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Lactobacillus plantarum USM8613 Aids in Wound Healing and Suppresses Staphylococcus aureus Infection at Wound Sites

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Abstract

This study aimed to elucidate the targets and mechanisms of anti-staphylococcal effects from bioactive metabolites produced by lactic acid bacteria. We aimed to better understand the safety and efficacy of these bioactive metabolites in in vivo systems, typically at topical sites. The cell-free supernatant and protein-rich fraction from *Lactobacillus plantarum* USM8613 inhibited staphyloxanthin biosynthesis, reduced ($p < 0.05$) the cell number of *Staphylococcus aureus* by 10^6 CFU/mL and reduced biofilm thickness by 55% in *S. aureus*-infected porcine skins. Genome-wide analysis and gene expression analysis illustrated the production of several plantaricins, especially the plantaricins EF and JK that enhanced the anti-staphylococcal effects of *L. plantarum* USM8613. In vivo data using rats showed that the protein-rich fraction from *L. plantarum* USM8613 exerted wound healing properties via direct inhibition of *S. aureus* and promoted innate immunity, in which the expression of β-defensin was significantly ($p < 0.05$) upregulated by 3.8-fold. The protein fraction from *L. plantarum* USM8613 also significantly enhanced ($p < 0.05$) the production of cytokines and chemokines through various stages of wound recovery. Using Δ*atl S. aureus*, the protein-rich fraction from *L. plantarum* USM8613 exerted inhibitory activity via targeting the *atl* gene in *S. aureus*. Taken altogether, our present study illustrates the potential of *L. plantarum* USM8613 in aiding wound healing, suppressing of *S. aureus* infection at wound sites and promoting host innate immunity.

Keywords *Lactobacillus plantarum* USM8613 · *Staphylococcus aureus* · Skin · Wound healing · Innate immunity

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Introduction

Over the past 100 years, changes in society and technology have led to lifestyle changes and improvement of many basic life needs globally. Simultaneously, these changes have resulted in new diseases and modification of existing dermatoses. The discovery and extensive use of antibiotics has generated various antibiotic-resistant variants, giving rise to new health risks [1]. *Staphylococcus aureus* is a transient opportunistic skin pathogen of humans and other animals. *S. aureus* is well equipped with various virulence factors that cause mild to severe infections, ranging from cutaneous to systemic infections. In addition to virulence factors, *S. aureus* also contains several surface components such as Protein A and extracellular adhesion proteins which facilitate *S. aureus* to evade the inhibitory actions of antibiotics and recognition by the host immune system [2, 3]. The emergence of antibiotic-resistant strains has further reduced treatment and therapeutic options [4]. Hence, there is a need for natural alternative compounds against *S. aureus*.

Lactic acid bacteria (LAB) are Gram-positive, non-sporulating, microaerophilic bacteria that produce lactic acid as the main end product of carbohydrate fermentation. LAB have a long history of use in the food industry due to their ability to exert various beneficial effects [5, 6]. In addition to its additive effect on food content, the intake of LAB also confers health benefits to the host via an improved gut ecosystem, reduced serum cholesterol and enhanced host immune system. Among them, members of the genera *Lactobacillus* are the most commonly and commercially used. Despite the long-term use of these beneficial lactobacilli in the food industry, it was not until recently that the use of lactobacilli was extended to improve dermal health. Several studies have suggested the use of these beneficial LAB to maintain cutaneous homeostasis and improve regulation of the skin immune system [7, 8].

Although LAB have been widely documented to produce antimicrobial substances, antimicrobial peptides such as bacteriocins remain the most extensively studied compounds. Bacteriocins are generally cationic and constitute a heterogenous group of peptides that could be classified into five main classes based on their molecular size, chemical structure, antimicrobial spectrum, mode of action and target cell receptors [9]. The bacteriocins produced by *Lactobacllus plantarum* are termed plantaricins. The plantaricin locus contains five operons: the *plnABCD* operon encodes for the signal-transducing pathway, the *plnEFI* and *plnJKLR* operons encode for bacteriocins and its immunity proteins, *plnGHSTUVW* operon encodes for an ABC transport system that processes and secretes peptides containing double-glycine N-terminal leaders, and lastly, the *plnMNOP* operon encodes genes with unknown functions in bacteriocin synthesis [10]. In the past, antimicrobial peptides produced by *Lactobacillus lactis* such as nisin, which has been extensively used for food preservation in over 50 countries, have been applied as biopreservatives in the food industry [11]. Antimicrobial peptides are now used for applications such as incorporation into nanofibre scaffolds for medical applications beyond the food industry [12], demonstrating its therapeutic potential for pharmaceutical demands.

Meanwhile, limited data are available on topical applications of LAB that specifically target *S. aureus* in an in vivo model, towards both *S. aureus* cells and/or the infected host. *Lactobacillus plantarum* USM8613 has demonstrated promising anti-staphylococcal activity via inhibitory metabolites that are proteinaceous in nature (data not shown). However, the efficacy, safety and immune-modulating effects of *L. plantarum* USM8613 in the presence of opportunistic *S. aureus* remain largely unknown. Thus, we aimed to elucidate these properties using ex vivo and in vivo wound models.

Materials and Methods

Bacterial Cultures

L. plantarum USM8613 was isolated from a meat product (pork minced meat) purchased at a local market (Penang, Malaysia), following the methods from Najjari et al. [13]. It was activated successively three times in sterile de Man, Rogosa and Sharpe (MRS) broth (HiMedia, Mumbai, India) at 37 °C before experimental use. A clinical isolate of *S. aureus* (identified via 16s rDNA) was obtained from the Penang General Hospital (Penang, Malaysia). The *atl* null (Δ*atl*) and its wild-type (WT) *S. aureus* strains were obtained from Hiroshima University, Japan. *S. aureus* was activated in trypticase soy broth (TSB; HiMedia) at 37 °C for 24 h prior to use.

Cell-Free Supernatant and Crude Protein Fractionation

L. plantarum USM8613 (grown overnight for 20 h at 37 °C) was diluted (OD600nm = 1.0) with MRS broth (HiMedia) prior to centrifugation at 3500×*g* for 15 min at 4 °C to obtain cell-free supernatant (CFS). The CFS was neutralised to pH 7.0 using 1.0 M NaOH and then filter-sterilised through 0.22 μm mixed cellulose filter membrane prior to use (Merck Millipore, Darmstadt, Germany). Crude protein was extracted using an ammonium sulphate (at 80% saturation) precipitation method with stirring at 4 °C for 18 h. The mixture was centrifuged at 8000×*g* for 10 min at 4 °C and the pellet was suspended with 1.0 M ammonium acetate buffer, pH 6.5 in a ratio of 1:1 (*v/w*) [14]. Then, the solution was filtered and the protein content was determined via the Bradford assay [15]. The crude protein obtained is then designated as a protein-rich fraction. Unfermented MRS broth fractionated in a similar manner was used as a control. Anti-staphylococcal activity against *S. aureus* was tested using the 96-well microtitre plate assay following the method from Romero-Tabarez et al. [16].

Staphyloxanthin Biosynthesis Inhibition Assay

Inhibition of staphyloxanthin biosynthesis was qualitatively determined according to the method by Liu et al. [17]. Briefly, 15 mL of trypticase soy agar (TSA; HiMedia) supplemented with 10% (*v/v*) unfermented MRS broth (HiMedia) was poured into a Petri dish and left to be solidified. Half of the agar was cut and dug out using a sterilised scalpel and tweezer. Then, 7.5 mL of TSA supplemented with 10% (*v/v*) CFS from *L. plantarum* USM8613 was added into the excavated area. *S. aureus* was streaked on both of the half area separately and incubated at 37 °C for 24 h.

Agarose Gel Electrophoresis

The DNA of *L. plantarum* USM8613 was extracted using a QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer's protocol. Polymerase chain reaction (PCR) was performed using a *Taq* PCR Master Mix Kit (Qiagen) and a Biometra Tone Thermal Cycler (Analytik Jena, Jena, Germany). The PCR amplicons were subjected to 2.0% (*w/v*) agarose gel electrophoresis at 100 V for 60 min. The gel was visualised using the GeneGenius Imaging System (Syngene, Cambridge, UK). The primers used are listed in Table S1.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Total RNA of *L. plantarum* USM8613 (grown overnight for 20 h at 37 °C) was extracted using RNeasy Mini kits (Qiagen) and converted to cDNA using SuperScript™ III Reverse Transcriptase (Life Technologies, CA, USA). Equal amounts of cDNA were used for qRT-PCR using QuantiTech SYBR® Green PCR master mix (Qiagen). The reactions were performed using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, CA, USA). All primer sequences are listed in Tables S1.

Porcine Skin

The dorsal skin part of a 60-kg domestic farm pig was obtained from a local market (Penang, Malaysia). The body hair on the skin was removed using a scalpel and the skin was cleaned using 70% (*v/v*) isopropanol (QRec, Malaysia). The skin samples were then cut into 4-cm^2 (2 cm × 2 cm) pieces. The 4-cm^2 skin samples were subjected to different treatments: (a) inoculated with 200 μL of unfermented MRS broth (HiMedia) and incubated at 37 °C for 24 h; (b) inoculated with *S. aureus* (10^6 CFU/mL) in 200 μL of unfermented MRS and incubated at 37 °C for 24 h; and (c) inoculated with *S. aureus* (10^6 CFU/mL) in 200 μL of protein-rich fraction of *L. plantarum* USM8613 prior to incubation at 37 °C for 24 h.

Confocal Scanning Laser Microscopic Analysis

The porcine skin samples were fixed in 10% (*v/v*) neutral buffered formalin for 24 h in separated glass Petri dishes. Following fixation, the skin samples were washed and dehydrated through an ascending series of ethanol, specifically 30%, 50%, 70%, 90% and 100% (*v/v*), and finally kept at − 20 °C. The dehydrated skin samples were then re-wetted via soaking in sterilised distilled water prior to the staining process. The rehydrated skin samples were then stained with 1% (*v/v*) acridine orange (AO) dye (Sigma-Aldrich, Steinheim, Germany) for 15 min, followed by washing with PBS (10 mM, pH 7.4) for 30 min. The skin samples were further stained with 1% (*v/v*) 2-(4-amidinophenyl)-1H-indole-6- carboxamide (DAPI; Sigma-Aldrich) for 30 min, and washed again with PBS (10 mM, pH 7.4) for 30 min. The skin samples were then examined using a confocal scanning laser microscope (CSLM; Eclipse-Ti, NY, USA). The thickness of the biofilm was quantified using the COMSTAT programme written as a script in the MATLAB 5.2 software suite [18].

Microbial Enumeration

The total microbial count of the porcine skins was enumerated via the spread plate method. Briefly, the porcine skin was immersed in 1 mL PBS (10 mM, pH 7.4) and homogenised using the Seward Stomacher 400C (Cole-Palmer, IL, USA) at 160 rpm for 30 s. The homogenised solution was obtained and a tenfold serial dilution was performed. One hundred microlitres of each sample dilution was spread onto mannitol salt agar (MSA; HiMedia). Three replicates were carried out for each dilution, and the agar plates were incubated at 37 °C for 24 h. The colonies were counted and the results were tabulated.

Animals

Thirty-six male Wistar rats weighting 150–200 g each were obtained from the Animal Research Unit and Service Centre (ARASC), Universiti Sains Malaysia (USM), and were housed individually in polypropylene cages under controlled light conditions with a 12-h light-dark cycle at 22 ± 2 °C and 50 ± 5% humidity. Water and food were provided to animals ad libitum. The 36 rats were divided into treatment and control groups with $n = 18$ rats in each group. The experimental protocol for treating animals was approved and in accordance with the guidelines recommended by the USM Committee on Animal Care; reference number: USM/Animal Ethics Approval/2012/(81)(433).

Formulation of Skin Ointment

A 10% (*v/w*) formulated ointment was prepared by mixing 50 μL of the protein-rich fraction from *L. plantarum* USM8613 with 500 mg of yellow soft paraffin [19]. Control ointment was prepared by mixing the protein-rich fraction precipitated from unfermented MRS broth with yellow soft paraffin (Unicorn Petroleum Industries, Mumbai, India).

Wounding and Infection

The animals were anaesthetised using ketamine/xylazine (75 mg/kg; 10 mg/kg). Using a sterile scalpel, a full

thickness wound of size 1.5 cm × 1.5 cm was created by removing the skin on the shaved dorsal part of the animals, at a depth down to the underlying fascia of the panniculus carnosus muscle. Wound sites were inoculated with 50 μL (10^6 CFU/mL) of virulent *S. aureus*. On the following day, the wounds of *L. plantarum* USM8613-treated rats were topically treated with 10% formulated protein-rich fraction of *L. plantarum* UMS8613 ointment, while control group rats were topically treated with 10% formulated control ointment. The wounds were then covered with plain gauze and reinforced with cohesive bandage. The ointment was reapplied twice per day. A total of three rats from each group were sacrificed at intervals of 4 days after treatment (days 1, 4, 8, 12 and 16). Blood samples were collected and the entire wound tissues were cut out and stored at −80 °C until further analysis. The expression of β-defensin at the wound sites was determined via the qRT-PCR method. Wound sites were evaluated at days 1, 4, 8, 12 and 16. The decrease in wound diameter during the healing process was measured with a calliper, with the percentage of wound closure (wound contraction) calculated as [19]:

$$(\text{Area of origin wound} - \text{Area of actual wound})$$
$$/\text{Area of original wound} \times 100\%.$$

Histological Analysis

Fresh tissue samples were collected and fixed in 10% (*v/v*) neutral formalin buffer for 24 h at 4 °C. Conventional techniques of paraffin wax sectioning and haematoxylin-eosin staining were used for histological studies. The formalin-fixed tissues were dehydrated via series of ethanol [30%, 50%, 70%, 90% and 100% (*v/v*)] and cleared in xylene [100% (*v/v*); QRec] and embedded in paraffin wax (Sigma-Aldrich). Sections of 5–8-μm thickness were cut using a microtome (Leica Biosystems, Nussloch, Germany) and stained using aqueous haematoxylin and eosin (H&E). The prepared slides were viewed under a light microscope (Nikon Instruments, NY, USA).

Serum Cytokine Analyses

Blood samples were collected in vacutainer tubes (Becton Dickinson, NJ, USA) and centrifuged at 3500×*g* for 10 min at 25 °C to obtain serum. The levels of interleukin-4 (IL-4), interleukin-6 (IL-6), transforming growth factor-β (TGF-β), interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) in the blood serum were determined using a commercial ELISA kit (R&D System Inc., MN, USA) according to the manufacturers' protocol.

MMP Gelatine Zymography

The presence of matrix metalloproteinases (MMPs) on the granulation tissue was analysed by gelatine zymography [19].

Transmission Electron Microscopic Analysis

The activated Δ*atl* and WT *S. aureus* cells were diluted to OD600nm = 0.3 (10^6 CFU/mL) and treated with 5 mL of 200 AU/mL protein-rich fraction in a 1:1 ratio at 37 °C for 5 h until the mid-exponential growth phase. The cells were collected via centrifugation (2200×*g* at 25 °C for 15 min) and washed twice with 0.1 M phosphate buffer (pH 7.2). The cell pellets obtained were pre-fixed with McDowell-Trump fixative for 24 h at 4 °C. The fixed samples were washed twice with 0.1 M phosphate buffer, followed by post-fixation in 1% osmium tetroxide (Sigma-Aldrich) for 1 h at 25 °C. Cell pellets were subsequently encapsulated in 2% (*w/v*) batch agar and cut into 1 mm × 5 mm rectangular shapes and dehydrated through a series of ethanol washes [50%, 75%. 95%, and 100% (*v/v*)] and a 100% (*v/v*) acetone wash prior to being embedded in Spurr resin. Five pieces of each sample were embedded in gelatine capsules filled with Spurr resin and polymerised at 60 °C for 24 h. Ultra-thin sections (70 to 90 nm) were collected onto uncoated 300-mesh nickel grids and stained using 2% (*v/v*) uranyl acetate and lead citrate. Samples were viewed and captured using an EF-TEM Libra 120 (Carl Zeiss, Oberkochen, Germany).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Total RNA of tissue samples, WT *S. aureus* and Δ*atl S. aureus* (grown overnight for 20 h at 37 °C) were extracted using RNeasy Mini kits (Qiagen) and converted to cDNA using SuperScript™ III Reverse Transcriptase (Life Technologies, USA). Equal amounts of cDNA were used for qRT-PCR using QuantiTech SYBR® Green PCR master mix (Qiagen). The reactions were performed using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). All primer sequences are listed in Tables S2, S3 and S4.

Statistical Analyses

All data were presented as the mean ± standard deviation from triplicate runs ($n = 3$), unless stated otherwise, and analysed using SPSS© version 20.0. One-way ANOVA was used to determine the significant differences between means at a significance level of $\rho < 0.05$. Tukey's test was used to perform multiple comparisons between means. The independent *t* test was used to determine the significant differences between two means at a given time point with a significance level of $\rho < 0.05$.

Results

Anti-staphylococcal Activity of CFS and Plantaricin Genes

S. aureus that grew on TSA supplemented with 10% (*v/v*) unfermented MRS broth appeared as golden-carotenoid pigmentation colonies but lost the golden-carotenoid pigment and grew as pale-white colonies in TSA supplemented with 10% of CFS from *L. plantarum* USM8613 (Fig. 1A). The ability of CFS to inhibit and remove biofilm formed by *S. aureus* was demonstrated using *S. aureus*-infected porcine skins. The number of viable *S. aureus* cells was significantly lower ($\rho < 0.05$) in the CFS treatment group (10^4 CFU/mL) compared to the unfermented MRS treatment group (10^{10} CFU/mL) (Fig. 1B). In addition, the biofilm formed was also significantly ($\rho < 0.05$) thicker in the unfermented MRS control group (118.92 ± 2.87 µm) in comparison to the CFS treatment group (53.67 ± 5.56 µm) (Fig. 1C).

Gel electrophoresis revealed the presence of structural *pln* genes such as *plnA*, *plnC*, *plnEF*, *plnJ* and *plnK*, and non-structural *pln* genes such as *plnB*, *plnD*, *plnG*, *plnI*, *plnN* and *plnS* in *L. plantarum* USM8613. Meanwhile, the non-structural *plnNC8* and structural *plnW* genes were both not detected (Fig. 2A). The positive response regulator PlnC was upregulated ($\rho < 0.05$) while the negative response regulator PlnD was suppressed (based on expression level = 1.00; $\rho < 0.05$; Fig. 2B). Gene expression of *plnB* and *plnEF* was neither upregulated nor suppressed, while the *plnJ* and *plnK* genes were significantly upregulated ($\rho < 0.05$) and *plnI* and *plnS* were significantly downregulated ($\rho < 0.05$; Fig. 2B).

Anti-staphylococcal Activity in Porcine Skin

The viable cell count of *S. aureus* in porcine skin treated with the protein-rich fraction of CFS from *L. plantarum* USM8613

was lower ($\rho < 0.05$) as compared to the porcine skin treated with the protein-rich fraction of unfermented MRS control at all time points studied (Fig. 3A). The protein-rich fraction also reduced the thickness of biofilm formed as compared to the control group at all time points studied ($\rho < 0.05$; Fig. 3B). Confocal images of the porcine skin treated with the MRS control revealed a thick layer of biomass on the surface, covering the porcine basal skin layer, as compared to porcine skin treated with the protein-rich fraction at all time points studied (Fig. 3C). In addition, colonies of *S. aureus* (represented as red coloured spots and indicated with white arrows) were observed in the protein-rich fraction-treated group, but not in the MRS control group (Fig. 3C).

Wound Healing, Inhibitory and Inflammatory Properties

After 1 day, the population of *S. aureus* maintained at 10^6 CFU/g in control rats, but reduced to 10^5 CFU/g in rats treated with the protein-rich fraction of CFS from *L. plantarum* USM8613. Subsequent reductions were observed over time for both treatment groups. By day 12, the population of *S. aureus* was reduced to 10^4 CFU/g in the control rats while the *L. plantarum* USM8613-treated rats showed a reduction of 50% as compared to the initial inoculum to reach 10^3 CFU/g (Fig. 4A). The *L. plantarum* USM8613-treated rats also showed an enhanced wound contraction percentage (54%) compared to their control counterparts (43%; $\rho < 0.05$) by day 4, although all rats showed increased wound contractions over time (Fig. 4B). Rats in the control group only achieved a wound contraction percentage of above 50% by day 8 (79%).

The production of β-defensin at wound sites of the *L. plantarum* USM8613-treated rats were upregulated ($\rho < 0.05$) in the initial stages of infection and healing, with a marked peak of 3.83-fold higher expression by day 4 as
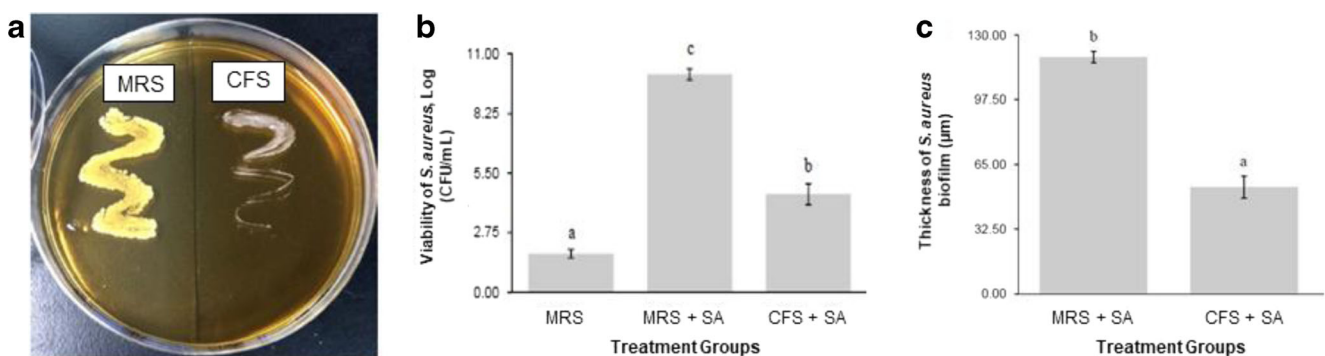

Fig. 1 (**A**) Staphyloxanthin production of *S. aureus* illustrated on TSA supplemented with 10% (*v/v*) of MRS and cell-free supernatant of *L. plantarum* USM8613 (CFS). (**B**) Number of viable *S. aureus* cell count on porcine skin samples inoculated with 200 µL of unfermented MRS, *S. aureus* (10^6 CFU/mL) in 200 µL of unfermented MRS or,

S. aureus (10^6 CFU/mL) in 200 µL of CFS prior to incubation at 37 °C for 24 h. (**C**) Thickness of biofilm formed on *S. aureus*-infected porcine skins. Results are expressed as mean ± standard deviation of means (*n* = 3). [a, b]Means between different sample groups were significantly different ($\rho < 0.05$)
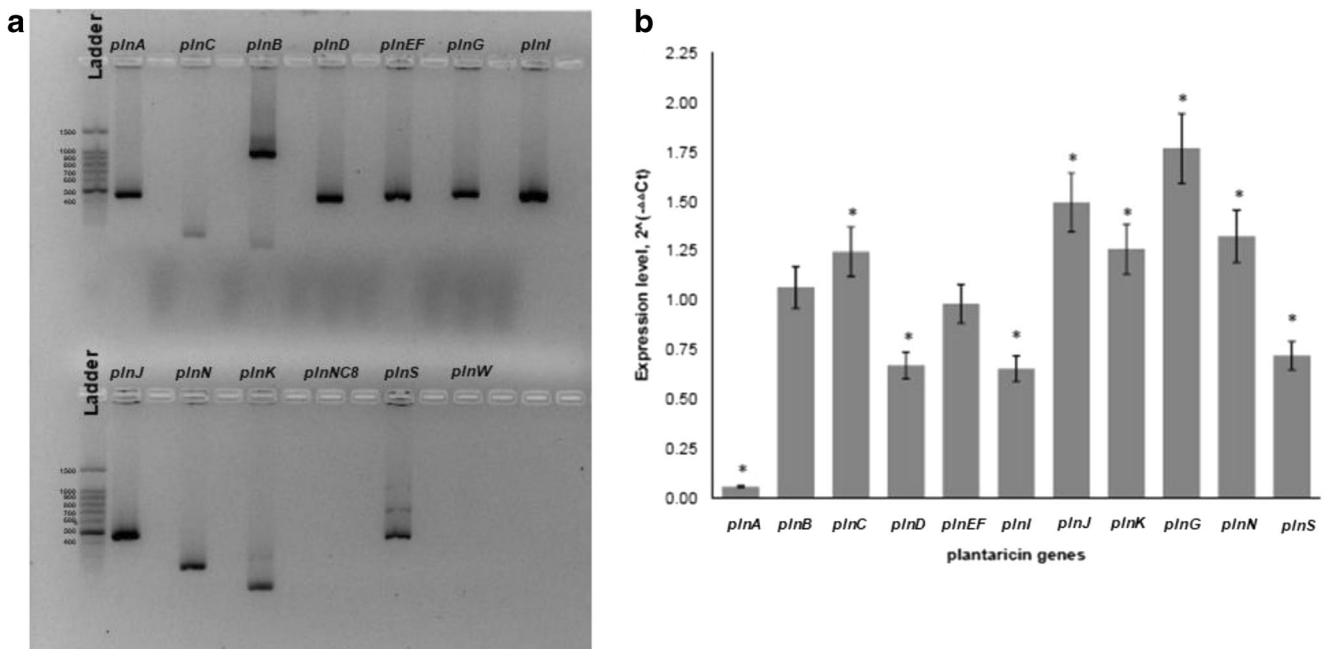
Fig. 2 (**A**) Gel electrophoresis of PCR amplicons of plantaricin genes (*pln*) in *L. plantarum* USM8613. (**B**) Gene expression levels of plantaricin genes in *L. plantarum* USM8613. Results are expressed as mean ± standard deviation of means ($n = 3$). *Means between different sample groups were significantly different ($\rho < 0.05$)

compared to the control group (Table 1). The expression of β-defensin of the *L. plantarum* USM8613-treated rats decreased over time (days 4–12) and was significantly lower compared to the control group at day 16 ($\rho < 0.05$).

H&E staining of skin biopsies showed that the protein-rich fraction from CFS of *L. plantarum* USM8613 accelerated keratinocyte migration over the wound edge towards the centre area over time (days 4–16; Fig. 5a–e) and achieved better wound closure and complete re-epithelisation by day 12 (Fig. 5d). Rats in the control group displayed slower wound closure over time (Fig. 5f–j), where complete re-epithelisation was only observed by day 16 (Fig. 5j). In addition, the presence of inflammatory cells remained visible at wound sites of the control rats at day 16 (Fig. 5j) as compared to day 12 of *L. plantarum* USM8613-treated rats (Fig. 5c).

In the early days of wound recovery (days 1–4), rats treated with the protein-rich fraction from CFS of *L. plantarum* USM8613 showed higher concentrations of Il-4, IL-6 and TGF-β at wound sites as compared to the control group (Fig. 6a, c and e), but showed lower concentrations of TNF-α than the control group during the final stages of wound recovery (day 16; Fig. 6b). The concentrations of IFN-γ at wound sites were increased after day 1 and decreased over time in both groups, but the *L. plantarum* USM8613-treated group decreased more by day 8 as compared to the control group, which only decreased by day 12 (Fig. 6d). Meanwhile, the levels of MMPs in wound sites of *L. plantarum* USM8613-treated rats were gradually reduced over time but this was not observed in the control rats (Fig. 7).

Autolysis Mechanisms

The gene expression of global regulators such as *sigB*, *agrA* and *ROT* in Δ*atl S. aureus* were unaffected upon treatment with the protein-rich fraction from CFS of *L. plantarum* USM8613 (Fig. 8a), while *TRAP* expression was significantly upregulated ($\rho < 0.05$). The same phenomena were observed in the pathogenicity factors of Δ*atl S. aureus*, in which expression of genes *hlb*, *clfB*, *icaA* and *lytM* were unaffected by the protein-rich fraction from *L. plantarum* USM8613 (Fig. 8b), while only the expression of the pathogenicity factor *spa* gene, which encodes for protein A, was suppressed ($\rho < 0.05$) upon treatment with the protein-rich fraction from CFS of *L. plantarum* USM8613 (Fig. 8b). Under TEM, the control Δ*atl S. aureus* was distinguishable by its rough outer surface, in contrast with the smooth outer surface appearance of the WT *S. aureus* (Fig. 8c). *L. plantarum* USM8613 treated-WT *S. aureus* also failed to maintain an intact cell wall, where leakage of the intracellular content was observed (Fig. 8c).

Most of the autolysis-related genes studied were expressed normally and not affected by the protein-rich fraction in both Δ*atl* and WT *S. aureus*. The expression of the *mgrA* gene was the only one that was upregulated, while the expression of *atl* was not detected in Δ*atl S. aureus* upon protein-rich fraction treatment ($\rho < 0.05$). Meanwhile, expression of *norA*, *cidA* and *sigB* in WT *S. aureus* was upregulated and that of *sarA* and *agrA* was downregulated ($\rho < 0.05$) upon treatment with
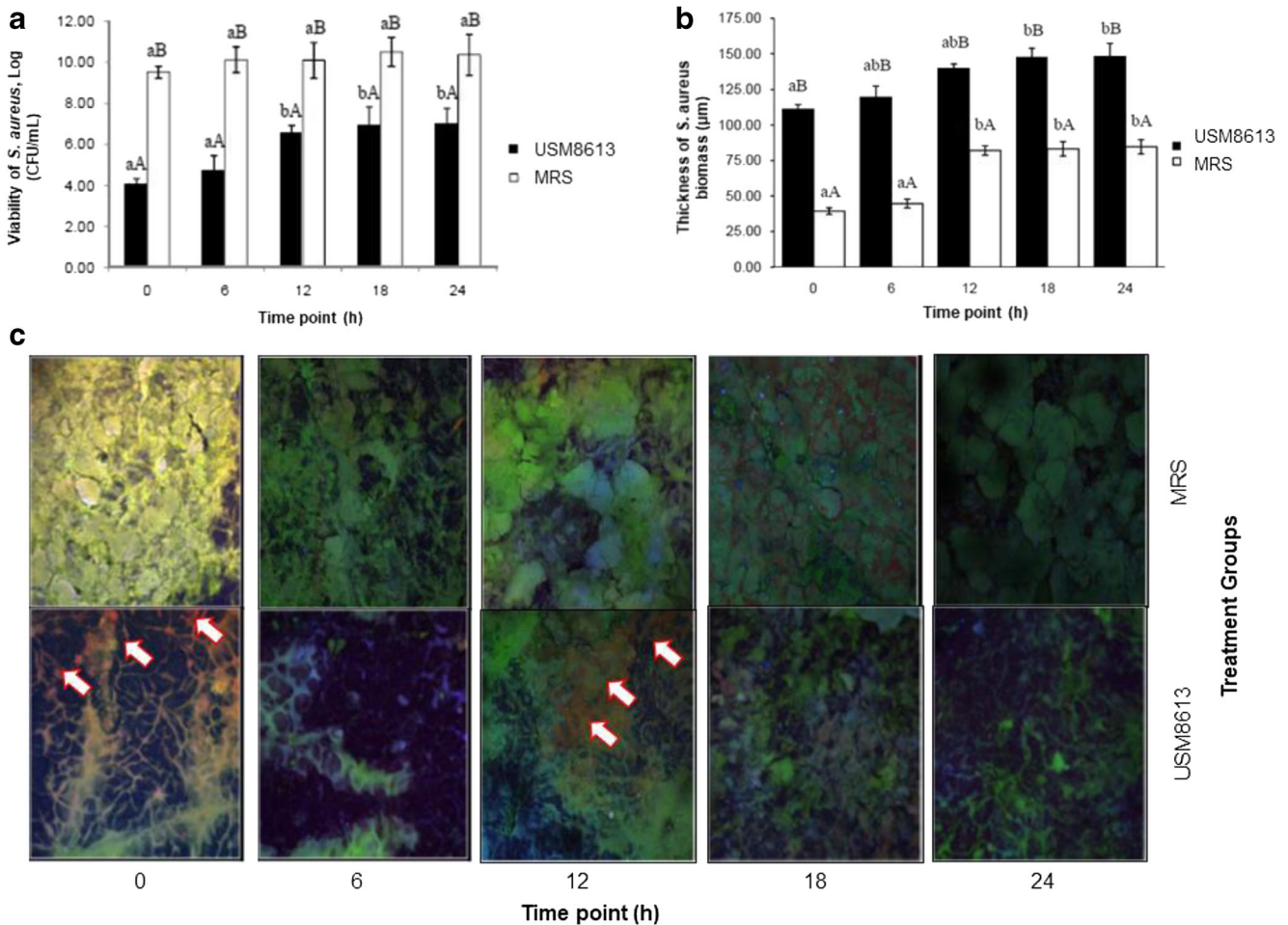
Fig. 3 (**A**) Viable cell count and (**B**) biofilm thickness of *S. aureus* on porcine skin samples upon treatment with the protein-rich fraction from unfermented MRS or CFS of *L. plantarum* USM8613. (**C**) Confocal micrographs of porcine skin cells (blue) stained with DAPI were differentiated from *S. aureus* cells (red) and its product (green) which was stained using AO dye. Magnification: 100x. Results are expressed as mean ± standard deviation of means ($n = 18$). [a, b]Means within the same sample group and [A, B]means between different treatment groups at the same time point were significantly different ($ρ < 0.05$)
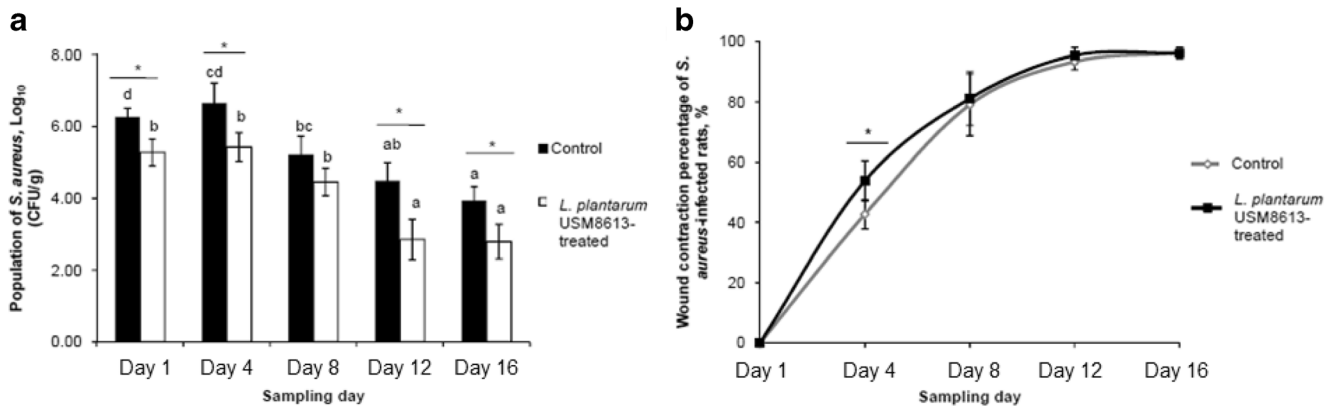
Fig. 4 (**A**) Population of *S. aureus* cells at wound sites (1.5 cm × 1.5 cm) of infected rats treated with 500 mg of yellow soft paraffin containing 50 μL of the protein-rich fraction from CFS of *L. plantarum* USM8613 or empty paraffin (control), applied twice daily over 16 days. (**B**) Wound contraction percentage of *S. aureus*-infected rats. Results are expressed as mean ± standard deviation of means ($n = 18$). *Means between treatment groups within the same sampling day are significantly different ($ρ < 0.05$); [a, b, c, d]Means between sampling day within the same group were significantly different ($ρ < 0.05$)

Table 1 Relative quantification of β-defensin from wound sites in *S. aureus*-infected rats using comparative CT method

Sampling day	Control rats	L. plantarum USM8613-treated rats
Day 1	1.00 ± 0.17[b]	2.26 ± 0.11[a]
Day 4	1.00 ± 0.07[b]	3.83 ± 0.27[a]
Day 8	1.00 ± 0.15[a]	0.78 ± 0.08[a]
Day 12	1.00 ± 0.09[a]	0.67 ± 0.16[a]
Day 16	1.00 ± 0.14[b]	0.29 ± 0.16[a]

Results are tabulated as mean ± standard deviation of means ($n = 18$). [a,] [b] Means between treatment groups within the same sampling day were significantly different ($\rho < 0.05$)

the protein-rich fraction of CFS from *L. plantarum* USM8613 (Fig. 9).

Discussion

Biofilm formation is a virulence factor in the pathogenesis of staphylococcal infections, contributing to over 80% of bacterial infections, and is one of the strategies used by *S. aureus* to survive in various environments [20]. *L. plantarum* USM8613 inhibited staphyloxanthin biosynthesis and reduced biofilm formation by *S. aureus*. Staphyloxanthin serves as a reactive oxygen radical scavenger that contributes to the survival of *S. aureus* during stress and host response. Since staphyloxanthin is membrane-bound, it primarily protects the membrane lipid against reactive oxygen radical threat and also protects the protein and DNA from free radicals [21]. By inhibiting staphyloxanthin biosynthesis,

L. plantarum USM8613 could compromise the survival of *S. aureus* under stress conditions.

Another proposed mechanisms involved the inhibition of quorum sensing, namely, the *agr* system and expression of the *ica* operon. The *ica* operon encodes for the production of polysaccharide intercellular adhesion, which is crucial for biofilm formation [22]. The upregulation of *plnJ* and *plnK* genes indicates the ability of *L. plantarum* USM8613 to produce the two-peptide plantaricin JK, which exerts activity via complementary actions of two different peptides and facilitates initial contact with the negatively charged membrane of *S. aureus* via electrostatic interaction [23]. Plantaricin JK permeabilises the inner membrane, leading to influx and efflux of various molecules across the transmembrane barrier, and altering electrical potential and pH gradients across the membrane, leading to cell death [24].

In addition to inhibition of the growth and pathogenicity of *S. aureus*, *L. plantarum* USM8613 also improved wound healing properties. Wound healing is a complex biological process that consists of four phases: the formation of blood clots, inflammation, proliferation and maturation. Blood clots are formed to seal and protect against further fluid loss and potential infections, while inflammation at wound sites in the early stages of infection triggers the wound healing process and blocks the invasion of pathogens [25]. The protein-rich fraction from *L. plantarum* USM8613 stimulated the production of β-defensin at wound sites. β-defensin, which is a group of small (3–5 kDa), cationic, cysteine-rich β-sheet peptides, plays an important role in cutaneous host immune defences. It confers a broad spectrum of antibacterial properties by disrupting membrane integrity leading to pathogenic cell lysis [26].
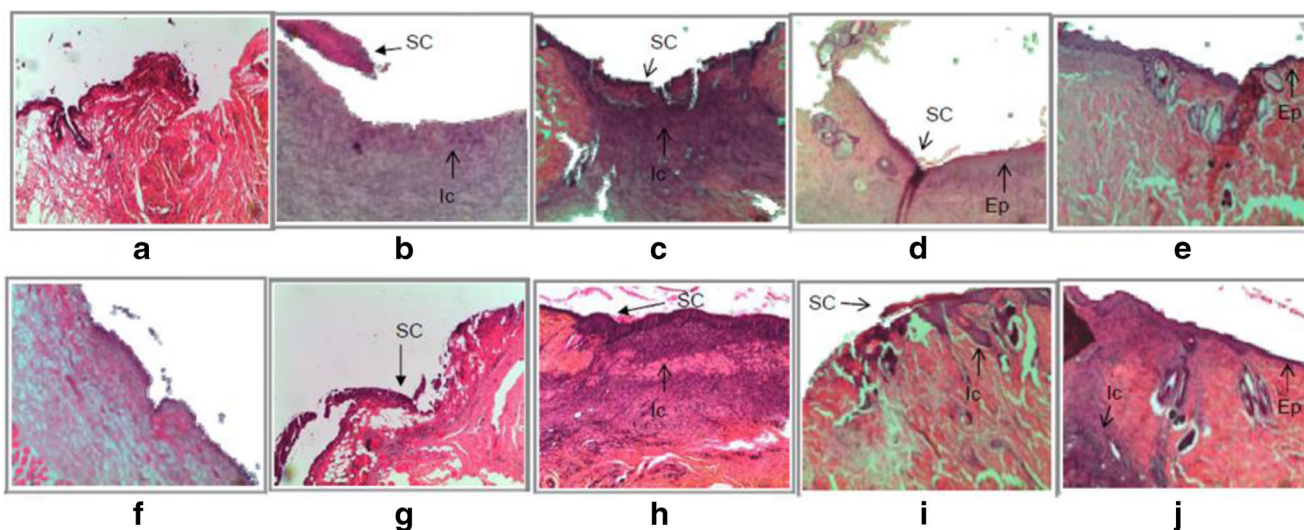
Fig. 5 Haematoxylin and eosin (H&E)-stained sections of the granulation tissue at wound sites from rats treated with 500 mg of yellow soft paraffin containing 50 μL of the protein-rich fraction from CFS of *L. plantarum* USM8613 or empty paraffin (control), applied twice daily over 16 days. *L. plantarum* USM8613-treated rats at (**a**) day 1, (**b**) day 4, (**c**) day 8, (**d**) day 12 and (**e**) day 16, and control rats at (**f**) day 1, (**g**) day 4, (**h**) day 8, (**i**) day 12 and (**j**) day 16. Magnification: × 400; Scab (SC), re-epithelialisation (Ep) and inflammatory cells (Ic)
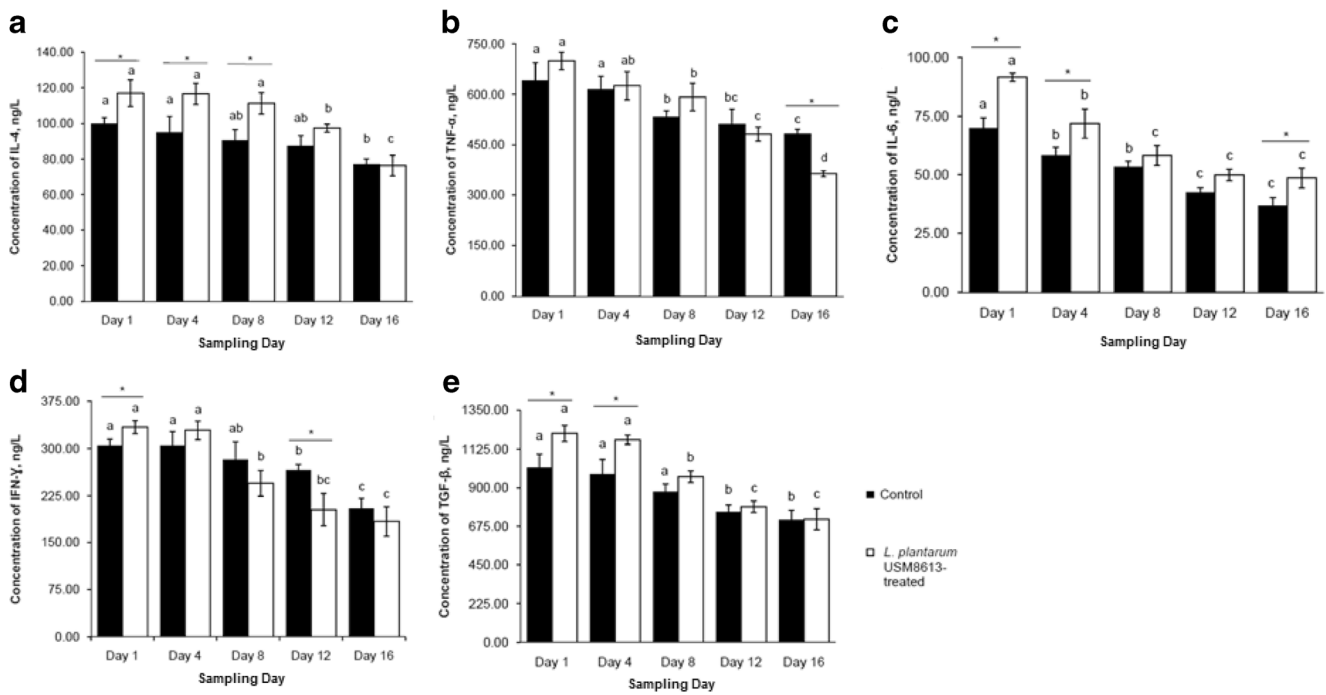
Fig. 6 Production of various cytokines, (**A**) interleukin (IL)-4, (**B**) tumour necrosis factor-α (TNF-α), (**C**) IL-6, (**D**) interferon-γ (IFN-γ) and (**E**) transforming growth factor-β (TGF-β) at wound sites of infected rats treated with 500 mg of yellow soft paraffin containing 50 μL of the protein-rich fraction from CFS of *L. plantarum* USM8613 or empty paraffin (control), applied twice daily over 16 days. Results are expressed as mean ± standard deviation of means ($n = 18$). *Means between treatment groups with the same sampling day were significantly different ($ρ < 0.05$); [a, b, c]Means between sampling days within the same group were significantly different ($ρ < 0.05$)

In addition to β-defensin, *L. plantarum* USM8613-treated rats also showed peak production of TNF-α, IL-4 and IL-6 during the early stages of wound healing, followed by a gradual decrease of TNF-α towards the end of the study. As a pro-inflammatory cytokine, TNF-α initiates the pro-inflammatory cascade and induces IL-1β synthesis to start the inflammatory phase. TNF-α gradually decreases in the latter stages of wound healing to prevent overexpression, which causes a delay in the wound healing process [27]. Meanwhile, IL-4 aids in the recruitment of mast cells and other inflammatory cells to wound sites [25], and IL-6 is expressed in the early stages of wound healing to influence the accumulation of neutrophils [27]. TH1 cells, NK cells and macrophages often produce IFN-γ, an immunomodulator that activates cytotoxic activity to kill foreign invaders [25]. In both groups, the production of IFN-γ was elevated in the initial inflammatory phase to aid against *S. aureus* infection. The levels of IFN-γ gradually decreased over time upon the clearance of *S. aureus* infections in the later stages, as overproduction of IFN-γ causes negative feedback and interrupts the wound healing process [28].
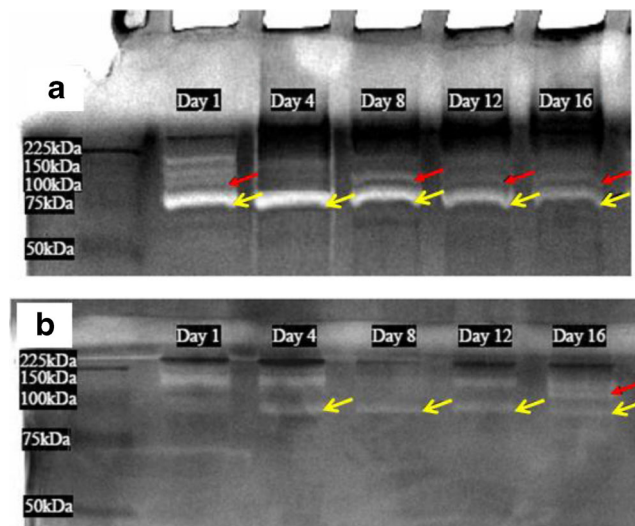
During the proliferation phase, the number of macrophages decreases at wound sites, while migration and proliferation of keratinocytes, fibroblasts and capillary endothelial cells results in re-epithelialisation and tissue granulation in close context with neovascularisation [28]. Several growth factors such as TGF-β and MMPs are secreted to stimulate proliferation, synthesis of extracellular matrix proteins and angiogenesis. TGF-β is a key regulator for the proliferation of keratinocytes at wound edges for re-epithelialisation by increasing collagen synthesis [25]. TGF-β transformed wound clots produced by activated fibroblasts into myofibroblasts, generating a strong contractile force for wound closure [25]. The levels of TGF-β were higher in *L. plantarum* USM8613-treated rats in the
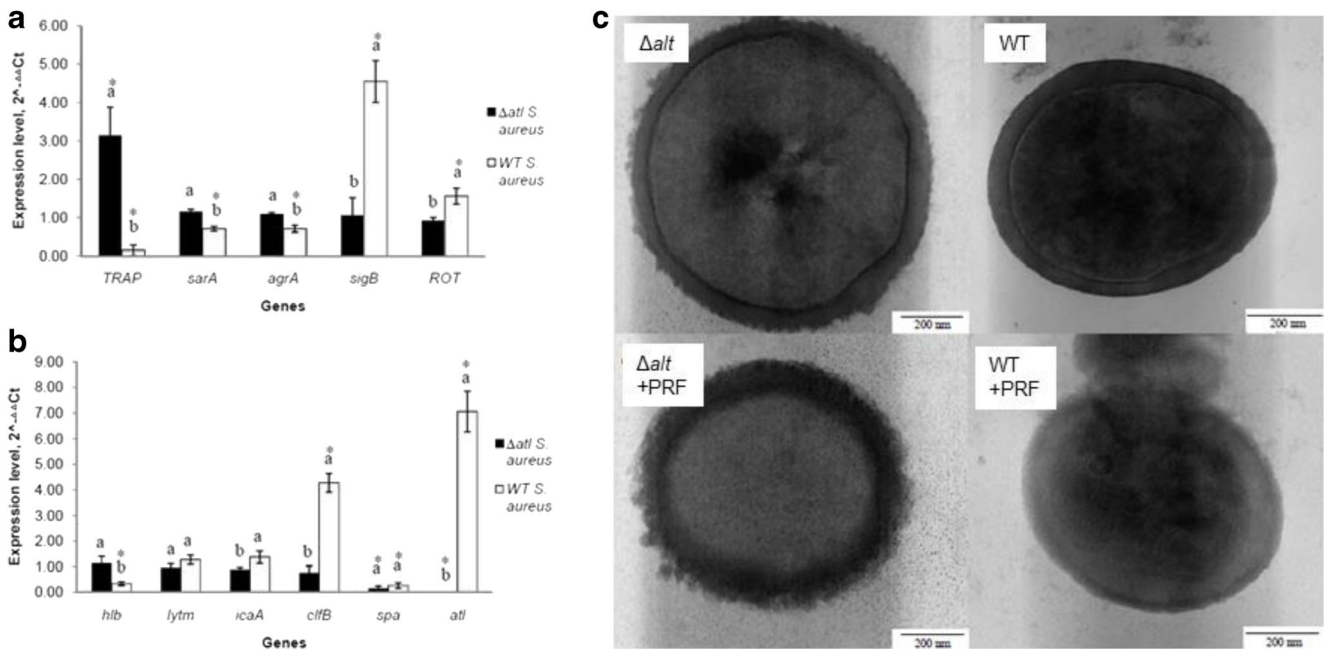

Fig. 7 Matrix metalloproteinases (MMPs) zymogram at wound sites of infected rats treated with 500 mg of yellow soft paraffin containing 50 μL of the protein-rich fraction from CFS of *L. plantarum* USM8613 (**A**) or empty paraffin (control; **B**), applied twice daily over 16 days. (Red arrows) MMP-9, 92 kDa; (yellow arrows) MMP-8, 75 kDa

Fig. 8 Gene expression of (**A**) global regulators and (**B**) pathogenicity factors in Δatl and WT S. aureus upon treatment with the protein-rich fraction from CFS of L. plantarum USM8613. C) TEM images of Δatl and WT S. aureus cells that were untreated (top images) and upon treatment with the protein-rich fraction from CFS of L. plantarum USM8613 until the mid-exponential phase (5 h; bottom images).

Magnification: 25,000X. Results are expressed as mean ± standard deviation of means (n = 3). [a, b]Means between Δatl and WT S. aureus within the same gene were significantly different (ρ < 0.05). *Means between treated-S. aureus and the untreated control (expression level = 1.00) were significantly different (ρ < 0.05)

early stages of wound recovery than in the control rats, indicating a faster wound healing effect. During the maturation phases, epidermal migration ceases and a new stratified epidermis with underlying basal lamina is re-established from the outer margins of the wound inwards. A reduction in MMP-9

levels during the late stages of wound recovery as observed in L. plantarum USM8613-treated rats is crucial, as prolonged and excessive production of MMP-9 and TNF-α would result in negative feedback, leading to a loss of keratinocytes and disordered wound healing [29]. The overall antimicrobial and
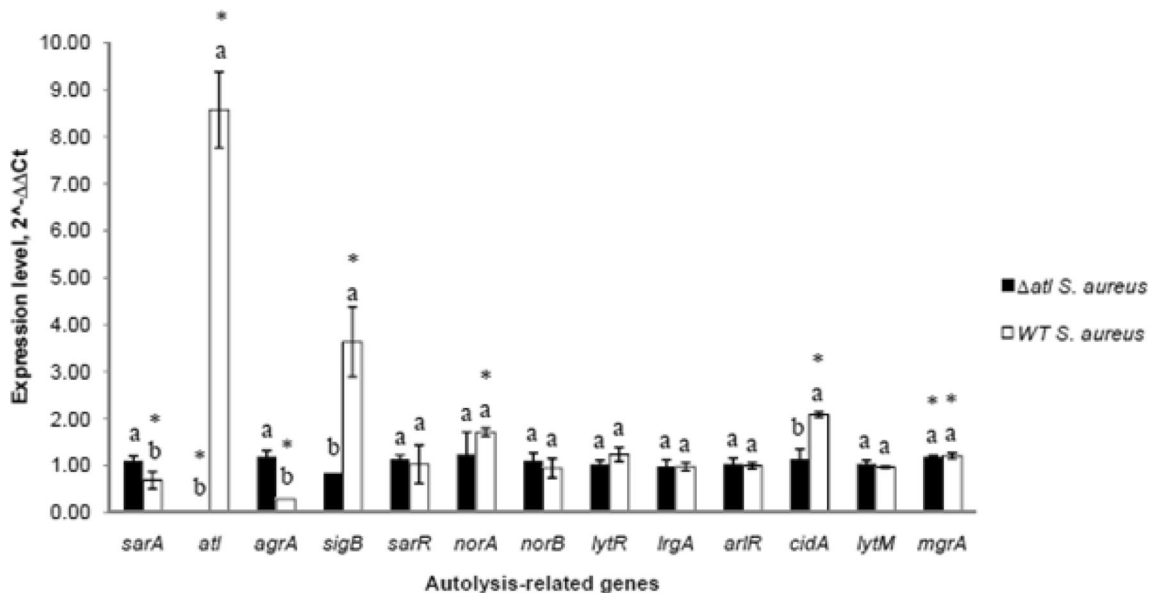

Fig. 9 Expression of autolysis-related genes in Δatl and WT S. aureus upon treatment with the protein-rich fraction from CFS of L. plantarum USM8613. Results are expressed as mean ± standard deviation of means (n = 3). [ab]Means between Δatl and WT S. aureus within the same gene

were significantly different (ρ < 0.05). * Means between treated S. aureus and the untreated control (expression level = 1.00) were significantly different (ρ < 0.05)
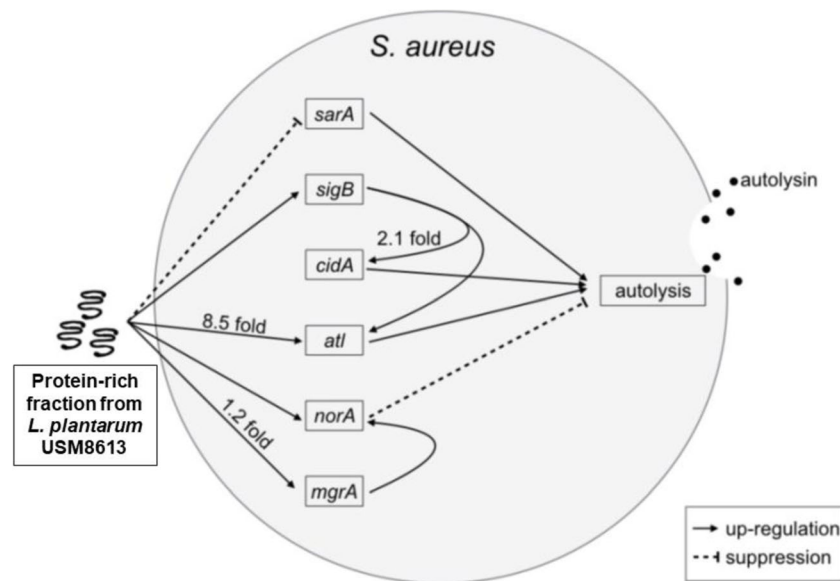
Fig. 10 Overall effect of the protein-rich fraction from *L. plantarum* USM8613 on the autolysis pathways of *S. aureus*. Upon treatment, the global regulator, *sarA* was suppressed, leading to activation of the autolysis process and cell death in *S. aureus*. Induction of stress (*sigB* gene) by the protein-rich fraction led to the expression of the *cidA* gene that eventually caused autolysis in *S. aureus*. Expression of the major autolysin *atl* gene was upregulated by 8.5-fold upon protein-rich fraction treatment, contributing to major cell death in *S. aureus* via autolysis. Expression of the global regulator *mgrA* gene and the efflux system *norA* gene was upregulated by 1.2- and 2.4-fold, respectively, to counteract the protein-rich fraction-induced autolysis in *S. aureus*, but it was insufficient to overcome cell death through autolysis as the *atl* gene was upregulated by 8.5 fold

wound healing-promoting activity of the protein fraction from *L. plantarum* USM8613 in *S. aureus*-infected rats is summarised in Fig. S1.

During cell division of *S. aureus*, the *atl* gene product appears as a ring structure on the septal region of the cell surface for cell separation. A proper balance between cell wall synthesis and degradation by autolysin during bacteria growth is needed to prevent autolysis of the cell due to overproduction of autolysin [30]. Treatment with a lytic concentration of penicillin reportedly induced autolysis of *S. aureus* cells while penicillin-induced autolysis was eliminated upon treatment with antisera against the *atl* gene product [31], illustrating the physiological involvement of the *atl* gene in autolysis. Hence, the role of the *atl* gene during autolysis, as induced by the protein-rich fraction from *L. plantarum* USM8613, was investigated using an *atl* null mutant strain of *S. aureus*.

Only the *TRAP* gene showed upregulated expression in Δ*atl S. aureus*. The same phenomena were observed in the pathogenicity factors of Δ*atl S. aureus*, in which gene expression of *hlb*, *clf*, *icaA*, and *lytM* were unaffected by the protein-rich fraction from *L. plantarum* USM8613. The results obtained indicated a directional relationship between the regulatory and downstream effector genes, in which the unaffected gene expression of *sigB* and *agrA* in Δ*atl S. aureus* enables their effector pathogenicity factors to express normally. Meanwhile, expression of the pathogenicity factor *spa* gene, which encodes for protein A, was suppressed after treatment

with the protein-rich fraction from *L. plantarum* USM8613, resulting in reduced pathogenesis in Δ*atl S. aureus*.

After treatment with the protein-rich fraction from *L. plantarum* USM8613, most of the autolysis-related genes (except *mgarA*) in both Δ*atl* and WT *S. aureus* were expressed normally and not affected by the protein-rich fraction from *L. plantarum* USM8613. The *mgrA* gene played an important role in suppressing the expression of autolysins to maintain the balance between cell wall synthesis and lysis for cell survival [32]. The *norA* and *norB* efflux pump system, which is regulated by *mgrA*, is another defence mechanism of *S. aureus* that enables cell adaptation to various environmental stresses [33]. In the current study, expression of both *norA* and *norB* of Δ*atl S. aureus* was unaffected, while expression of *norA* in WT *S. aureus* was upregulated upon treatment with the protein-rich fraction from *L. plantarum* USM8613. This indicated that upregulation of the *mgrA* gene was insufficient to upregulate expression of the *norA* and *norB* genes in Δ*atl S. aureus* upon treatment with the protein-rich fraction of *L. plantarum* USM8613.

Under the stresses of the stationary phase, environmental and host signals, *sar* proteins alter the expression and transcription of the *agr* system, virulence determinants and autolysis in *S. aureus* cells [34]. Downregulation of *sarA* expression in WT *S. aureus* indicated the ability of the protein-rich fraction from *L. plantarum* USM8613 to affect major regulators and effector genes in WT *S. aureus* towards autolysis, whereas gene expression of *sarA* in Δ*atl S. aureus* was

unaffected by the protein-rich fraction from *L. plantarum* USM8613, enabling all of the autolysis-related genes to express normally. The *atl* gene also directly affected the high expression levels of the *sigB* gene in WT *S. aureus*. In addition to the major autolysin *atl* gene, the *cidABC* operon has reportedly also enhanced murein hydrolase activity and eventually cell death [35]. The expression of the *cidABC* operon is dependent on the stress regulator, *sigB* [36]. This indicates that the high expression level of the *cidA* gene in WT *S. aureus* was attributed to upregulation of the *sigB* gene upon treatment with the protein-rich fraction from *L. plantarum* USM8613, while such a phenomenon was absent in Δ*atl S. aureus*. The overall effect of the protein-rich fraction from *L. plantarum* USM8613 on the autolysis pathways of *S. aureus* is summarised in Fig. 10.

Conclusions

The protein-rich fraction from *L. plantarum* USM8613 exerted wound healing properties via inhibition of opportunistic *S. aureus* and promotion of innate immunity via stimulated expression of β-defensin at wound sites. The protein fraction from *L. plantarum* USM8613 also enhanced the production of cytokines and chemokines (TNF-α, IFN-γ, IL-4, IL-6, TGF-β and MMPs) through various stages of wound recovery starting from inflammation to re-epithelialisation, resulting in faster wound recovery. The protein-rich fraction from *L. plantarum* USM8613 also reduced the survival and resistance of *S. aureus* via autolysis pathways. The results from this study suggest the possible use of *L. plantarum* as a natural alternative for treatment of *S. aureus* dermal infections and wound healing amid concerns of antibiotic resistance.

Compliance with Ethical Standards

The experimental protocol for treating animals was approved and in accordance with the guidelines recommended by the USM Committee on Animal Care; reference number: USM/Animal Ethics Approval/2012/ (81)(433).

Competing Interests The authors declare that they have no competing interests.

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