Assessment of *Saccharomyces boulardii* effect on rats *Staphylococcus aureus* induced skin infection: an *in-vivo* study

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ABSTRACT: A concerning global health issue is the growing incidence of antibiotic resistance in skin and soft tissue infection. This study aimed to assess the healing potential of topically applied *Saccharomyces boulardii* on excisional skin wound infected with *Staphylococcus aureus* using a rat model. The wounded animals were divided into three groups; group 1: uninfected untreated, group 2: infected untreated and group 3: infected *S. boulardii*-treated. Treatment commenced 2 days post infection. Parameters studied were wound microbial load, rate of wound contraction, serum C-reactive protein (CRP) level and rat β-defensin 1 (RBD-1) gene expression. Staphylococcal bacterial load decline was significantly ($p < 0.05$) higher in group 3 when compared to group 2 at day 5, 6 and 7 accompanied by a growing of *S. boulardii* load on the animals of group 3. A complete wound closure (100%) was monitored in study group 3 at day 7 in comparison to the control groups, where partial healing was achieved (67.10 and 91.74% in group 1 and 2 respectively). No significant difference of CRP level among the three groups was encountered indicating no influence of *S. boulardii* on this protein. RBD-1 gene expression in group 3 was found higher than the two control groups ($p < 0.05$ and < 0.01 in comparison to group 1 and group 2 respectively). Topical application of *S. boulardii* was shown able to enhance wound healing and competitively exclude the infecting pathogen. The increased antimicrobial peptide gene expression in *S. boulardii* treated group might contribute to its healing potential.

KEYWORDS: Bacterial load; C-reactive protein; defensin; gene expression; *Saccharomyces boulardii*; skin wound; *Staphylococcus aureus.*

1. INTRODUCTION

The process of wound healing should normally pass through a number of physiological events starting with inflammation, followed by processes of re-epithelisation and granulation tissue construction and accompanied by remodeling of the extracellular matrix [1]. Having the skin as the first line body immune defensive mechanism, it is documented that any forms of skin injury is inclined to get infected. The rates of skin infections have been reported to be the most common infection in hospital settings [2,3,4]. Bacterial wound infections have been shown to slow-down the process of wound healing by promoting proteolysis, easing microbial colonization and perpetuation of inflammation either directly or indirectly [5,6,7]. Therefore, control of inflammation and bacterial wound occupation play a key aspect of effective wound management.

One of the frequently isolated and clinically relevant bacteria from infected wounds is *S. aureus* [8,9]. *S. aureus* has been shown to be problematic in clinical ward settings due to its ability to acquire antibiotics resistance and to form biofilms on wound surfaces resulting in difficult to eradicate chronic infection [10]. The empiric therapy of treating *S. aureus* consists of systemic administration of antibiotics such as daptomycin, linezolid, or vancomycin [11,12]. However, this regime has faced the dilemma of resistance in addition to the antibiotics induced side effects.

Recently, the yeast *Saccharomyces boulardii* has attracted the interest of the authors for its antimicrobial activity and immune modulatory effects [13,14,15,16,17]. Although the antimicrobial potential of *S. boulardii* and other yeasts has not been explored entirely, it has been proposed to be attributed to competitively exclude pathogenic bacteria [15,17], the secretion of hydrogen peroxide and organic acids [18], and the production of the antimicrobial beta glucan [19]. Since bacterial colonization influences wound inflammation and the latter plays a crucial role in wound healing, alterations of the bacterial wound colonizers might demonstrate a

powerful therapeutic strategy. This is particularly possible since a number of studies have documented the effectiveness of other probiotic bacteria in reducing inflammation and enhancing wound closure [20,21].

Antimicrobial peptides, namely defensins, are small host peptide molecules with antimicrobial and immune balancing activities against pathogenic infections. β-defensin, a member of the defensins family, locates primarily in the epithelial surfaces of the skin and other tissues [22]. A number of studies have shown that probiotics including *S. boulardii*, can potentially induce the production of β-defensin from epithelial cells as a mechanism to hamper pathogens proliferation and contributes to barrier integrity [23]. However, up to our knowledge, the role of *S. boulardii* on gene expression or production of β-defensin associated with skin infection has not been studied yet. Given the aforesaid activities and the positive outcomes of other studies, it seems plausible to assess *S. bouladii* as a potential infected-wound management approach using an *in vivo* rodent model.

2. RESULTS

2.1. Animal survival and microbiology

No fatalities were reported throughout the entire period of the experiment due to skin infection or anesthesia. Skin swabs cultured on mannitol salt agar revealed a trend of comparable *S. aureus* growth on the wounds surface of both group 2 and 3 since day 1 post inoculation (3.24 ± 1.73 and 3.31 ± 1.55 log10 colony forming unit (CFU)/mL respectively) until day 3 (4.63 ± 2.17 and 4.44 ± 2.34 log10 CFU/mL respectively) when treatment commenced (Figure 1a).

Following treatment of group 2 with phosphate buffer saline (PBS) and group 3 with the yeast suspension, a decline in the wound bioburden was monitored in the two groups since day 4 (3.30 ± 1.95 and 2.39 ± 1.77 log10 CFU/mL respectively) until the end of the experiment at day 7 (1.30 ± 1.17 and 0.0 ± 0.0 log10 CFU/mL respectively). However, this decline was significantly ($p < 0.05$) higher in group 3 when compared to group 2 at day 5, 6 and 7 (Figure 1a). *S. boulardii* wound load was exemplified in figure 1b. Although non-significant ($p > 0.05$), a trend of rising in the probiotic load was documented since day 4 and continued for the following two days to start declining in day 7 of the experiment.
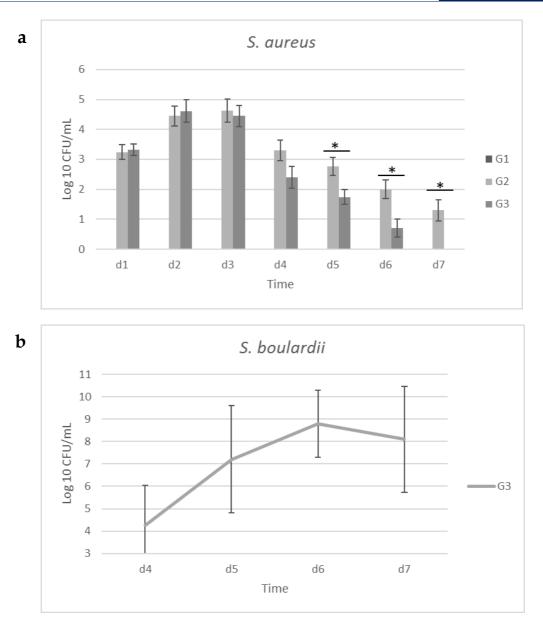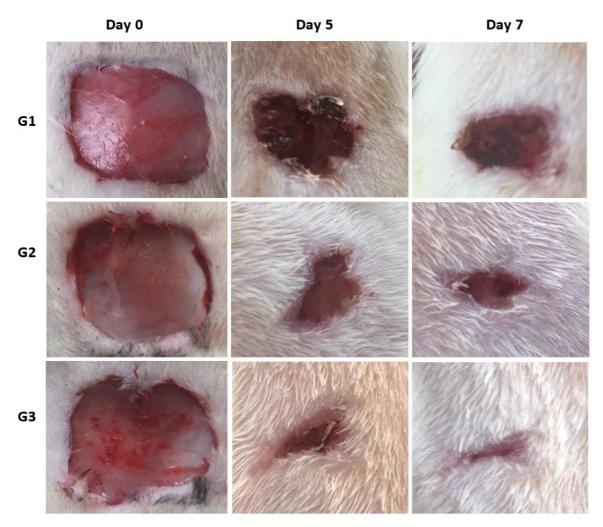
a

b

Figure 1. Microbial viable cells recovery during the experiment period. **a)** *S. aureus* cells recovery from wound swab. **b)** *S. boulardii* cells recovery from wound swab. *; $p < 0.05$ using unpaired *t*-test. G1; group 1, G2; group 2, G3; group 3, d; day, CFU; Colony Forming Unit.

2.2. Wound contraction

The rate of wound healing was measured as the percentage of wound contraction and represented in the table of figure 2. The average number of days required for complete healing with no residual wound in any of the three groups was seven days. Therefore, termination of experiment was decided at day 7. As shown in figure 2, a complete closure of the induced wounds in animals of group 3 treated with *S. boulardii* was recorded on day 7 (% of wound contraction = 100%). However, the rate of wound contraction in the uninfected untreated group 1 rats on day 7 was 67.10 %. Infected wounds of group 2 rats treated with PBS demonstrated a healing tendency that was higher than wound healing in animals of group 1 but less than that of group 3 (% of wound contraction=91.74).

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	Rate of wound contraction (%)		
	Day 0	**Day 5**	**Day 7**
G1	0.00 ± 0	27.44 ± 4.2	67.10 ± 1.4
G2	0.00 ± 0	75.42 ± 2.5	91.74 ± 2.7
G3	0.00 ± 0	82.34 ± 3.7	100.00 ± 0.0
	% calculated according to Eq. 1.		

Figure 2. Effect of *S. boulardii* treatment on wound contraction of group 3 (G3) in comparison to the negative control groups (G1 and G2). A complete closure of the induced wound was achieved on day 7 of the animals group treated with *S. boulardii*. Photos are representative of the experimental animals employed in three study groups. Data in the table are expresed as mean ± standard error of the mean (SEM).

2.3. C-reactive protein

Blood levels of CRP (Figure 3) demonstrated no significant differences among the three animals' groups at day 1 post wound creation and infection ($p > 0.05$). Similarly, no significant differences were encountered among the three groups at day 7 of the experiment ($p > 0.05$). Acute phase protein levels declined at day 7 in the three groups when compared to day 1. The decline was significant in group 2 and group 3 ($p < 0.05$) in comparison to the corresponding group at day 1. Data were represented in figure 3 as mean ± standard error of the mean (SEM).
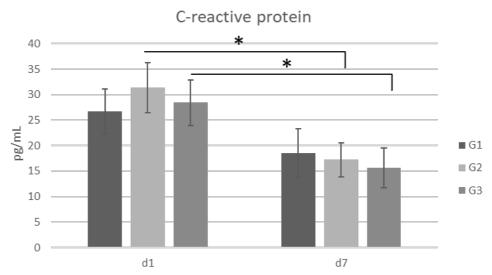
Figure 3. C-reactive protein levels in the three animal groups. No significant differences were encountered among the three groups at day 1 and day 7. Significant decline was reported at day 7 in comparison to day 1 in group 2 and 3. No significance; $p > 0.05$, *; $p < 0.05$ using Tukey's one way ANOVA test. G1; group 1, G2; group 2, G3; group 3.

2.4. β-defensin 1 gene analysis

The expression of rat β-defensin (RBD) -1 was analyzed by quantitative polymerase chain reaction (qPCR) of the extracted skin tissue of the three study groups at the end of the experiment (day 7). RBD-1 gene expression was quantified as relative levels. Our results demonstrated that the relative expression level of RBD-1 gene was enhanced in group 3 supplied with the topical yeast suspension in comparison to the control groups; infection control (group 1) and treatment control (group 2). This higher level of gene expression in group 3 was statistically significant ($p < 0.05$ and < 0.01 in comparison to group-1 and group-2 respectively). Data were expressed as mean ± SEM as shown in figure 4.
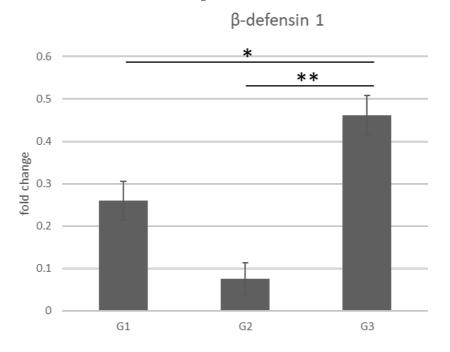

Figure 4. Comparison of the relative expression of RBD-1 in rat skin of the three groups. Error bars ± SEM are shown. Asterisks represent statistical significance determined by Tukey's one way ANOVA test (*$p < 0.05$; **$p < 0.01$). G1; group 1, G2; group 2, G3; group 3.

3. DISCUSSION

The Bacterially colonized wounds enforce an unexpected load to the patients and health care system, with *S. aureus* being one of the most common bacterial isolates [2,3,8,9.12]. As a probiotic, the yeast *S. boulardii*, taken orally or topically, has been reported to exert beneficial effects on the host biological systems [15]. In this study, a rodent *S. aureus* infected-wound model was developed to assess the wound healing potential of topical viable *S. boulardii* treatment. This potential was evaluated microscopically and macroscopically. Effect of *S. boulardii* on the antimicrobial peptide RBD-1 gene expression was also assessed.

Our results demonstrated an improved healing rate in wounds treated with the yeast. An eradication of the colonizing pathogen; *S. aureus* was also found significant in animals treated with the yeast suspension. A chief finding of the current study was the enhanced relative gene expression of RBD-1 in the group treated with the tested suspension. However, no influence of *S. boulardii* on the acute phase protein (CRP) was reported in comparison to the control groups.

The acute response proteins family, of which CRP is a member, is released in response to injury, infections, inflammatory or neoplastic disorders [32]. Therefore, it was supposed reasonable to assess its levels in response to wound healing status. Meta-analysis of 20 studies suggested that probiotics treatment may dramatically diminish the acute phase reactive protein C [33]. Although current serum CRP levels were found diminished in accordance with wounds contracture and healing, measurements did not reveal a significant influence encountered by treatment with *S. boulardii*. This might be due to the local topical effect of the yeast or that the decline was not substantial to be measured.

Studies have documented a wound healing activity of different probiotics, mechanically by reducing inflammation and subsequently reduce wound closure time [34,20,21,35]. Cicatrizing activity of *S. boulardii* on the induced rat dorsal wounds was also estimated and found efficacious. Comparable to our finding, a healing effect of *Lactobacillus plantarum* on wound induced on rat skin was reported by Heydari et al. [21]. However, the wound closure time was prolonged in Heydari's study to 21 days compared to 7 days in the current study. This might be due to experimental differences since no infection was induced in the formed wounds. It might also be due to difference in the commensal microbiota of the experimental animals employed in the study [36]. Similarly, a novel nitric oxide-producing lactic acid bacteria formulated as skin patches was found effective in treating infected wounds in a rabbit model [37]. In contrast, a preliminary study conducted by Jessica et al. 2016 [14] has revealed a lack of acute wound healing potential of *S. boulardii* applied topically on porcine wounds. The authors recommended further investigations using different animal wound healing models. This might be due to the differences of the inflammatory patterns of different animal-models and the variances of their skin microbiota. It might also be due to the different dosing and dose frequency followed in the two studies.

One of the proposed and investigated mechanisms involved in probiotics health-beneficial effects is the competitive exclusion of pathogenic bacteria from binding site [15,17]. A decline in the staphylococcal wound bed load was found accompanying the increase in wound yeast load (Figure 2). The decline in the pathogenic bacterial count may be justified by the competitive displacement by the treating *S. boulardii*. Similar to the current verdict, *S. boulardii* was found able to competitively hamper the adhesive virulence of *Citrobacter rodentium* ameliorating the experimentally induced colitis in a study conducted by Wu et al 2007 [38].

Considering the impact of *S. boulardii* on the molecular mechanisms involved in the process of infected wound healing, we focused on the gene expression of the antimicrobial peptide RBD-1. This innate peptide has shown a broad-spectrum of activity against a wide range of pathogenic bacteria, viruses and fungi. Its effect has been particularly noticed at the epithelial interfaces [39]. The faster healing rate encountered in *S. boulardii* treatment group might be due to such enhancing effect on RBD-1 as a potential mechanism of action. In accordance with our finding, a significant increase in the production of the intestinal murine β-defensin was monitored in response to treatment with *S. boulardii* following *Helicobacter suis* infection [23].

4. CONCLUSION

In summary, the finding of this study clearly demonstrates the healing potential of topically applied *S. boulardii* suggesting employing it as a candidate for further applications and investigations. A competitive exclusion of the colonizing pathogenic bacteria and the enhancing antimicrobial peptide gene expression has been proposed as a possible mechanism of the obtained healing capacity of the studied yeast.

5. MATERIALS AND METHODS

The study was approved by the Institutional Animal Care and Use Committee of the College of Veterinary Medicine/University of Mosul; Ref: UM.VET.2022.02

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5.1. Bacterial suspension

Staphylococcus aureus was used to induce skin wound infection. The pathogenic strain colonies were allowed to grow in brain heart infusion broth to the mid-exponential phase aerobically at 37 ˚C. The bacterial pellet was obtained by centrifugation at 6000 × *g* for 5 min. The obtained pellet was washed twice with PBS and resuspended in the same buffer up to an optical density at 620 nm equivalent to a final concentration of 10^8 CFU/mL. *S. boulardii*, previously isolated from a commercial product [13], grew on Sabouraud's agar (SAB; Oxoid, Hampshire, UK) for 24 h and colonies were collected and resuspended in PBS to a final colony forming unit of 10^8 per mL [24].

5.2. Animals and wound creation

Eighteen adult male albino rats in a good health status weighing between 200-250 g were randomly selected and included in the study. They were housed individually, fed with rat pellets *ad libitum* and watered with tap water at constant temperature (around 22 ˚C) and humidity according to the guidelines of the Institutional Animal Care and Use committee of the College of Veterinary medicine. The animals were randomly grouped into three groups of 6 animals each; group 1 (G1)- rats with uninfected wounds left untreated to the end of the experiment, group 2 (G2)- rats with infected wound treated with PBS and group 3 (G3)- rats with infected wound treated with *S. boulardii* suspension. Rats of the three groups were initially anaesthetised by intraperitoneal injection of ketamine (25 mg/kg) and xylazine (5 mg/kg) [25]. The dorsal surface hair of the animals was trimmed with an electric clipper and rubbed with 70% alcohol. Wounds were created according to a rat excisional wound model designed by Nayak et al. 2007 with minor modifications [26]. Briefly, two full thickness excision wounds of an approximate area of 15 mm x 20 mm and 2 mm depth were made by sharply removing rat's dorsum skin and panniculus carnosus using saw-like forceps, a surgical blade and scissors. The resultant defects were left opened entirely and treated according to the labelled group.

5.3. Treatment

Animals of group 2 and 3 were infected by applying 0.5 mL of the prepared *S. aureus* suspension in PBS (1×10^8 CFU/mL) into each back wound. The animals were visually inspected at daily basis to ensure purulent wounds were developed. Two days after inducing the infection, treatment was started. Infected wounds of group 2 animals were treated with topically applying 0.5 mL sterile PBS once a day for five days. Group 3 animals received a single dose of 0.5 mL topical *S. boulardii* suspension in PBS (1×10^8 CFU/mL) for five days. Following treatment, wounds were left unveiled to the environment. Swab was taken from each wound of the three groups on daily basis and cultured on mannitol salt agar and SAB agar to follow *S. aureus* and *S. boulardii* growth respectively. At the end of the treatment period, animals were humanely euthanized. One full-thickness biopsy of around 10 mm x 10 mm area of the skin involving the wound were obtained from each wound at the end of the experiment. This tissue sample was used for gene expression analysis.

5.4. Investigational monitoring

Wounds were monitored on daily basis and photographed. Wounds' edges were outlined and dimensions were recorded using graduated ruler on the day of wound creation (day 0), 3rd day after treatment started (day 5) and on the end of the experiment (day 7) when whole closure of the wound was achieved. Wound healing was traced by calculating the rate of wound contraction according to the following formula [27]:

% Wound contraction=Healed area˚/Total wound area*100 ……….[Eq. 1]

[˚; Healed area= Original wound area-Present wound area].

5.5. C-reactive protein

For assessment of the acute inflammatory response in the study groups, serum C-reactive protein (CRP) level was measured [28]. Two time points were selected; one at day 1 after wound creation and infection and one at the end of the experiment before animals' scarification. The animals were anesthetized as explained above. Blood sample was withdrawn from the orbital venous plexus into plain centrifuge tube [29]. Blood was allowed to clot and serum was separated by centrifugation and kept frozen until running the test. The protein was measured using an enzyme-linked immunosorbent assay (ELISA). Instructions from kit's manufacturer were followed (CRP: Boster Biological Technology Co., Ltd., CA).

5.6. Rat β-defensin 1 gene expression

The obtained skin tissue biopsies were frozen and then homogenized in TriReagent® (Molecular Research Center, Inc., Cincinnati) using a Tissue TearorTM electric homogenizer. Later, DNA was extracted using AddPrep Genomic DNA Extraction Kit (Republic of Korea) following the manufacturer's instruction. DNA concentration was determined using IMPLEN GMBH Nano drop (nanophotometer) device. Quantitative PCR technique (qPCR) was used to assess the relative expression of the gene rat β-defensin 1 (RBD-1). Briefly, DNA (100 ng/mL in nuclease-free water) was added to the corresponding wells of chilled 96 PCR plate (Sigma, UK) and kept in ice bath until use. Reaction mix was prepared using the GoTaq® Probe (Promega), qPCR Master Mix, PCR primers and nuclease-free water. This mix was then added to the corresponding wells of the reaction plate containing the DNA template. Reverse transcription product was used for each sample to amplify RBD-1 using Stratagene Mx3005P real-time heat processor (German Ecosystem) and qPCR software MxPro3005P. The reference gene used for this experiment was the house-keeping D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. For RBD-1, the forward (5′ to 3′) primer was GGACGCAGAACAGATCAATACCGA and the reverse primer (5′ to 3′) was TCTTCAAACCACTGTCAACTCCTG. For the house-keeping reference GAPDH gene, forward (5′ to 3′) primer was AGACAGCCGCATCTTCTTGT and the reverse primer (5′ to 3′) was CTTGCCGTGGGTAGAGTCAT [30,31].

5.7. Statistical analysis

Unpaired *t*-test (GraphPad InStat 3 (v3.06)) was used to evaluate statistical difference between tests' means of the recovered viable microbial cells of group 2 and group 3. Tukey's one way ANOVA test (GraphPad InStat 3 (v3.06)) was used to assess the statistical difference in CRP concentrations and RBD-1 gene expression among the three animals' groups. Data were expressed as mean ± standard error of the mean (SEM) where relevant.

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