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Inhibition of *Staphylococcus aureus* by crude and fractionated extract from lactic acid bacteria

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**Research Article**

**Abstract**

Increasing levels of antibiotic resistance by *Staphylococcus aureus* have posed a need to search for non-antibiotic alternatives. This study aimed to assess the inhibitory effects of crude and fractionated cell-free supernatants (CFS) of locally isolated lactic acid bacteria (LAB) against a clinical strain of *S. aureus*. A total of 42 LAB strains were isolated and identified from fresh vegetables, fresh fruits and fermented products prior to evaluation of inhibitory activities. CFS of LAB strains exhibiting a stronger inhibitive effect against *S. aureus* were fractionated into crude protein, polysaccharide and lipid fractions. Crude protein fractions showed greater inhibition against *S. aureus* compared to polysaccharide and lipid fractions, with a more prevalent effect from *Lactobacillus plantarum* 8513 and *L. plantarum* BT8513. Crude protein, polysaccharide and lipid fractions were also characterised with glycine, mannose and oleic acid being detected as the major component of each fraction, respectively. Scanning electron microscopy revealed roughed and wrinkled membrane morphology of *S. aureus* upon treatment with crude protein fractions of LAB, suggesting an inhibitory effect via the destruction of cellular membrane. This research illustrated the potential application of fractionated extracts from LAB to inhibit *S. aureus* for use in the food and health industry.

**Keywords:** lactobacilli, antimicrobials, anti-staphylococcal

1. Introduction

Staphylococcal infections represent a grave threat to humans. Over the past few decades, *Staphylococcus aureus* has gained much attention as one of the most common etiological agents of skin and soft tissue infections (Anderson et al., 2008; Charlier et al., 2009). In recent years, *S. aureus* has evolved into drug-resistant virulent variants (MRSA) leading to increased complications in treatment (Chambers, 2005). Thus, the use of new and novel antimicrobial substances is needed to treat *S. aureus* infections via non-antibiotic measures.

Lactic acid bacteria (LAB) are a group of non-spore forming Gram-positive bacteria with documented gut health potential. Numerous strains of LAB have great potential beyond gut well-being, including use as food biopreservatives and improving hypercholesterolaemia (Liong et al., 2007; Rattanachaikunsopon and Phumkhachorn, 2010). Recently, it has been promoted that LAB have a great potential to promote skin health (Oh et al., 2006). LAB are currently seen as a feasible alternative to decolonise MRSA and treat staphylococcal skin infections, as LAB have a long history of safe use without increasing the risk of multidrug resistance of the pathogen. LAB have been reported to inhibit *S. aureus* isolated from foods and human vaginal tract (Charlier
et al., 2009), mainly via the production of antimicrobial compounds, such as organic acids, diacetyl, hydrogen peroxide and antimicrobial peptides (Karska-Wysocki et al., 2009). For instance, the LAB bacteriocin nisin was found to be active against numerous food pathogens, including S. aureus, and has been employed as a food biopreservative to enhance food safety (Karam et al., 2013). Recently, LAB bacteriocins have been reported to promote wound healing via reduction of bacteria-induced inflamed acne lesions (Oh et al., 2006), thus leading to increasing interest in the utilisation of LAB in dermatology. For instance, bacteriocin from Lactococcus sp. HY 449 was able to inhibit the growth of skin inflammatory bacteria, such as S. aureus, Staphylococcus epidermidis, Streptococcus pyogenes, and Propionibacterium acnes (Oh et al., 2006).

Many studies on the antimicrobial properties of LAB were attributed to organic acids and protein-based extracts. However, to our knowledge, little information is available on polysaccharide- and/or lipid-based antimicrobial substances produced by LAB. The aim of this study was to isolate and identify potential LAB inhibitory against a model food and dermal pathogen, S. aureus. In addition, the protein-, polysaccharide- and lipid-based cell-free supernatants (CFS) of LAB were fractionated, evaluated and characterised.

2. Materials and methods

Isolation of lactic acid bacteria

LAB strains were isolated from locally fermented products, fresh fruits and vegetables (Penang, Malaysia). Food samples were homogenised with sterilised distilled water in a blender (Waring, East Windsor, NJ, USA), serially diluted with peptone water (Merck, Darmstadt, Germany). The bacterial isolates were enumerated using the pour plate method with De Man, Rogosa and Sharpe agar (MRS; Biomark, Maharashtra, India) supplemented with 3% (v/v) L-cysteine hydrochloride (HiMedia, Mumbai, India). Plates were incubated at 37 °C and colonies were examined via Gram staining. Gram-positive rods or cocci were selected, cultured and stored at -20 °C in sterile glycerol (40% v/v) (Yeo and Liong, 2009). Cultures were activated successively three times in MRS broth supplemented with 3% (v/v) L-cysteine hydrochloride at 37 °C for 24 h prior to further analysis.

Identification of lactic acid bacteria

Total genomic DNA of each isolate was extracted using a commercial DNA extraction kit (DongSheng Biotech, Guangzhou, China P. R.) and used as a template for polymerase chain reaction (PCR) amplification. PCR primers used were 16Sf (5'-GCTGGCGGCATGCTTAACACAT-3') and 16Sr (5'-GGAGGTTGATCCAGCGCGAGGT-3'). Amplification was performed in a MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA) with the programme as described by Ruiz et al. (2000). The sequencing of purified PCR products was performed by the Centre for Chemical Biology (CCB, Universiti Sains Malaysia, Malaysia), and nucleotide sequences of the 16S rRNA gene were analysed using the BLAST program from NCBI (http://www.ncbi.nlm.nih.gov/).

Antimicrobial activity of cell-free supernatant

CFS from identified LAB strains were prepared by centrifugation and evaluated for inhibition against a clinical isolate of S. aureus (General Hospital, Penang, Malaysia) as a target pathogen using the microtitre plate assay as described by Holo et al. (1991). Unfermented MRS broth was used as a control. Microplates were incubated for 20 h at 37 °C. Growth inhibition of S. aureus was measured spectrophotometrically at 600 nm by using a HALO MPR-96 Microplate Reader (Dynamica, Zug, Switzerland) at time intervals of 2 h (Turcotte et al., 2004). LAB strains exhibiting a statistically stronger inhibition against S. aureus were selected for further analysis. To exclude antimicrobial effects attributed to organic acids, CFS of the selected LAB strains was adjusted to pH 6.5 with 1 M NaOH. The antimicrobial activity of neutralised CFS was then determined and the growth inhibition of S. aureus was calculated as:

\[
\% \text{ of growth } OD_{600 \text{ nm}} = \frac{\text{growth } OD_{600 \text{ nm}} \text{ of samples}}{\text{growth } OD_{600 \text{ nm}} \text{ of control}} \times 100%.
\]

Neutralised MRS broth was used as a control.

Determination of acetic and lactic acid

The concentration of organic acids in CFS produced by selected LAB strains was determined according to the method described by Dubey and Mistry (1996) using a high performance liquid chromatography (HPLC) system equipped with an ultraviolet-visible detector (Shimadzu, Kyoto, Japan) set at 220 nm and a Luna C18(2) column (150×4.6 mm, 5 µm; Phenomenex, Torrance, CA, USA). HPLC grade acetic and lactic acid (Sigma-Aldrich, Steinheim, Germany) were used as standards (Yeo and Liong, 2009).

Fractionation of cell-free supernatant

Protein fractionation was performed by adding solid ammonium sulphate (80% (w/v) saturation) with constant stirring and left to stand for 24 h at 4 °C (Ivanova et al., 2000). The precipitates were recovered by centrifugation and evaluated for inhibition against a clinical isolate of S. aureus (General Hospital, Penang, Malaysia) prior to use. Polysaccharide fractionation was performed by adding cold ethanol (99.5%; QRec, Rawang, Malaysia) to CFS at 1:3 ratio (v/v) and left to stand for 24 h
at 4 °C (Orsod et al., 2012; Pandey et al., 2010). The crude polysaccharide fractions were recovered by centrifugation (7,000×g, 20 min, 4 °C), the pellets were resuspended in sterile deionised water at 1:1 ratio (w/v). Lipid fractionation was performed as described by Bligh and Dryer (1959) with some modifications, whereby chloroform (99.5%):methanol (99.9%) solvent (1:2, v/v) (Qrec, Selangor, Malaysia) was added to CFS at 1:3 ratio (v/v) and vortexed. Chloroform and distilled water were subsequently added to the mixture at 1:1 ratio (v/v) and vortexed. The mixture was centrifuged at 1000×g for 5 min at 4 °C and the crude lipid fraction (bottom layer) was collected and dried under nitrogen flux. The dried crude lipid fraction was resuspended in 500 µl sterile distilled water prior to use. In the same way, MRS broth was fractionated for use as a control. The microtiter plate assay described above was used to determine the antimicrobial activity of each fraction against S. aureus.

Characterisation of fractionated cell-free supernatant

Fractionated CFS from LAB strains with stronger antimicrobial activities were characterised. Freeze-dried crude protein fractions were evaluated for amino acid composition according to the method as described by Bidlingmeyer et al. (1984) using a reversed phase high performance liquid chromatography (RP-HPLC) system on a Superdex ODS column (5 µm, 4.61×50 mm, Shiseido, Tokyo) equipped with an ultraviolet-visible detector (JASCO UV detector Model Uvidec-100-VI; Japan Spectroscopic, Tokyo, Japan). A standard amino acid mixture was used (Sigma-Aldrich). The crude polysaccharide fraction was first hydrolysed in boiling water with 2 M HCl at 100 °C for 3 h, followed by neutralisation prior to determination of monosaccharide composition (Levander et al., 2001). An HPLC system equipped with a refraction index detector (Shimadzu) and a Waters Sugar Pak 1 column (300×6.5 mm, 5 µm; Waters Corporation, Milford, MA, USA) was used to analyse the monosaccharide concentrations. Column temperature was set at 85 °C with a degassed mobile phase of filtered deionised water; the flow rate was 0.2 ml/min. HPLC grade monosaccharide standards kit (Sigma-Aldrich) was used as a standard. The crude lipid fraction was converted to fatty acid methyl esters through sodium methoxide catalysis. A gas chromatography-mass spectrophotometer system (GC-MS) (GCMS-QP2010 Ultra; Shimadzu) equipped with a BPx-70 capillary column (60 m × 0.25 mm, i.d., 0.25 µm film; SGE, Ringwood, Australia) was used for the quantification of fatty acids. The initial oven temperature was 50 °C, increased to 160 °C at a rate of 4 °C/min (maintained for 10 min), followed by an increase to 200 °C at a rate of 1 °C/min (maintained for 2 min), and finally increased to 210 °C at a rate of 10 °C/min (maintained for 10 min). Helium gas was used as the carrier at a flow rate of 0.8 ml/min with split ratio 1:50. The injector temperature was 210 °C. The mass spectra were recorded at an ionisation 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 to 550 m/z with a scan speed of 10,000. The injection volume was 10 µl. Supelco 37 Component FAME Mix (Sigma-Aldrich) was used as a standard.

Scanning electron microscopy

Morphological changes of S. aureus upon treatment with the protein fraction of CFS of selected LAB strains for 20 h at 37 °C was examined using scanning electron microscope as previously described by Lye et al. (2010). Untreated cells of S. aureus were used as a control.

Statistical analysis

Data analysis was carried out with SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA). Repeated measures analysis of variance (ANOVA) was used for time-based analysis. One-way ANOVA was used to determine significant differences between means at a significance level of α=0.05. Tukey’s test was used to perform multiple comparisons between means. All data were presented as mean ± standard deviation from three separate runs.

3. Results

Isolation and identification of lactic acid bacteria

A total of 42 bacterial cultures were isolated from fresh vegetables (n=22), fresh fruits (n=13) and fermented products (n=7), 39 of which were Gram-positive bacteria, and identified via 16S rRNA sequencing. The LAB isolates mainly consisted of Lactobacillus (74.4%), followed by Weissella (15.4%), Leuconostoc (5.1%) and Pediococcus (5.1%), with species such as Lactobacillus brevis, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus paracasei, Lactobacillus plantarum, Leuconostoc mesenteroides, Pediococcus pentosaceus, Weissella cibaria and Weissella confusa.

Antimicrobial activity of isolates

CFS of LAB isolated from the three food sources exhibited inhibitory effects (P<0.05) against S. aureus (Figures 1A, B and C). Five LAB strains exhibited a statistically stronger antimicrobial activity (P<0.05) against S. aureus compared to the other strains and were selected for subsequent analysis: L. fermentum 1912 and 8513 (isolated from fermented products), L. plantarum 8513 and BT8513 (isolated from fresh fruits), and W. cibaria 8513 (isolated from fresh vegetables).
Figure 1. Antimicrobial activity of cell-free supernatant of lactic acid bacteria isolated from (A) fermented products, (B) fresh fruits and (C) fresh vegetables against growth of *Staphylococcus aureus*. Each point represents the mean of triplicates from three separate runs. Error bars represent standard deviation of the means. Means with different superscript letters are significantly different from one another (P<0.05).
Inhibition of Staphylococcus aureus

Acetic and lactic acids

All the five strains studied produced both lactic and acetic acids, the concentration of lactic acid being higher than that of acetic acid (P<0.05; Table 1). *L. plantarum* 8513 and BT8513 produced a higher amount of lactic acid (P<0.05) than the other strains with a concentration of 10.796 and 10.813 mg/ml, respectively. *L. fermentum* 1912 produced a greater amount of acetic acid (4.156 mg/ml) than the other strains studied (P<0.05).

Antimicrobial activity of neutralised cell-free supernatant

Neutralised CFS of *L. fermentum* 1912, *L. fermentum* 8513, *L. plantarum* 8513 and *L. plantarum* BT8513 exerted inhibitory effects against *S. aureus* growth, with more prevalent effects of *L. plantarum* 8513 and BT8513 (P<0.05; Figure 2). This illustrated the possibility of these strains to produce other non-acid antimicrobial metabolites.

Antimicrobial activity of fractionated cell-free supernatant

All the three fractions of CFS (crude protein, polysaccharide and lipid fractions) from *L. plantarum* 8513 and BT8513 exhibited significant inhibitory effects (P<0.05) against *S. aureus* growth compared to the control (Figure 3). The crude protein fraction exhibited a significantly more prevalent inhibitory effect than the polysaccharide and lipid fractions (Figure 3).

Partial characterisations of fractionated cell-free supernatant

A total of 16 amino acids were detected in the crude protein fraction of CFS from *L. plantarum* 8513 and BT8513 (Figure 4). The control (unfermented MRS) fractionated in the same way contained a higher amount of amino acids, such as leucine, isoleucine, phenylalanine, alanine, proline, threonine and valine compared to the protein fraction from

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**Table 1. Concentration of organic acids in De Man, Rogosa and Sharpe broth fermented by strains of lactic acid bacteria at 37 °C for 24 h.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of organic acid (mg/ml) 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactic acid</td>
</tr>
<tr>
<td><em>Weisella cibaria</em> 8513</td>
<td>6.239±0.037cdA</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em> 8513</td>
<td>5.990±0.019dkA</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> 8513</td>
<td>10.796±0.074AB</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> BT8513</td>
<td>10.813±0.246AB</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em> 1912</td>
<td>6.430±0.063AB</td>
</tr>
</tbody>
</table>

1 Each value is expressed as mean ± standard deviation of the mean from three separate runs. Means in the same column with different lowercase superscript letters and means in the same row with different uppercase superscript letters are significantly different (P<0.05).
L. plantarum 8513 and BT8513, ranging from 0.02 to 1.30%. On the other hand, the protein fraction from L. plantarum 8513 and BT8513 contained higher amounts of serine, histidine, arginine, tyrosine and methionine compared to the control, ranging from 0.20 to 1.00%. Glycine was the major component in the crude protein fractions of all samples studied and was prevalent from L. plantarum 8513 (P<0.05).

Upon fractionation, glucose and mannose were the only monosaccharides detected in the polysaccharide fractions studied. The control (unfermented MRS) contained 0.103 mg/ml glucose and 30.061 mg/ml mannose (Table 2). Arabinose, ribose, fructose, galactose and xylose were not detected in the samples. The concentrations of glucose and mannose were lower upon fermentation by L. plantarum 8513 and BT8513. The glucose concentration...
Inhibition of *Staphylococcus aureus*

Table 2. Monosaccharide composition (mg/ml) of the crude polysaccharide fraction extracted from *Lactobacillus plantarum* strains.1

<table>
<thead>
<tr>
<th>Strains</th>
<th>Glucose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. plantarum</em> 8513</td>
<td>0.041±0.002bB</td>
<td>15.882±0.570bA</td>
</tr>
<tr>
<td><em>L. plantarum</em> BT8513</td>
<td>0.039±0.009bB</td>
<td>11.230±0.551bA</td>
</tr>
<tr>
<td>Control2</td>
<td>0.103±0.019gaB</td>
<td>30.061±0.771aA</td>
</tr>
</tbody>
</table>

1 Each value is expressed as mean ± standard deviation of the mean from three separate runs. Means in the same column with different lowercase superscript letters and means in the same row with different uppercase superscript letters are significantly different (P<0.05).

2 Control = unfermented MRS fractionated in the same way as the *L. plantarum* strains.

4. Discussion

LAB are widely distributed in fermented products, fresh fruits and vegetables with a broad spectrum of inhibition against food spoilage microorganisms (Trias et al., 2008). *S. aureus*, a Gram-positive opportunistic pathogen, was chosen as a target pathogen due to its infectious nature and increasing antibiotic resistance, which has led to complications in treatment. LAB have been used to control the growth of *S. aureus* via growth inhibition and biofilm formation (Ammor et al., 2006), mainly attributed to the production of bacteriocins, hydrogen peroxide and organic acids (Karska-Wysocki et al., 2009).

In the present study, CFS of LAB isolated from fermented products, fresh fruits and vegetables inhibited *S. aureus* growth as early as the log phase. It is assumed that it is attributed to the production of inhibitory metabolic end products. *L. plantarum* BT8513 and 8513 produced the highest amount of lactic acid among the strains studied, indicating that the antimicrobial activity might be dominated by organic acids. LAB produce lactic and acetic acid as their main metabolites during carbohydrate fermentation; the production is growth dependent (Liong and Shah, 2005). Organic acids exert antimicrobial effects via acidifying the environment and altering the membrane potential, which leads to reduced intracellular pH and inhibition of active transport and cellular metabolic functions (Ross et al., 2002). To evaluate the effects of non-acid antimicrobial compounds, CFS was neutralised. Our data showed that neutralised CFS of *L. plantarum* 8513 and BT8513 remained strong in inhibiting *S. aureus* growth, indicating the presence of non-acid inhibitory compounds.

CFS of *L. plantarum* 8513 and BT8513 were further fractionated to evaluate the potential of antimicrobial compounds originating from lipids, proteins and/or polysaccharides. All the fractions studied inhibited the growth of *S. aureus*, with a more prevalent effect from the crude protein fraction. This may be attributed to the presence of antimicrobial peptides, including bacteriocins. Certain strains of LAB can produce bacteriocins with a wide antibacterial spectrum against Gram-positive and

**Figure 5. Scanning electron microscope images of *Staphylococcus aureus* treated with (A) protein fraction of *Lactobacillus plantarum* 8513 and (B) protein fraction of *L. plantarum* BT8513, and (C) untreated *S. aureus*. Magnification 20,000×.**
Gram-negative bacteria, such as *Listeria monocytogenes*, *S. aureus*, *Escherichia coli* and *Salmonella* (Atanassova et al., 2003; De Vuyst and Leroy, 2007). Glycine was detected as the major component in the crude protein fractions of CFS from *L. plantarum* 8513 and BT8513. Rogne et al. (2009) described the importance of glycine residues for the antimicrobial activity of plantaricin J and K. Replacing the individual glycine residues with other residues generally reduced antimicrobial activities. Plantaricin 1.25β produced by *L. plantarum* TMW1.25 and the anti-*Listeria* bacteriocin, plantaricin C19 produced by *L. plantarum* C19 were reported as rich in glycine (Atrih et al., 2001), while glycine-rich features were also associated with the characteristics for bacteriocins (Remiger et al., 1999).

### Table 3. Fatty acids composition of crude lipid fractions extracted from *Lactobacillus plantarum* strains.\(^1\)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Strains of LAB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>L. plantarum 8513</em></td>
</tr>
<tr>
<td></td>
<td><em>L. plantarum BT8513</em></td>
</tr>
<tr>
<td></td>
<td><em>Control</em>(^2)</td>
</tr>
<tr>
<td>C6:0 (hexanoic acid)</td>
<td>nd(^3)</td>
</tr>
<tr>
<td>C8:0 (octanoic acid)</td>
<td>0.092±0.080(^{eA})</td>
</tr>
<tr>
<td>C10:0 (decanoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C11:0 (undecanoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C12:0 (dodecanoic acid)</td>
<td>7.518±0.215(^{cA})</td>
</tr>
<tr>
<td>C13:0 (tridecanoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C14:0 (myristic acid)</td>
<td>1.239±0.043(^{eA})</td>
</tr>
<tr>
<td>C14:1 (myristoleic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C15:0 (pentacontanoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C15:1 (cis-10-pentadecanoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C16:0 (palmitic acid)</td>
<td>17.415±0.334(^{bA})</td>
</tr>
<tr>
<td>C16:1 (palmitoleic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C17:0 (heptadecanoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C17:1 (cis-10-heptadecenoic acid)</td>
<td>0.006±0.006(^{fA})</td>
</tr>
<tr>
<td>C18:0 (stearic acid)</td>
<td>6.05±0.116(^{fA})</td>
</tr>
<tr>
<td>C18:1N9 (elaidic acid)</td>
<td>0.018±0.031(^{fA})</td>
</tr>
<tr>
<td>C18:1n9c (oleic acid)</td>
<td>66.635±1.011(^{aB})</td>
</tr>
<tr>
<td>C18:2N6 (linoleic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C18:3N3 (γ-linolenic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C18:3N6 (linolenic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C20:0 (arachidic acid)</td>
<td>0.507±0.036(^{fA})</td>
</tr>
<tr>
<td>C20:1 (cis-11-eicosanoic acid)</td>
<td>0.368±0.035(^{fA})</td>
</tr>
<tr>
<td>C20:2 (cis-11,14-eicosadienoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C21:0 (heneicosanoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C20:3N6 (cis-8,11,14-eicosatrienoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C20:4N6 (arachidonic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C20:3N3 (cis-11,14,17-eicosatrienoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C22:0 (behenic acid)</td>
<td>0.002±0.003(^{fA})</td>
</tr>
<tr>
<td>C22:1N9 (erucic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C20:5N3 (cis-5,8,11,14,17-eicosapentaenoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C23:0 (tricosanoic acid)</td>
<td>0.003±0.005(^{fA})</td>
</tr>
<tr>
<td>C22:2 (cis-docosadienoic acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C24:0 (tetracosanoic acid)</td>
<td>0.002±0.004(^{fA})</td>
</tr>
<tr>
<td>C24:1 (cis-tetracosenate acid)</td>
<td>nd</td>
</tr>
<tr>
<td>C22:6N3 (docosahexaenoic acid)</td>
<td>nd</td>
</tr>
</tbody>
</table>

\(^1\) Each value is expressed as mean ± standard deviation of the mean from three separate runs. Means in the same column with different lowercase letters and means in the same row with different uppercase letters are significantly different (P<0.05).

\(^2\) Control = unfermented De Man, Rogosa and Sharpe broth fractionated in the same way as the *L. plantarum* strains.

\(^3\) nd = not detected.
Hence, we postulate that glycine as the major amino acid residue attributed to the significant antimicrobial activity of *L. plantarum* 8513 and BT8513. Furthermore, the amino acid composition of the protein fractions from both strains confirmed the hydrophobic character of the active molecule, as amino acids, such as leucine, phenylalanine, glycine, alanine, proline, valine and isoleucine, were prevailing. Hydrophobicity is a common feature of several potent bacteriocins isolated from *L. plantarum* (Muriana and Klaenhammer, 1991).

Apart from proteins, crude polysaccharide fractions of CFS from *L. plantarum* 8513 and BT8513 also inhibited the growth of *S. aureus*. This might be attributed to the production of exopolysaccharides (EPS). EPS from LAB was reported to exert anti-tumour and immunostimulatory activities and also antimicrobial activity against food pathogenic bacteria (Chabot et al., 2001; Fanning et al., 2012). The sugar monomers, modes of linkage, branching and substitution, which are unique in exerting functionality, might attribute to the actions of EPS. For instance, EPS from *Lactobacillus kefiransacchari* ATCC 43761 consisting of equal amounts of glucose and galactose could increase mucosal response in mice (Chabot et al., 2001). However, studies on the antimicrobial properties of EPS from *L. plantarum* are limited. In the present study, the crude polysaccharide fractions of CFS from *L. plantarum* 8513 and BT8513, which consisted mainly of mannose and glucose at concentrations lower than the control, exhibited an inhibitory effect against *S. aureus*. It is postulated that the arrangement of the sugar monomers outweighed the importance of concentration in exerting antimicrobial effects. Further investigations are needed to elucidate this issue.

Lipid fractions of CFS from *L. plantarum* 8513 and BT8513 also exhibited inhibition against *S. aureus*, suggesting the presence of antimicrobial fatty acids. Generally, long- and medium-chain fatty acids and monoglycerides are more prevalent to exhibit antimicrobial effects compared to short-chain fatty acids, mainly via growth inhibition or direct bactericidal effects (McGaw et al., 2002). In the present study, oleic acid (C18:1n9c) was the main fatty acid detected. Oleic acid has been reported to be bactericidal against antibiotic-resistant *S. aureus* (Shina et al., 2007). Both *L. plantarum* 8513 and BT8513 were grown under the same conditions but the lipid fraction of CFS from *L. plantarum* BT8513 contained a higher oleic acid concentration, which subsequently inhibited *S. aureus* better than *L. plantarum* 8513. To date, this study is the first to demonstrate the inhibitory effect against *S. aureus* of a lipid fraction from LAB strains dominated by oleic acid.

Scanning electron microscopy revealed morphological changes of *S. aureus* upon exposure to the protein fraction from *L. plantarum* 8513 and BT8513 compared to the control. Bacteriocins and/or antimicrobial peptides could increase membrane permeability of susceptible bacteria leading to cellular deformation and cell leakage, accompanied by the release of intracellular components to the environment, thus resulting in cell lysis and eventual death (Dalie et al., 2010; Klayraung and Okonogi, 2009). We postulate that this effect is illustrated by the extracellular debris present in treated cells but not in control cells. In accordance with many other bacteriocins of *L. plantarum*, such bacteriocidal effects were also revealed by plantaricin MG, a bacteriocin active against *Salmonella typhimurium*, and plantaricin 149 (Gong et al., 2010; Lopes et al., 2009). This microscopic evaluation justified that the CFS protein fraction from *L. plantarum* 8513 and BT8513 exerted antimicrobial effects on *S. aureus* via membrane disruption and permeability.

5. Conclusions

LAB strains were successfully isolated from local fermented products, fresh vegetables and fruits, and identified via 16S rRNA gene sequencing analysis. CFS of *L. plantarum* 8513 and BT8513 exerted a strong inhibitory effect against *S. aureus*, with a more prevalent effect of the CFS protein fraction. The results of the present study suggested that these LAB strains produced proteinaceous antimicrobial compounds inhibiting *S. aureus* via membrane disruption, as confirmed by scanning electron microscopy. These findings offer a possibility for the protective use of LAB against infections associated with *S. aureus*. Nevertheless, further studies are crucial to identify the proteinaceous compounds and envisage the mechanisms involved.

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