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ORIGINAL ARTICLE

# Activity of crude and fractionated extracts by lactic acid bacteria (LAB) isolated from local dairy, meat, and fermented products against *Staphylococcus aureus*

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**Abstract** This study aimed to evaluate anti-staphylococcal properties of crude and fractionated extracts of lactic acid bacteria (LAB) isolated from local meat, dairy, and fermented products. A total of 36 LAB isolates were obtained and identified via 16S rDNA sequencing. Cell-free supernatant (CFS) of all isolates exhibiting a statistically significant inhibition against *Staphylococcus aureus* ($\rho$<0.05), with six LAB isolates exhibiting a more prevalent inhibition. The inhibition effects of cell wall and intracellular extracts from the six prevalent isolates were evaluated. *Lactobacillus plantarum* USM8613 was the most prominent isolate with both CFS and cell wall extract exhibiting the most prevalent inhibition against *S. aureus*. Scanning electron micrographs showed alteration of *S. aureus* membrane morphology upon CFS treatment, suggesting an anti-staphylococcal effect via membrane destruction. Confocal laser scanning micrographs showed inhibition against biofilm formations by *S. aureus* in porcine skins upon CFS treatment. The CFS from *L. plantarum* USM8613 was separated into protein, lipid, and polysaccharide fractions for evaluation of anti-staphylococcal activity and chemical characterization. All fractions inhibited growth of *S. aureus* ($\rho$<0.05), with protein fractions exhibiting stronger inhibition effect. Data from our present study showed that extracts from LAB could be applied as biopreservatives in the food industries and/or as an antimicrobial agent against bacterial infections for cosmeceutical and pharmaceutical uses.

**Keywords** Lactic acid bacteria · *Lactobacillus* · *Staphylococcus aureus* · Antimicrobials

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## Introduction

Lactic acid bacteria (LAB) consist of Gram-positive bacteria that ferment carbohydrates into lactic acid and energy, and the most commonly recognized genera are *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, and *Pediococcus* (Jay 2000; Holzapfel et al. 2001). They are widely distributed and may appear as indigenous microflora in raw meats, dairy products, beverages, and bakery products (Jay 2000; O'Sullivan et al. 2002). Although LAB have exhibiting various health properties to human, they also play an important role in food industries as they have been applied in many foods stuffs either as starters or as preservatives (Caplice and Fitzgerald 1999).

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Meanwhile, *Staphylococcus aureus* is a transient and opportunist pathogen of humans and various animals. *S. aureus* could also present in various foods and is easily transmitted via skin-fomite contact (Tang et al. 2014). *S. aureus* is well armed with a variety of virulence factors such as surface proteins, enzymes, and various toxins, causing mild to severe infection from cutaneous to systemic infections. Despite these virulence weapons, the emergence of multi-resistant *S. aureus,* which shields it from the action of antibiotics, further complicates treatment and reduces therapeutic options (Foster 2005). Therefore, a natural antibacterial substance is required to substitute the use of synthetic antibiotics and overcome the threat from *S. aureus*.

LAB isolated from natural resources may have commercial purposes as they extend the shelf life of foods through the production of various antimicrobial substances such as short chain fatty acids, diacetyl, hydrogen peroxide, bacteriocin and bacteriocin-like compounds, and enzymes that liberate antimicrobial-bioactive peptides from existing protein sources (Liong and Shah 2005; Tan et al. 2013). Previous study showed a proline-rich antimicrobial peptide liberated by proteinases of *Lactobacillus helveticus* PR4 against caseins from buffalo milk possessed inhibitory activity towards *S. aureus* (Minervini et al. 2003). LAB also produce exopolysaccharide (EPS) that has been reported to be beneficial in modulating lactose intolerance, enhancing immunity, reducing mutagenic enzymes, and has anti-tumor and blood cholesterol lowering properties (de Roos and Katan 2000; Vinderola et al. 2006; Patel et al. 2012). Recently, EPS produced by bifidobacterium has been shown to exhibit antimicrobial activities against several types of food spoilage and pathogenic bacteria (Wu et al. 2010). However, the direct antimicrobial effect of EPS, typically those produced by LAB, against pathogenic bacteria such as *S. aureus* has yet to be discovered. Some microbicidal fatty acids have also been reported to exert protection against skin and mucosal infections by inhibiting against a broad range of microorganisms, ranging from bacteria such as *S. aureus* (more susceptible against Gram-positive than Gram-negative bacteria), fungi such as yeast, to viruses such as herpes simplex virus and parainfluenza virus (Osk Thorgeirsdottir et al. 2006; Hilmarsson et al. 2007; Thormar and Hilmarsson 2007). Unsaturated fatty acids such as palmitoleic acid (C16:1) from LAB has been shown to exert antibacterial activity against Gram-positive bacteria such as *S. aureus* (Kristmundsdóttir and Skulason 2011).

Despite vast reports attributing the inhibiting effects of LAB against *S. aureus* to the metabolic end products of LAB, to our knowledge, little information is available on the fractionated extracts of LAB. Therefore, the present study aimed to isolate LAB from local meats, dairy, and fermented products, followed by identification via 16S rDNA sequencing. In addition, the antimicrobial properties of cell-free supernatant (CFS), intracellular extracts, and cell wall components of LAB were also evaluated. CFS was fractionated into protein, polysaccharide, and lipid fractions for antimicrobial evaluations and chemical characterization.

## Materials and methods

### Isolation of LAB

Dairy products, meat products, and fermented products were purchased from markets in Penang, Malaysia. These food samples were homogenized, serially diluted using peptone water (Merck, Germany) and enumerated via the pour plate method at 37 °C using De Man-Rogosa-Sharpe (MRS) agar (Biomark$^{TM}$, India) supplemented with 3 % (v/v) of L-cysteine hydrochloride. Single colonies were isolated prior to Gram staining. Gram-positive rods or cocci with chain arrangement were selected for further identification. The selected isolates were stored in 40 % (v/v) glycerol (QReC, Malaysia) at −20 °C while the working cultures were maintained on MRS agar at 4 °C. Cultures were activated three successive times prior to use.

### Identification of LAB

Sequencing of 16S rDNA was performed to identify the selected isolates. DNA extraction was performed using the Quick Genomic DNA Extraction kit (DongSheng Biotech, China). PCR amplification of 16S rDNA was performed using the universal 16S rDNA primer, with the forward sequence 5′-GCTGGCGGCATGCTTAACACAT-3′ and reverse primer sequence 5′-GGAGGTGATCCAGCCGCAGGT-3′, in a thermal cycler (Bio-Rad, USA) (Ruiz et al. 2000). The program for amplification consisted of (i) denaturation at 95 °C for 5 min, (ii) 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 1 min, extension at 72 °C for 2 min, (iii) final extension at 72 °C for 4 min, and finally maintained at 4 °C until further use. The PCR amplicons were subjected to agarose gel electrophoresis at 100 V for 60 min. The gel was visualized using the GeneGenius Imaging System (Syngene, UK). PCR products were purified using PCR and DNA Fragment Purification Kit (DongSheng Biotech, China) and sequencing was performed by Center for Chemical Biology (Universiti Sains Malaysia, Malaysia). The nucleotide sequences of the isolates were analyzed using the BLAST program from NCBI (http://www.ncbi.nlm.nih.gov).

### Antimicrobial assay

Activated LAB cultures were diluted ($OD_{600nm}$=1.0) with MRS broth (Biomark$^{TM}$, India) prior to centrifugation at 3,500 × *g* for 15 min at 4 °C to obtain cell-free supernatant (CFS). A clinical isolate of *Staphylococcus aureus* (General

Hospital, Penang, Malaysia) was selected as the target pathogen, and was maintained in Trypticase Soy Broth (TSB; Himedia, India) at 37 °C for 24 h prior to use. *S. aureus* culture was diluted with TSB ($OD_{600nm}$=0.3; $10^6$ CFU $mL^{-1}$). CFS was dispensed into each well of a 96-well microplate (100 μL) followed by addition of the diluted *S. aureus* culture (100 μL). Control contained diluted *S. aureus* culture and unfermented MRS broth. Microplates were incubated at 37 °C and inhibition was measured at time intervals of 2 h until 20 h using the HALO MPR-96 Microplate Reader (Dynamica, Switzerland) (Lash et al. 2005).

LAB isolates that exhibiting a statistically stronger inhibition against *S. aureus* were selected to evaluate the antimicrobial activity of their cell wall and intracellular extracts. Prior to antimicrobial assay, overnight cultures of LAB were standardized to $OD_{600nm}$=1.0. Cell pellets from the standardized cultures were collected and washed thrice with phosphate buffered saline (PBS, 10 mM, pH 7.4), re-suspended in PBS and sonicated for 30 min in an ice bath, followed by centrifugation at 3,500 × *g* for 15 min at 4 °C. Intracellular extracts were obtained from the supernatant while cell wall fraction was obtained from the washed cell pellet. The microtiter plate assay as described above was used to determine the antimicrobial activity of each extract against *S. aureus*. The overall growth of *S. aureus* was calculated as:

$$\%\text{ of Growth} = \left(\text{Growth } OD_{600nm} \text{of samples} \big/ \text{Growth } OD_{600nm} \text{of control}\right) \times 100$$

Unfermented MRS broth was used as control. LAB that exhibits a statistically stronger inhibition against *S. aureus* was selected for subsequent analyses.

### Scanning electron microscopy

Morphology changes of *S. aureus* upon treatment with CFS produced by the selected LAB with stronger inhibitive effect for 20 h at 37 °C were observed using a scanning electron microscope (SEM). Untreated cells of *S.* aureus were used as control. The cell pellet was obtained via centrifugation, followed by fixation with McDowell Trump fixative (4 % formaldehyde, Sigma-Aldrich; 1 % glutaraldehyde, Sigma-Aldrich) and 1 % (w/v) osmium tetraoxide (Sigma-Aldrich). The pellet was subsequently dehydrated using a series of ethanol and hexamethyldisilazane (Supelco, Bellefonte, PA, USA). The dried cells were then mounted on a SEM specimen stub, coated with gold in a sputter coater (Polaran, Walford, UK), and examined under SEM (Leo Supra, Oberkochen, Germany).

### Ex vivo examination

Porcine skin samples were collected from the dorsal part of domestic farm pig (60 kg) and cut into 4 $cm^2$ sized pieces. The body hair was removed using scalpel and sterilized using 70 % isopropanol. These 4 $cm^2$ skin samples were subjected to different treatments: a) a positive control group, where porcine skin was injected with 200 μL of *S. aureus* ($10^6$ CFU $mL^{-1}$) together with blank MRS broth and incubated at 37 °C for 24 h; b) the treatment group, where porcine skin was injected with 200 μL of *S. aureus* ($10^6$ CFU $mL^{-1}$) and incubated at 37 °C for 24 h, followed by treatment with 200 μL of CFS of the selected LAB with stronger inhibitive effect and further incubation at 37 °C for 24 h; and c) the prevention group, where porcine skin was injected with 200 μL of *S. aureus* ($10^6$ CFU $mL^{-1}$) and 200 μL of CFS prior to incubation at 37 °C for 24 h.

Upon incubation, all skin samples were fixed with 10 % formalin and dehydrated with an ascending series of ethanol (30 % to 100 %). The dehydrated skin samples were re-wetted via soaking in sterilized distilled water prior to stain with 1 % acridine orange dye (AO) (Sigma-Aldrich, Steinheim, Germany) for 15 min, followed by washing with PBS (10 mM, pH 7.4) for 30 min, 1 % 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) (Sigma-Aldrich, Steinheim, Germany) for 30 min, and PBS again for 30 min. Skin samples were subsequently examined using a confocal scanning laser microscope (Eclipse-Ti, Nikon, NY, USA).

### Fractionation of CFS and antimicrobial activity

CFS of the selected LAB with stronger inhibitive effect was adjusted to pH 7.0 using 1.0 M sodium hydroxide (NaOH) prior to separation into protein, polysaccharide, and lipid fractions. Extraction methods have been optimized and validated prior to use (data not shown).

In protein fractionation, solid ammonium sulfate was added slowly up to 80 % saturation while stirring at 4 °C for 18 h. The mixture was centrifuged at 8,000 × *g* for 10 min at 4 °C and the pellet was suspended with ammonium acetate buffer pH 6.5 in a ratio of 1:1 (v/w). The solution was then filtered and protein content determined via the Bradford assay (Bradford 1976).

Prior to polysaccharide fractionation, CFS was added with ethanol (99.5 %) at a ratio of 1:3 (v/v) and left to stand overnight at 4 °C. The mixture was then centrifuged at 7,000 × *g* for 20 min at 4 °C, followed by washing with deionised water:ethanol at a ratio of 1:3 (v/v). The crude polysaccharide pellet was collected and re-suspended in sterile deionised water in ratio 1:1 (w/v) and quantified via the phenol-sulphuric assay (Dubois et al. 1956).

The lipid fraction was extracted using the method of Bligh and Dyer (1959). Briefly, CFS was mixed with chloroform:methanol (1:2, v/v) at a ratio of (1:3; v/v) and vortexed. Chloroform (99.5 %) was added to the mixture at a ratio of 1:0.4 (v/v) followed by distilled water at a ratio of 1:0.4 (v/v). The mixture was centrifuged at 1,000 × *g* for 5 min

at 4 °C. The chloroform layer containing the lipid fraction was collected and concentrated under nitrogen flux and quantified via the phospho-vanilin assay (Knight et al. 1972).

Unfermented MRS broth fractionated in the similar manner for all fractions was used as control. All fractions were standardized in concentration and used for antimicrobial analyses using the microtiter plate assay as describe above.

### Characterization of crude protein, lipid, and polysaccharide fractions of CFS

The protein fraction was freeze-dried prior to amino acid composition analysis using an amino acid mixture as standard (Sigma-Aldrich, Steinheim, Germany). Briefly, protein hydrolysates were derived with phenyl isothiocyanate (PITC) and separated by RP-HPLC on a Superiodex ODS column (5 μm, 4.61 x 50 mm, Shiseido, Tokyo) with a Hitachi L-6200 Intelligent Pump (Hitachi, Tokyo) and a JASCO UV detector Model Uvidec-100-VI (Japan Spectroscopic, Tokyo). The column was equilibrated with solvent A (94 % 0.14 M sodium acetate buffer, pH 5.4) and elution was performed using a gradient from solvent A to B (40–60 % acetonitrile in water). The column was maintained at 38 °C, the flow rate was 1.0 mL $min^{-1}$, and the derivatives were detected at 254 nm (Bidlingmeyer et al. 1984).

The extracted lipid fraction was esterified to fatty acid methylesters (FAMEs) through sodium methoxide catalysis according to the method of Christie (1989). Briefly, FAME was determined by gas chromatography-mass spectrophotometer, using a Shimadzu GCMS-QP2010 Ultra (Shimadzu Corp., Kyoto, Japan) equipped with a BPX-70 capillary column (60 m x 0.25 mm, i.d., 0.25 μm film; SGE, Ringwood, Australia). The initial oven temperature (50 °C) was increased to 160 °C at a rate of 4 °C $min^{-1}$ (held for 10 min), to 200 °C at rate of 1 °C $min^{-1}$ (held for 2 min), and finally to 210 °C at a rate of 10 °C $min^{-1}$ (held for 10 min). Helium gas was used as the carrier at a flow rate of 0.8 mL $min^{-1}$ with split ratio of 1:50. The injection volume was 10 μL at an injector temperature of 210 °C. The mass spectra were recorded at ionization energy of 0.97 kV with an ion source temperature of 200 °C. The mass scan was performed at a range of 29 m $z^{-1}$ to 550 m $z^{-1}$ with a scan speed of 10,000. Supelco® 37 Component FAME Mix (Sigma-Aldrich, Steinheim, Germany) was used as a standard.

The polysaccharide fraction was hydrolyzed to monosaccharides using 1 M $H_2SO_4$ at 80 °C for 2 h, prior to neutralization using 1 M NaOH. The monosaccharides were quantified using a high-performance liquid chromatography (HPLC) system, equipped with a refraction index (RI) detector (Shidmazu, Kyoto, Japan) and a Waters Sugar Pak-1 column (300 mm x 6.5 mm, 5 μm; Waters Corporation, Milford, MA, USA). Samples were eluted at a running temperature of 85 °C, in degassed sterile deionized water at a flow rate of 0.2 mL $min^{-1}$. HPLC grade mix monosaccharides (Sigma-Aldrich, Steinheim, Germany) were used as standards.

### Statistical analysis

Data were presented as mean±standard deviation from triplicate analyses and analyzed using SPSS© version 19.0 (IBM© SPSS© Statistics, USA). A repeated measure analysis of variance (ANOVA) was used for the time-based analyses. One-way ANOVA was used to determine the significant differences between means at a significance level of $\rho<0.05$. Turkey's test was used to perform multiple comparisons between means.

## Results

### Isolation and Identification of LAB

In this study, a total of 40 isolates were obtained from dairy products (13 isolates), meat products (16 isolates), and fermented products (11 isolates). Upon screening, 36 (90 %) isolates were Gram-positive and rod-shaped, while four isolates (10 %) were Gram-negative. All of the Gram-positive isolates were identified via 16S rDNA sequencing as *Lactobacillus fermentum* (17 isolates), *Lactobacillus plantarum* (six isolates), *Lactobacillus brevis* (six isolates), *Lactobacillus casei* (two isolates), *Lactobacillus paracasei* (two isolates), *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus garvieae,* and *Weissella confusa* (one isolate each). In this study, all of the isolates possessed 83–100 % similarity with coverage ranging from 96 to 100 % (NCBI database).

### Antimicrobial activity

CFS from all of the isolated LAB (from dairy, meat, and fermented products) exhibited statistically significant ($\rho<0.05$) antimicrobial effects against *S. aureus* compared to the control (Fig. 1a to c). LAB obtained from each of the isolation sources with a significantly stronger antimicrobial activity were selected for further evaluation. The selected isolates were *L. plantarum* USMT8313, *L. plantarum* USM8313, *L. plantarum* USM8613, *L. casei* USM1912, *L. plantarum* USM8913, and *L. fermentum* USM1415.

Cell wall extracts of the selected LAB isolates exhibited a statistically significant ($\rho<0.05$) antimicrobial effect against *S. aureus* compared to the control (Fig. 2), with a more prevalent effect from *L. plantarum* USM8613 ($\rho<0.05$) compared to the other isolates studied. Meanwhile, the intracellular extracts of LAB exhibited insignificant antimicrobial activity against *S. aureus* compared to the control (Fig. 2).

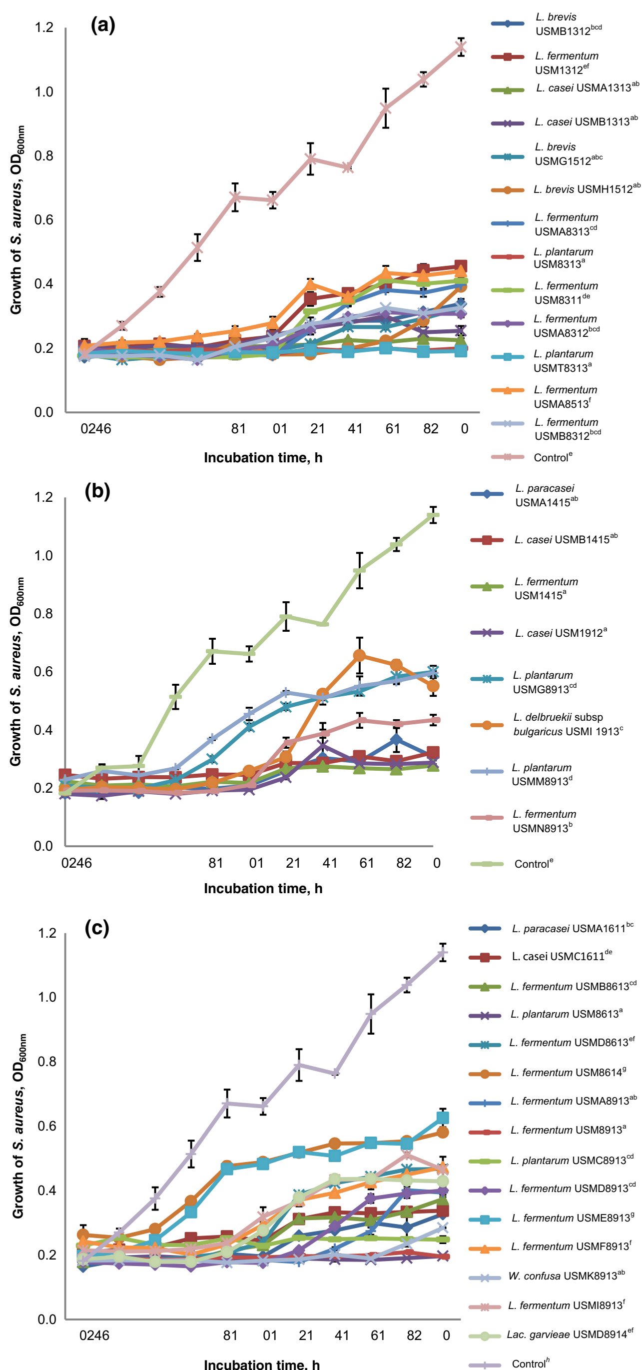


**Fig. 1** Antimicrobial activity of CFS of LAB isolated from (**a**) dairy products, (**b**) fermented products, and (**c**) meat products against *S. aureus*. Results are expressed as mean±standard deviation of mean ($n$=3). [abcdefgh] Mean values for antimicrobial activity of isolated LAB with different lowercase letters are significantly different ($\rho$<0.05). Control: unfermented MRS

## Scanning electron microscopy

Considering that CFS exhibits a stronger antimicrobial effect compared to cell wall and intracellular extracts ($\rho$<0.05) (Fig. 2), while *L. plantarum* USM8613 was the most prominent isolate with a higher inhibitory activity compared to the other isolates studied for both CFS and cell wall extracts ($\rho$<0.05) (Fig. 1a to c), the CFS of *L. plantarum* USM8613 was used for subsequent analyses.

SEM micrographs revealed that the cells of *S. aureus* treated with unfermented MRS broth showed regular and smooth surfaces (Fig. 3a). In contrast, cells of *S. aureus* treated with CFS of *L. plantarum* USM8613 exhibiting rough cell surfaces, depository of extracellular debris, with bulb-like appearance and ruptured cells (Fig. 3b).

## Ex vivo examination

Confocal image of uninfected porcine skin (Fig. 4a) revealed a clear surface with obvious basal layer, while the positive control porcine skin (Fig. 4b) exhibited a thick layer of biomass on the surface covering the basal layer of porcine skin. Meanwhile, although the formation of biomass could be observed on porcine skin from the prevention group (Fig. 4c), the depth of biomass was less compared to that from the positive control (Fig. 4b). The formation of biomass by *S. aureus* on porcine skin was thinner in the treatment group (Fig. 4d) as compared to the positive control and prevention groups, with an observable basal layer of the porcine skin. In addition, single colonies of *S. aureus* (represented by red spots) could be observed in both the prevention and treatment groups, but not in the control group.

## Antimicrobial activity of fractionated CFS

Considering that the CFS from *L. plantarum* USM8613 exhibited significant antimicrobial activities in in vitro, neutralized CFS, and ex vivo analyses, it was subsequently separated into protein, lipid, and polysaccharide fractions for further analysis. Prior to fractionation, CFS from *L. plantarum* USM8613 was neutralized (pH 7) in order to eliminate possible primary antimicrobial effects from actions of organic acids, and to evaluate possible antimicrobial effects from non-acid metabolites. Lactic acid (10.21±0.04 mg mL$^{-1}$) and acetic acids (3.41±0.01 mg mL$^{-1}$) were produced by *L. plantarum* USM8613, as quantified by HPLC. Upon fractionation, crude protein fraction of CFS from *L. plantarum* USM8613 marked a concentration of 92 μg mL$^{-1}$, polysaccharide fraction with 0.2 mg mL$^{-1}$ of total polysaccharide content and 0.3 μg mL$^{-1}$ of total lipid content in crude lipid fraction. All three fractions showed a statistically significant ($\rho$<0.05) antimicrobial effect against the growth of *S. aureus* compared to the control with higher inhibitory effect from crude protein fractions (Fig. 5).

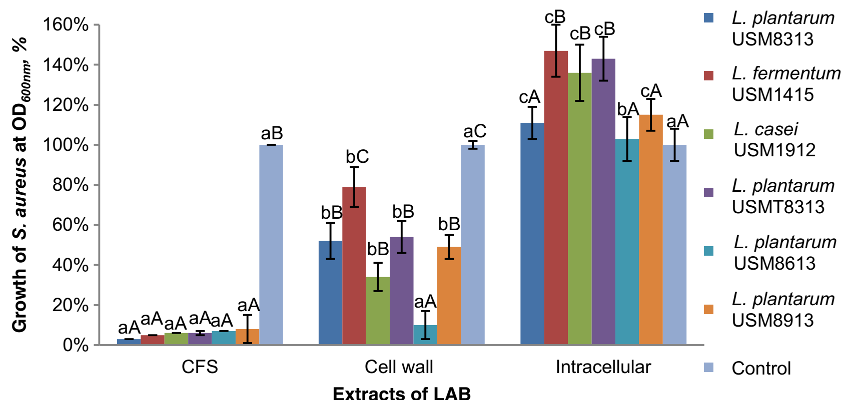


**Fig. 2** Antimicrobial activity of intracellular and cell wall extracts of LAB against *S. aureus*. Results are expressed as mean±standard deviation of mean ($n$=3). [abc] Means among different extracts within the same LAB with different lowercase letters are significantly different ($\rho$<0.05); [ABC] Means among different isolates of LAB within the same type of extract with different uppercase letters are significantly different ($\rho$<0.05). Control: unfermented MRS

Characterization of crude protein, lipid and polysaccharide fractions

The crude protein fraction of *L. plantarum* USM8613 contained a higher amount of glycine (22.65 %), followed by alanine (11.47 %) compared to the other amino acids detected (Fig. 6). A higher amount of glycine (0.98 %), arginine (0.78 %), glutamine (0.36 %), methionine (0.22 %), serine (0.21 %), asparagine (0.17 %), and lysine (0.08 %) were detected compared to the control. On the other hand, the concentrations of leucine (0.78 %), threonine (0.58 %), phenylalanine (0.56 %), isoleucine (0.45 %), valine (0.28 %), alanine (0.07 %), tyrosine (0.04 %), proline (0.03 %), and cysteine (0.01 %) were lower compared to the control.

FAME profiles (Table 1) showed that lipid fraction of CFS from *L. plantarum* USM8613 contained a significantly higher amount of unsaturated fatty acids (69.61 %) than saturated fatty acids (30.39 %) ($\rho$<0.05). Among all unsaturated fatty acids determined, oleic acid was most prevalent in the lipid fraction of *L. plantarum* USM8613, at 3.37 % higher than the control ($\rho$<0.05).

HPLC analysis of sugars showed that only glucose and mannose were present in the polysaccharides fraction of CFS from *L. plantarum* USM8613 (Table 2). The concentration of glucose and mannose in the polysaccharide fraction of *L. plantarum* USM8613 was lower than the control ($\rho$<0.05).

## Discussion

LAB are characterized as Gram-positive, non-sporing, microaerophilic bacteria which produce lactic acid as the main end product of the carbohydrate fermentation, and have been well-documented for exerting various health benefits (Kandler 1983). *Staphylococcus aureus* was chosen as a model pathogen due to its nature as a food-borne pathogen causing staphylococcal food-poisoning via the production of enterotoxin. *S. aureus* is also a transient pathogenic organism of the skin, and an opportunistic pathogen that expresses a vast variety of potential virulence factors such as surface proteins, exotoxins and enzymes, leading to a variety of cutaneous to systemic infections, including staphylococcal scalded skin syndrome, bacteraemia, toxic shock syndrome, and infections on medical implants and devices (Foster 2005).

In this study, CFS of the selected LAB isolates exhibited stronger antimicrobial activities than cell wall and intracellular

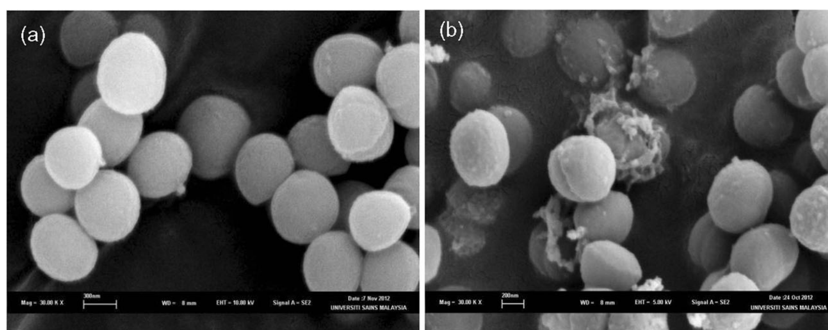


**Fig. 3** Scanning electron microscope image of *S. aureus* in the (**a**) control and (**b**) upon treatment with CFS of *L. plantarum* USM8613. *S. aureus* ($OD_{600nm}$=0.3; $10^6$ CFU $mL^{-1}$) was incubated with CFS of *L. plantarum* USM8613 at a ratio of 1:1 (v:v) at 37 °C for 20 h

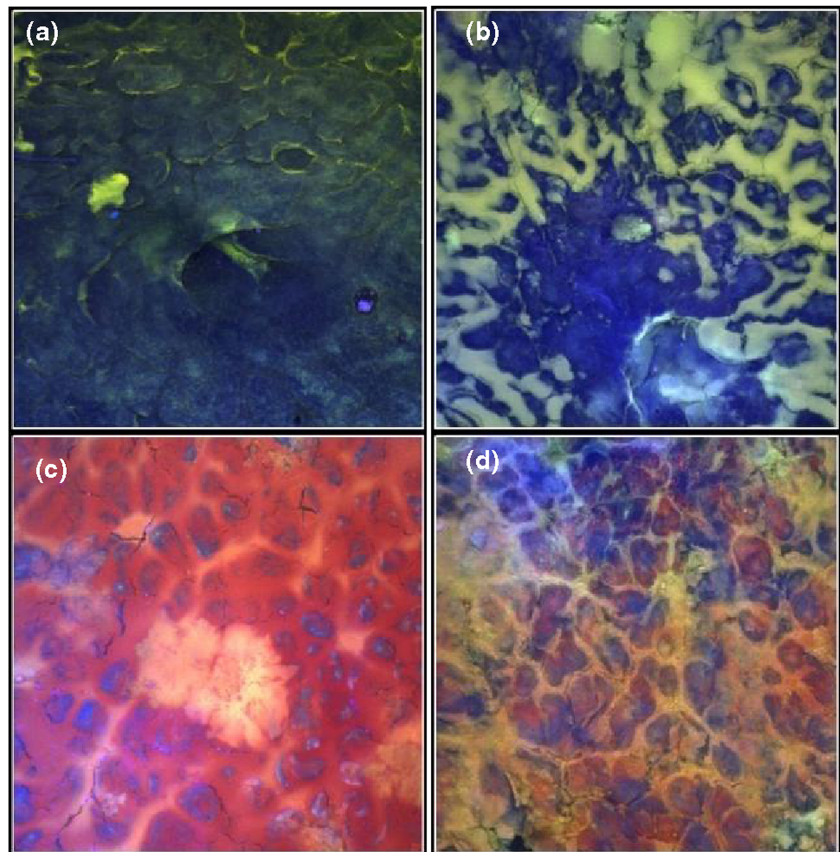


**Fig. 4** Confocal micrograph of (**a**) uninfected porcine skin, (**b**) positive control (porcine skin injected with 200 µL of *S. aureus* ($10^6$ CFU $mL^{-1}$) together with unfermented MRS, incubated at 37 °C for 24 h, (**c**) prevention group (porcine skin injected with 200 µL of *S. aureus* ($10^6$ CFU $mL^{-1}$) and 200 µL of CFS from *L. plantarum* USM8613 prior to incubation at 37 °C for 24 h, (**d**) treatment group (porcine skin injected with 200 µL of *S. aureus* ($10^6$ CFU $mL^{-1}$), incubated at 37 °C for 24 h prior to treatment with 200 µL of CFS from *L. plantarum* USM8613 followed by further incubation at 37 °C for 24 h. Porcine skin cells (blue) stained with DAPI were differentiated from *S. aureus* cells (red) and its product (green) which was stained using acridine orange dye. Magnification: 100x

extracts. This may have been due to the presence of antimicrobial substances such as lactic acid, hydrogen peroxide, bacteriocin, and bacteriocin-like compounds, that are mainly secreted out into the environment (Reid et al. 2004). In addition to CFS, cell wall extracts from selected isolates of LAB also inhibited the growth of *S. aureus*. This may be attributed to the various membrane fatty acids such as palmatic, stearic, plamitoleic, and oleic acids that are present in the cell wall extracts (Hofmann et al. 1957). *L. plantarum* USM8613 was the only isolate that possessed both CFS and cell wall extracts which exhibited stronger inhibition against *S. aureus*. Palmitic, stearic, oleic, and palmatitoleic acid have been reported to exert antimicrobial effects against various pathogenic bacteria including methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, and *Yersinia enterocolitica* (Bergsson et al. 2001; Altieri et al. 2009). However, intracellular extracts of LAB did not inhibit the growth of *S. aureus*. Fructose, glucose, proline, glutamate, and alanine are well

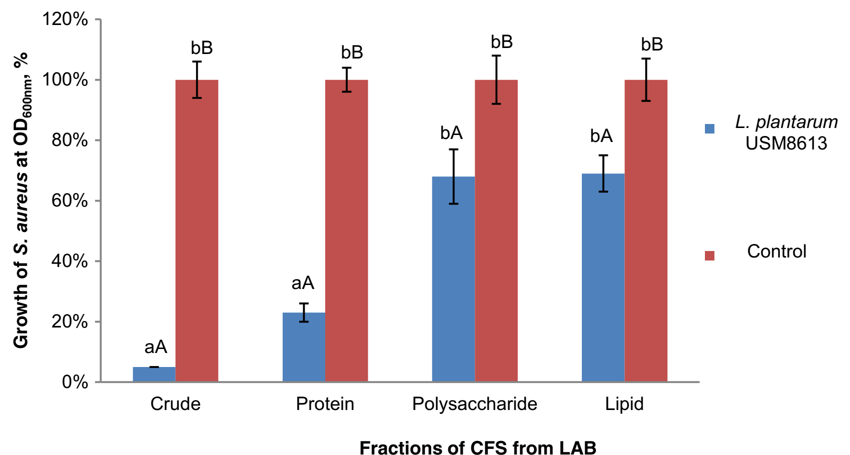


**Fig. 5** Antimicrobial activities of fractionated CFS from *L. plantarum* USM8613 against *S. aureus*. Results are expressed as mean±standard deviation of mean ($n$=3). [abcd] Means among different fractions with different lowercase letters are significantly different ($\rho$<0.05); [AB] Means among different samples within the same fraction, with different uppercase letters are signficantly different ($\rho$<0.05). Control: fractions obtained from unfermented MRS

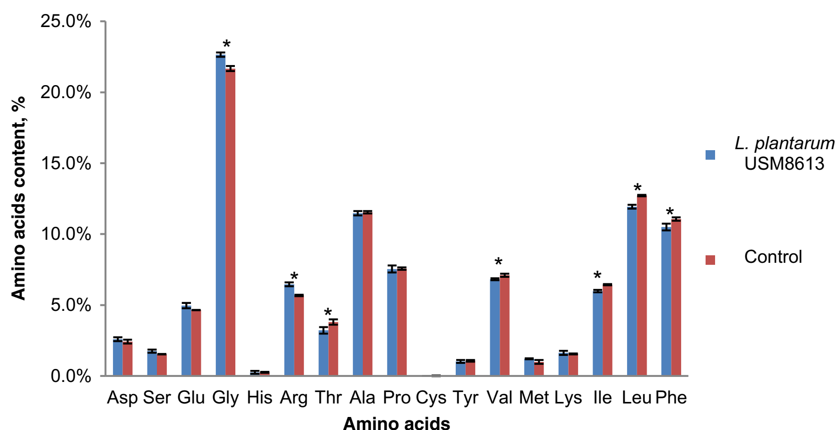


**Fig. 6** Amino acid composition of crude protein fraction from CFS of *L. plantarum* USM8613. Results are expressed as mean± standard deviation of mean ($n$=3). * Means between *L. plantarum* USM8613 and control are significantly different ($\rho$<0.05). Control: protein fraction of unfermented MRS

documented to be present intracellularly and could act as growth nutrients instead (Glaasker et al. 1996; Patel et al. 2012).

The cellular morphology of *S. aureus* demonstrated prevalent changes upon incubation with CFS of *L. plantarum* USM8613. Antimicrobial compounds from the CFS of *L. plantarum* USM8613 have caused pore formation on the cell membrane, resulting in leakage and rupture of the cells, and subsequently enhanced influx of extracellular matrix compounds intracellularly, leading to the swelling of cells. The morphology of rough surfaces accompanied by depository of extracellular debris, as in the treated cells, could also be attributed to the disruption of membrance integrity and/or loss of cellular electron-dense material on the surface, leading to lysis of the outer membrane (Klayraung and Okonogi 2009). Such bactericidal actions were also in accordance with CFS of *L. plantarum* DM5 and *L. fermentum* FTL 2311 and FTL 10BR against *S. aureus* (Klayraung and Okonogi 2009; Das and Goyal 2013). This microscopy study justified the bactericidal effects of CFS of *L. plantarum* USM 8613 was exhibited through membrane disruption.

Confocal laser scanning microcropy (CLSM) has been used in our present study, with benefits of direct evaluation of biological tissues at a cellular level without tissue processing. Biofilm formation is one of the common virulent factors expressed by *S. aureus*, and mainly comprises polysaccharides which were represented by green fluorescence on the surface of porcine skin upon non-specific binding with AO dye (Fig. 4). The DAPI dye stained nucleic acid of the porcine skin, providing a blue background against the red and green colour of stained *S. aureus* cells/colony and cellular products, respectively. The thinner formation of *S. aureus* biomass in the treatment and prevention group indicated that CFS from *L. plantarum* USM8613 inhibited the growth of *S. aureus* and subsequently reduced the production of biofilm. The greater inhibitory effect of the treatment group as compared to the prevention group indicated that CFS from *L. plantarum* USM8613 performed better in inhibiting infection. Matured biofilm has been reported to be a more vulnerable target for elimination, with anti-biofilm activity mainly derived from reduced intracellular communications among matured bacteria cells, in addition to the less-evolved chemical compositions of matured biofilms (Flemming and Wingender 2010; Stowe et al. 2011). Our findings are in agreement with many chemical compounds such as chitosan and amine oxides, which also exhibited a stronger inhibitory effect against matured biofilms (Fraud et al. 2005; Orgaz et al. 2011).

Crude protein fractions from CFS of *L. plantarum* USM8613 showed prevalent antimicrobial activity, possibly attributed to the presence of bioactive peptides and/or bacteriocin. LAB have been documented to produce various ribosomally synthesized peptides, commonly known as bacteriocins, to counteract against various pathogens and spoilage bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, and *Escherichia coli*, by altering the proton motive force (membrane potential and pH gradient) through poration on the cytoplasmic membrane (Bruno and Montville 1993; O'Sullivan et al. 2002). The amino acid profile revealed that within the protein fraction of CFS from *L. plantarum* USM8613, leucine, threonine, isoleucine, phenylalanine, and valine were highly consumed, yielding higher glycine and arginine contents. Various glycine-rich bacteriocins have been discovered, such as plantaricin 1.25β by *L. plantarum* TMW1.25, plantaricin C19 by *L. plantarum* C19, and the common plantaricin J and K (Remiger et al. 1999; Ehrmann et al. 2000; Atrih et al. 2001). These glycine residues play an important role in antimicrobial activity of these bacteriocins where replacement of glycine residue will significantly reduce their antimicrobial activity (Atrih et al. 2001). Hence, we postulate that glycine could be responsible and contribute to the significant antimcrobial activity of crude protein fraction of *L. plantarum* USM8613.

Crude lipid fractions of CFS from *L. plantarum* USM8613 were also capable of inhibiting the growth of *S. aureus*. Fatty acids such as palmatic, *cis*-vaccenic, capric, lauric, and stearic

**Table 1** Composition of fatty acids from the lipid fraction of CFS from *L. plantarum* USM8613

| Fatty acids | Amount of fatty acids (%) | |
|---|---|---|
| | *L. plantarum* USM8613 | Control* |
| C6:0 (Hexanoic acid) | Nd** | 0.00±0.01 |
| C8:0 (Octanoic acid) | 0.03±0.05 | 0.08±0.02 |
| C10:0 (Decanoic acid) | Nd | Nd |
| C11:0 (Undecanoic acid) | Nd | Nd |
| C12:0 (Dodecanoic acid) | 7.60±0.22 | 7.79±0.13 |
| C13:0 (Tridecanoic acid) | Nd | Nd |
| C14:0 (Myristic acid) | 0.91±0.12 | 0.98±0.09 |
| C14:1 (Myristoleic acid) | Nd | Nd |
| C15:0 (Pentadecanoic acid) | 0.03±0.04 | 0.03±0.04 |
| C15:1 (*cis*-10-Pentadecenoic acid) | Nd | Nd |
| C16:0 (Palmitic acid) | 15.59±0.22$^{B}$ | 18.57±0.47$^{A}$ |
| C16:1 (Palmitoleic acid) | 0.04±0.00 | 0.00±0.01 |
| C17:0 (Heptadecanoic acid) | Nd | 0.09±0.08 |
| C17:1 (*cis*-10-Heptadecenoic acid) | Nd | Nd |
| C18:0 (Stearic acid) | 5.78±0.28 | 6.06±0.36 |
| C18:1N9T (Elaidic acid) | 0.02±0.04 | 0.03±0.05 |
| C18:1N9C (Oleic acid) | 68.90±0.54$^{A}$ | 65.53±0.96$^{B}$ |
| C18:2N6T (Linolelaidic acid) | Nd | Nd |
| C18:2N6C (Linoleic acid) | 0.26±0.23$^{A}$ | 0.01±0.02$^{B}$ |
| C18:3 N6 ($\gamma$-Linolenic acid) | Nd | Nd |
| C18:3 N3 (Linolenic acid) | Nd | Nd |
| C20:0 (Arachidic acid) | 0.45±0.02 | 0.47±0.04 |
| C20:1 (*cis*-11-Eicosenoic acid) | 0.37±0.00 | 0.35±0.04 |
| C20:2 (*cis*-11,14-Eicosadienoic acid) | Nd | Nd |
| C21:0 (Heneicosanoic acid) | Nd | Nd |
| C20:3 N6 (*cis*-8,11,14-Eicosatrienoic acid) | Nd | Nd |
| C20:4 N6 (Arachidonic acid) | Nd | Nd |
| C20:3 N3 (*cis*-11,14,17-Eicosatrienoic acid) | Nd | Nd |
| C22:0 (Behenic acid) | Nd | Nd |
| C22:1 N9 (Erucic acid) | Nd | Nd |
| C20:5 N3 (*cis*-5,8,11,14,17-Eicosapentaenoic acid) | Nd | Nd |
| C23:0 (Tricosanoic acid) | Nd | Nd |
| C22:2 (*cis*-Docosadienoic acid) | Nd | Nd |
| C24:0 (Tetracosanoic acid) | Nd | Nd |
| C24:1 (*cis*-tetracosenoate acid) | Nd | Nd |
| C22:6 N3 (Docosahexaenoic acid) | Nd | Nd |
| Total saturated fatty acids | 30.39±0.43$^{bB}$ | 34.08±0.61$^{bA}$ |
| Total unsaturated fatty acids | 69.61±0.56$^{aA}$ | 65.92±0.96$^{aB}$ |

* Control: lipid fraction of unfermented MRS

** Nd: not detected

Results are expressed as mean± standard deviation of mean; $n$=3. $^{ab}$ Means between different fatty acids with different lowercase letters are significantly different ($\rho$<0.05); $^{AB}$ Means between *L. plantarum* USM8613 and control with different uppercase letters are significantly different ($\rho$<0.05)

acids have been reported to be produced by different strains of LAB, with antibacterial (*S. aureus* and *E. coli*), antiviral (enveloped viruses), and antifungal (*Candida albicans*) properties via disruption and disintegration of the cell membrane of pathogenic bacteria (Hofmann et al. 1957; Bergsson et al. 2001; Hilmarsson et al. 2007). Fatty acids also reportedly penetrated into inner cell membrane of pathogens and subsequently inhibited cellular enzyme activity, impaired nutrient uptake, and generated peroxidation and auto-oxidation degradation products (Schönfeld et al. 2000; Wojtczak and Więckowski 1999). Fatty acid profiles of CFS from *L. plantarum* USM8613 indicated that unsaturated fatty acids were at a higher concentration than their saturated counterparts, primarily attributed to oleic acid (C18:1n9C). Antimicrobial activity of unsaturated fatty acids against pathogenic bacteria has been reported,

**Table 2** Composition of monosaccharides from the polysaccharide fraction of CFS from *L. plantarum* USM8613

| Sample | Concentration of monosaccharides (mg mL$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Glucose | Mannose | Galactose | Fructose | Arabinose | Ribose | Xylose |
| *L. plantarum* USM8613 | 40.7±7.0$^{b}$ | 14.7±2.2$^{b}$ | Nd** | Nd | Nd | Nd | Nd |
| Control* | 102.9±19.0$^{a}$ | 28.5±2.8$^{a}$ | Nd | Nd | Nd | Nd | Nd |

* Control: polysaccharide fraction of unfermented MRS

** Nd: not detected

Results are expressed as mean±standard deviation of means (mg mL$^{-1}$); $n$=3.$^{ab}$ Means between *L. plantarum* USM8613 and control with different lowercase letters are significantly different ($\rho$<0.05)

mainly via direct bacteriocidal effects, while oleic acid was previously reported to possess bacteriocidal effects against *S. aureus* (McGaw et al. 2002; Shin et al. 2007). However, the oleic acid-rich antimicrobial lipid fraction produced by LAB which is active against *S. aureus* was first demonstrated in our study.

In addition, antimicrobial activity of fatty acids was reportedly enhanced through alteration of environmental factors such as lowering of environmental pH. An acidic environment could remove the permeability barrier of the outer membrane of pathogens, facilitating the influx of fatty acids into the cytoplasm (Thormar and Hilmarsson 2007). Our data showed that the crude CFS of *L. plantarum* USM8613 had a stronger antimicrobial activity than the crude lipid fractions (Fig. 5). We postulate that in addition to the individual inhibitory effect of lipids, the production of lactic and acetic acids by LAB may have exerted a synergistic effect in inhibiting the growth of *S. aureus*.

Certain strains of LAB could secrete polysaccharides into the environment, namely exopolysaccharides (EPS), which mainly consist of simple sugars, dextran, reuteran, kefiran, and levan (Patel et al. 2012). Kefiran, a heteropolysaccharide consisting of glucose and galactose produced by *Lactobacillus kefiri*, inhibited the growth of *S. aureus*, in addition to promoting wound healing (Rodrigues et al. 2005). These actions of EPS are predominately dependent on their sugar monomers, modes of linkage, branching, and substitution, which are unique in exerting functionality. Hence, we postulate that the arrangement of the sugar monomers outweighed the importance of concentrations in exerting an antimicrobial effect. More works are needed to elucidate this.

In conclusion, CFS from *L. plantarum* USM8613 exhibiting antimicrobial effects against *S. aureus*, with the crude protein fraction, yielded a stronger effect. Our results showed that compounds from *L. plantarum* USM8613, mainly proteinase compound, are capable of inhibiting *S. aureus* via membrane disruption and anti-biofilm as demonstrated by SEM and CLSM. Our findings could be potentially applied as biopreservatives in the food industries and/or as an antimicrobial agent against bacterial infections for cosmeceutical and pharmaceutical uses. Further studies are needed to characterize and identify the bioactive compounds which are responsible for antimicrobial effects and the possible mechanisms involved.

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