new frontier in food and pharmaceutical research. Critical Reviews in Food Science and Nutrition 2023; 63: 8375-8402. https://doi.org/10.1080/10408398.2022.2056727

Sanders ME. How do we know when something called "probiotic" is really a probiotic? A guideline for consumers and health care professionals. Functional Food Reviews 2009; 1: 3-12.

Savadogo A, Ouattara AC, Bassole HI, Traore SA. Bacteriocins and lactic acid bacteria-a minireview. African Journal of Biotechnology 2006; 5.

Sinha M, Sen CK, Singh K, Das A, Ghatak S, Rhea B, et al. Direct conversion of injury-site myeloid cells to fibroblast-like cells of granulation tissue. Nature Communications 2018; 9: 936. https://doi.org/10.1038/s41467-018-03208-w

Sorg H, Tilkorn D J, Hager S, Hauser J, Mirastschijski U. Skin wound healing: an update on the current knowledge and concepts. European Surgical Research 2017; 58: 81-94. https://doi.org/10.1159/000454919

Touré R, Kheadr E, Lacroix C, Moroni O, Fliss I. Production of antibacterial substances by bifidobacterial isolates from infant stool active against Listeria monocytogenes. Journal of Applied Microbiology 2003; 95: 1058-1069. https://doi.org/10.1046/j.1365-2672.2003.02085.x

Volk SW, Bohling MW. Comparative wound healing-are the small animal veterinarian's clinical patients an improved translational model for human wound healing research? Wound Repair and Regeneration 2013; 21: 372-381. https://doi.org/10.1111/wrr.12049

Wang D, Zhang N, Meng G, He J, Wu F. The effect of form of carboxymethyl-chitosan dressings on biological properties in wound healing. Colloids and Surfaces B: Biointerfaces 2020; 194: 111191. https://doi.org/10.1016/j.colsurfb.2020.111191

Wang F, Cerione RA, Antonyak MA. Isolation and characterization of extracellular vesicles produced by cell lines. STAR Protocols 2021; 2: 100295. https://doi.org/10.1016/j.xpro.2021.100295

Xu X, Wang R, Sun Z, Wu R, Yan W, Jiang Q, et al. Trehalose enhances bone fracture healing in a rat sleep deprivation model. Annals of Translational Medicine 2019; 7. https://doi.org/10.21037/atm.2019.05.73

Yin M, Wan S, Ren X, Chu C-C. Development of inherently antibacterial, biodegradable, and biologically active

chitosan/pseudo-protein hybrid hydrogels as biofunctional wound dressings. ACS Applied Materials & Interfaces 2021; 13: 14688-14699. https://doi.org/10.1021/acsami.0c21680

Yoshikawa Y, Morita M, Hosomi H, Tsuneyama K, Fukami T, Nakajima M, et al. Knockdown of superoxide dismutase 2 enhances acetaminophen-induced hepatotoxicity in rat. Toxicology 2009; 264: 89-95. https://doi.org/10.1016/j.tox.2009.07.017

Zhao J, Li J, Xu S, Feng P. Emerging roles of protein deamidation in innate immune signaling. J Virol 2016; 90: 4262-4268. https://doi.org/10.1128/JVI.01980-15