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ORIGINAL ARTICLE

Use of extracellular extracts of lactic acid bacteria and bifidobacteria for the inhibition of dermatological pathogen *Staphylococcus aureus*

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ABSTRACT

Background/Objectives: The application of lactic acid bacteria (LAB) and bifidobacteria, which exhibit therapeutic benefits, in dermatology, including treatment of skin infections specifically caused by *Staphylococcus aureus*, is new. The objectives of this study were to screen LAB and bifidobacteria for antimicrobial activity against *S. aureus* and to identify the antimicrobial compounds produced by LAB. In addition, the study aimed to inhibit the biofilm of *S. aureus* with extracellular extracts of LAB.
Methods: A total of 87 strains of LAB and three strains of bifidobacteria, grouped according to their respective origins, were screened for antimicrobial activity against *S. aureus* using the cell-free supernatant (CFS). Antimicrobial activity of the CFS was evaluated following neutralization, protease treatment, and protein precipitation treatment. Characterization was performed to identify the antimicrobial compounds in the CFS. Inhibition of the *S. aureus* biofilm was assessed with a crystal violet assay.
Results: LAB and bifidobacteria inhibited the growth of *S. aureus*, with percentage of growth inhibition ranging from 0.5% to 34.2%. All strains demonstrated a drastic reduction ($p < 0.05$) in growth inhibition upon neutralization. Antimicrobial compounds in the CFS were lactic acid, acetic acid, hydrogen peroxide, and diacetyl. The CFS of strain *Lactobacillus bulgaricus* FTDC 8611 significantly hindered ($p < 0.05$) the biofilm formation of *S. aureus*. Statistical analysis was performed with SPSS version 19.0.
Conclusion: LAB were able to produce antimicrobial compounds that inhibit *S. aureus*. The inhibitory action of the CFS was mainly due to the organic acids produced by LAB. Antimicrobial metabolites produced by LAB comprise lactic acid, acetic acid, hydrogen peroxide, and diacetyl. *S. aureus* was able to form a biofilm, which was successfully inhibited by the CFS of *L. bulgaricus* FTDC 8611.

Introduction

In the recent years, various therapeutic benefits of lactic acid bacteria (LAB) and bifidobacteria have been studied extensively, but their application in dermatology remains new. *Staphylococcus aureus* is Gram-positive cocci that have been identified to be most virulent among all staphylococcal species to cause skin and soft tissue infections, surgical site infections, and hospital-acquired bloodstream infections.[1] In some infections, survival, dissemination, and pathogenesis of staphylococci are supported by the formation of a biofilm.[2] Treating infections in which biofilms are involved is very difficult, and due to the emergence of methicillin-resistant *S. aureus*, alternative natural antibacterial therapies have gained much attention.[3,4]

LAB and bifidobacteria are useful for a variety of applications due to their therapeutic effects; these microorganisms are, in general, nonpathogenic, and thus have been assigned a "generally recognized as safe" status.[5] These microorganisms have been highlighted for their ability to treat acute infectious diarrhea, antibiotic-associated diarrhea, viral infections of the respiratory tract, inflammatory bowel disease, and cholesterol lowering.[6] In recent studies, beneficial effects of LAB and bifidobacteria that extend beyond the gut were uncovered, as these bacteria also demonstrated their potential in promoting dermal health and exerting cellular immunity response required for in skin defence.[7] Bioactive compounds that were found to be useful in dermatological applications included hyaluronic acid, peptidoglycan, lipoteichoic acid, and sphingomyelinase. These compounds were produced by LAB at an effective concentration to inhibit pathogens causing dermal illness.[7]

Conflicts of interest: The authors declare that they have no financial or non-financial conflicts of interest related to the subject matter or materials discussed in this article.

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Findings of extensive studies on these beneficial bacteria suggest that the therapeutic effects exhibited by these microorganisms are due to the secretion of various inhibitory compounds, particularly LAB, which can produce growth-inhibitive compounds such as lactic acid, acetic acid, bacteriocin, hydrogen peroxide, and diacetyl.[7–9] The unique ability of LAB and bifidobacteria to produce organic acids not only empower bacteria with the ability to preserve food, but also to inhibit pathogenic microorganisms by penetrating the targeted bacterial membranes and interfering with the essential metabolic functions.[9,10] Bacteriocins are another group of interesting natural antimicrobial compounds produced by LAB, which resemble antibiotics; they work by adhering to specific receptors on targeted bacterial cytoplasmic membrane and affect the metabolic activity within cells.[11]

Such evidence of therapeutic effects suggested that LAB and bifidobacteria could be used for inhibiting the growth as well as eradicating the biofilm of dermatological pathogen *S. aureus*. Thus, the aim of this study is to evaluate the antimicrobial activity of LAB and bifidobacteria, as well as the antimicrobial compounds produced in the extracellular extracts of these bacteria, against *S. aureus*.

Methods

Bacteria strains and media

A total of 87 strains of LAB and three strains of bifidobacteria and *S. aureus* were obtained from the Culture Collection Centre of Bioprocess Technology Division, School of Industrial Technology, Universiti Sains Malaysia (Penang, Malaysia). The LAB strains used in this study are listed in Table 1. De Man–Rogosa–Sharpe (MRS) broth (Biomark, Pune, India) was used for the incubation of LAB and bifidobacteria strains. LAB and bifidobacteria strains were activated using 10% (v/v) inoculum in MRS broth at 37°C for 24 hours under static conditions and were subcultured twice prior to use. These LAB and bifidobacteria strains were used as antimicrobial compounds producing strain. The pathogen *S. aureus* was activated in sterile tryptic soy broth (TSB) (Biomark) with 10% inoculum at 37°C for 24 hours. It was subcultured twice in TSB prior to use. All bacteria strains were preserved at −20°C in 40% glycerol.[12]

Screening for antimicrobial activity

Activated LAB and bifidobacteria strains were incubated in MRS broth for 20 hours at 37°C and standardized to an optical density (600 nm) of 1.000 ± 0.05 upon incubation. Cell cultures were centrifuged at 1100 × *g* for 10 minutes; next, the supernatants (extracellular extracts) were collected and subsequently filter sterilized. The resulting filtrates were designated as crude cell-free supernatant (CCFS). Each 100 μL of CCFS weighed 103 μg. The assay was carried out in 96-well microplates.[13] Activated *S. aureus* was grown in TSB at 37°C for 20 hours and standardized to an optical density (600 nm) of 0.300 ± 0.015. A total of 100 μL *S. aureus* cell suspension and 100 μL LAB CCFS were added to the 96-well microplate and incubated at 37°C for 20 hours. Turbidity was measured as the absorbance at 596 nm. A negative control was prepared using MRS broth to replace the LAB extracts. A positive control was prepared with streptomycin (10 μg/mL) to replace the LAB extracts. The percentage of growth inhibition was calculated based on the following equation[14]:

$$\%\, I = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100 \qquad [1]$$

where I (%) is the percentage of inhibition of the sample in relation to the growth control, OD control represents the changes in the

Table 1 Origin of lactic acid bacterial strain.

Category	No.	Source/origin	Strain
Dairy	A1	Cow milk	*Lactobacillus acidophilus* FTDC 2333
	A2	Cow milk	*L. acidophilus* FTDC 2131
	A3	Cow milk	*Bifidobacterium* FTDC 2142
	A4	Cow milk	*L. acidophilus* FTDC 2133
	A5	Cow milk	*Lactobacillus* FTDC 2113
	A6	Cow milk	*L. acidophilus* FTDC 1231
	A7	Cow milk	*L. acidophilus* FTDC 1733
	A8	Cow milk	*Lactobacillus bulgaricus* FTDC 1311
	A9	Cow milk	*L. bulgaricus* FTDC 1511
	A10	Cow milk	*Lactobacillus brevis* BD 1312b
	A11	Cow milk	*Lactobacillus fermentum* BD 1312
	A12	Cow milk	*Lactobacillus casei* BD 1313a
	A13	Cow milk	*Lactobacillus plantarum* BD 8313b
	A14	Cow milk	*L. plantarum* BD 8313c
	A15	Yogurt	*L. fermentum* BD 8312b
	A16	Cow milk	*L. casei* BD 1313b
	A17	Cow milk	*L. fermentum* BD 8313a
	A18	Yogurt	*L. fermentum* BD 8311
	A19	Cow milk	*L. fermentum* BD 8315
	A20	Yogurt	*L. fermentum* BD 8312a
	A21	Cow milk	*Bifidobacterium longum* FTDC 8843
	A22	Cow milk	*L. acidophilus* FTDC 8033
	A23	Cow milk	*L. bulgaricus* FTDC 8011
	A24	Cow milk	*L. acidophilus* FTDC 8833
	A25	Cow milk	*Bifidobacterium* FTDC 8943
	A26	Cow milk	*L. acidophilus* FTDC 8933
	A27	Cow milk	*L. bulgaricus* FTDC 8913
	A28	Cow milk	*Weissella cibaria* FTDC 8643
	A29	Cow milk	*Lactobacillus. gasseri* FTDC 8131
	A30	Cow milk	*L. bulgaricus* FTDC 8611
	A31	Cow milk	*L. bulgaricus* FTCC 0411
	A32	Cow Milk	*L. casei* FTCC 0442
	A33	Cow Milk	*L. acidophilus* FTCC 0291
Vegetable	B1	Old cucumber	*L. brevis* BD 1515
	B2	Tomato	*Lactobacillus paracasei* BD 1512e
	B3	Tomato	*L. plantarum* BD 1512f
	B4	Cabbage	*W. cibaria* BD 1512i
	B5	Cabbage	*L. fermentum* BD 1512j
	B6	Butterhead	*L. casei* BD 1513f
	B7	Butterhead	*W. cibaria* BD 1514h
	B8	Cabbage	*W. cibaria* BD 8513b
	B9	Spinach	*L. fermentum* BD 1512k
	B10	Spinach	*W. cibaria* BD 8513j
	B11	Spinach	*Weissella confusa* BD 8513k
	B12	Tomato	*L. fermentum* BD 8515a
	B13	Purple cabbage	*L. casei* BD 8513g
	B14	Purple cabbage	*L. fermentum* BD 8513h
Fruits	C1	Water chestnut	*L. casei* BD 1512a
	C2	Ciku	*L. casei* BD 1512b
	C3	Guava	*L. casei* BD 1512d
	C4	Dukung	*L. brevis* BD 1512m
	C5	Pineapple	*L. fermentum* BD 1512n
	C6	Water chestnut	*L. brevis* BD 1513a
	C7	Dukung	*L. casei* BD 1513c
	C8	Kiwi	*L. fermentum* BD 8513d
	C9	Kiwi	*L. plantarum* BD 8513e
	C10	Kiwi	*L. plantarum* BD 8513f
	C11	Mango	*L. fermentum* BD 8513r
	C12	Mango	*L. fermentum* BD 8513q
	C13	Pineapple	*L. fermentum* BD 8513a
Meat	D1	German sausage	*L. paracasei* BD 1611a
	D2	German sausage	*L. casei* BD 1611c
	D3	Pork minced meat	*L. plantarum* BD 8613c
	D4	BBQ pork sausage	*L. plantarum* BD 8913b

Table 1 (*continued*)

Category	No.	Source/origin	Strain
	D5	Cheesy pork sausage	*L. fermentum* BD 8913d
	D6	Pork minced meat	*L. fermentum* BD 8613b
	D7	BBQ pork sausage	*L. fermentum* BD 8913a
	D8	Chinese sausage	*L. plantarum* BD 8913c
	D9	Cheesy pork sausage	*L. fermentum* BD 8913e
	D10	Cheesy pork sausage	*L. fermentum* BD 8913f
	D11	Beef sausage	*W. confuse* BD 8913k
	D12	Beef sausage	*L. fermentum* BD 8913l
	D13	Chinese sausage	*Lactococcus gravieae* BD 8914d
Fermented food products	E1	Sourdough	*L. paracasei* BD 1415a
	E2	Sourdough	*L. casei* BD 1415b
	E3	Coconut milk	*L. brevis* BD 1512g
	E4	Coconut milk	*L. brevis* BD 1512h
	E5	Tosai	*L. brevis* BD 1912c
	E6	Kimchi	*L. fermentum* BD 1913a
	E7	Tempoyak	*Pediococcus pentosaceus* BD 1913b
	E8	Fermented bean curd	*L. fermentum* BD 8912
	E9	Sourdough	*L. fermentum* BD 1415c
	E10	Beancurd	*L. casei* BD 1511a
	E11	Tosai	*L. casei* BD 1912b
	E12	Pickle mustard	*L. fermentum* BD 1912d
	E13	Fermented olives	*L. fermentum* BD 8513c
	E14	Octopus	*L. fermentum* BD 8613d
	E15	Tosai	*Lactobacillus delbrueckii* subsp. *bulgaricus* BD 8913i
	E16	Tosai	*L. plantarum* BD 8913g
	E17	Pickled cucumber	*Leuconostoc. mesenteroides* BD 8914a

optical density of the growth control, and OD sample represents the changes in the optical density of the sample.

Determination of pH

Activated cultures of LAB were incubated in MRS broth at 37°C for 20 hours. The pH of bacterial cultures prior to and after fermentation was measured with a pH meter equipped with an automatic temperature compensation probe (CyberScan pH 510, EUTECH, Singapore).

Antimicrobial activity of neutralized CCFS

The pH of LAB supernatants (OD 1.0) was adjusted to 7.0 using 1.0 N HCl and 1.0 N NaOH. Neutralization was performed so that the number of hydrogen ions became equal to that of hydroxide ions in the CCFS, in order to rule out the effect of organic acids. Antimicrobial activity of the neutralized extracellular extracts was tested against *S. aureus* in a 96-well microplate, incubated at 37°C for 20 hours.[13]

Antimicrobial activity of neutralized and protease-treated CCFS

Supernatants of selected LAB strain (OD 1.0) were neutralized to pH 7.0 and filter sterilized using a 0.22 μm membrane filter. Trypsin (Sigma-Aldrich, Missouri, USA) and proteinase K (Sigma-Aldrich) were added to the CFS at a final concentration of 1 mg/mL. The mixture was incubated at 37°C for 1 hour. Upon incubation, the enzymes were heat-inactivated by boiling for 3 minutes.[15] The resulting enzyme-treated CFS was tested for antimicrobial activity against *S. aureus* in a 96-well microplate at 37°C for 20 hours.

Antimicrobial activity of precipitated protein fraction

Precipitated protein fractions of selected LAB strains were prepared using the modified ammonium sulfate precipitation method. Supernatants of LAB (OD 1.0) were adjusted to pH 7 and filter sterilized. Protein fraction was precipitated at 40% ammonium sulfate saturation. The mixture was incubated at 4°C for 24 hours with continuous stirring. The precipitated supernatant was centrifuged at 8590*g* for 1 hour at 4°C, and the collected precipitate was resuspended in 20 mL of 0.05 M sodium phosphate buffer, pH 7.0, prior to filter sterilization. The obtained protein fractions were subjected to antimicrobial activity assay against *S. aureus*.[16]

Determination of protein content

Protein concentrations in the extracellular extracts of LAB were determined using the Bradford method.[17] First, 100 μL of the sample was mixed with 100 μL of distilled water and 800 μL of Bradford reagent. The mixture was allowed to stand at room temperature for 5 minutes, and the absorbance of each sample was measured at 596 nm. The standard curve of protein concentration was constructed using a series of dilutions of the Bovine Serum Albumin (BSA) protein standard stock solution.

Determination of hydrogen peroxide

A spectrophotometric method was used to determine the concentrations of hydrogen peroxide in extracellular extracts of selected LAB, using o-dianisidine horseradish peroxidase.[18] A standard curve of hydrogen peroxide was prepared freshly, and absorbance was measured at 430 nm.

Determination of organic acids

Concentrations of lactic acid and acetic acid in extracellular extracts of selected LAB were determined using high-performance liquid chromatography (HPLC). The HPLC system (Shimadzu, Nakagyō-ku, Kyoto, Japan) consisted of an Aminex-HPX 87H column (300×7.8 mm^2; Bio-Rad, Hercules, CA, USA), and the temperature of the column was maintained at 35°C. An aliquot (20 μL) of the filtered samples was injected into the HPLC equipped with a UV absorbance detector (Shimadzu) set at 215 nm. Degassed 0.004 M H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/minute. HPLC-grade acetic acid and lactic acid (Sigma, Buchs, St. Gallen, Switzerland) were used as standards.[19]

Determination of diacetyl

A colorimetric reaction method, using creatine and α-naphthol in an alkaline medium, was employed to determine the concentrations of diacetyl in the extracellular extracts of selected LAB. The standard curve was prepared using fresh diacetyl (Merck, Hohenbrunn, Germany), and absorbance was measured at 525 nm. The diacetyl and the solution containing 3% NaOH and 3.5% α-naphthol were stored with light preservation.[19]

Quantitative in vitro biofilm formation of S. aureus

The *S. aureus* strain was grown in tryptic soy broth (TSB) at 37°C for 20 hours. The culture was standardized to an optical density (600 nm) of 0.300 ± 0.015 in TSB supplemented with 2% glucose (w/v) to maximize ica operon induction.[20] The biofilm was evaluated with a crystal violet assay using 1% crystal violet solution and 33% acetic acid. Upon incubation, the absorbance was measured at 596 nm.[21]

Statistical analysis

Data were analyzed using SPSS version 19.0 (IBM, New York, NY, USA). A one-way analysis of variance was performed to study

significant differences between sample means. A repeated-measure analysis of variance was used for time-based experiments. The level of significance was set at $\alpha = 0.05$. Data means were compared by Tukey's test. All experimental results were expressed as mean values obtained from three replicates ($n = 3$), unless stated otherwise.

Results

Screening of LAB and bifidobacteria for antimicrobial activity

Antimicrobial activities of LAB and bifidobacteria categorized according to their sources of origins were determined by measuring the growth inhibition percentage of *S. aureus* cultured in the presence of extracellular extracts of LAB and bifidobacteria in relation to the control (Figure S1 in the supplementary material online). All 87 strains of LAB and three strains of bifidobacteria were able to exhibit antimicrobial activity against the test organism, with growth inhibition recorded ranging from 0.5% to 34.2%.

Higher percentages of growth inhibition ($p < 0.05$) were demonstrated by five different strains of LAB from five different categories, namely, *Lactobacillus bulgaricus* FTDC 8611 (A30) from the dairy products category, *Weissella cibaria* BD 1514h (B7) from the vegetable category, *Lactobacillus fermentum* BD 1512n (C5) from the fruit category, *L. fermentum* BD 8913f (D10) from the meat products category, and *Lactobacillus casei* BD 1511a (E10) from the fermented food products category. Antimicrobial activities of these strains were evaluated by imposing different treatments on the extracellular extracts, while *L. bulgaricus* FTDC 8611 (A30), which exhibited a higher percentage of inhibition ($p < 0.05$) than other selected strains, was chosen for *S. aureus* biofilm inhibition analysis.

Effects of different treatments on the antimicrobial activity of LAB extracellular extracts (CFS)

Antimicrobial activities of the five selected LAB supernatants were tested with different treatments imposed on the extracts. Neutralization of the LAB supernatants resulted in a drastic reduction ($p < 0.05$) in the percentage of *S. aureus* growth inhibition as compared to the non-neutralized CFS (Figure 1). Among all strains, *L. bulgaricus* FTDC 8611 (A30) showed a higher drop (approximately 75% reduction; $p < 0.05$) in growth inhibition after the CFS was neutralized.

The neutralized and protease (proteinase K and trypsin)-treated LAB supernatants of all selected strains exhibited poorer growth inhibition ($p < 0.05$) of *S. aureus* than the untreated CFS (Figure 1). Among the selected strains, both *W. cibaria* BD 1514h (B7) and *L. fermentum* BD 8913f (D10) showed poorer growth inhibition ($p < 0.05$), as compared to their neutralized CFS.

In this study, *S. aureus* growth inhibition by the selected five protein-precipitated LAB supernatants was significantly higher ($p < 0.05$) than the growth inhibition by neutralized CFS and protease-treated CFS (Figure 2). These crude protein fractions significantly inhibited ($p < 0.05$) the growth of test microorganisms, with the percentage of inhibition ranging from 16.39% to 24.89%. However, this growth inhibition, when compared with the inhibition caused by nontreated CFS, was still significantly poor ($p < 0.05$).

Characterization of LAB extracellular extracts

Supernatants of the five selected LAB strains were characterized to determine the amount of antimicrobial agents present, including lactic acid, acetic acid, hydrogen peroxide, diacetyl, and protein (Tables 2 and 3).

Generally, the amount of lactic acid produced by the LAB strains was higher than that of acetic acid present in the extracellular extracts. Lactic acid comprised 69.39–73.45% of the total acids produced, whereas acetic acid comprised 28.57–30.61% (Table 2). Based on the data, *L. casei* BD 1511a (E10) produced a large amount ($p < 0.05$) of lactic acid compared to *W. cibaria* BD 1514h (B7), which was 11.11% higher. The strain *L. bulgaricus* FTDC 8611 (A30) was able to produce a higher concentration ($p < 0.05$) of acetic acid. The total amount of acids produced by the LAB strains was higher ($p < 0.05$) in the extracellular extracts of *L. bulgaricus* FTDC 8611 (A30) and *L. fermentum* BD 8913f (D10), with a total concentration of 0.049 mmol/mL. Data also demonstrated that the pH in the MRS growth medium of the five selected LAB strains was reduced significantly ($p < 0.05$) to approximately 4.0 upon incubation for 20 hours at 37°C.

All the strains of LAB tested produced hydrogen peroxide, at concentrations ranging between 0.003 mg/mL and 0.007 mg/mL, in the extracellular extracts (Table 3). Both *L. fermentum* BD 8913f (D10) and *L. fermentum* BD 1512n (C5) produced significantly large amounts ($p < 0.05$) of hydrogen peroxide, which were, respectively, 57.14% and 50.00% higher than the amount produced by *L. casei* BD 1511a (E10).

Results showed that five selected LAB strains produced a detectable amount of diacetyl but at a minimal concentration (Table 3). The varying concentrations of diacetyl estimated from these strains of LAB ranged from 0.171 ng/mL to 4.419 ng/mL in the extracellular extracts.

Based on the data, the LAB strains studied produced varying concentrations of protein, ranging from 0.119 mg/mL to 0.219 mg/mL, in the extracellular extracts (Table 3). Among all the strains, higher amounts ($p < 0.05$) of protein were produced by the strain *L. fermentum* BD 1512n (C5), whereas a lower protein content ($p < 0.05$) was observed in the extracellular extracts of *L. bulgaricus* FTDC 8611 (A30).

Quantitative in vitro biofilm formation of S. aureus and its inhibition by extracellular extracts of LAB

As observed during the study, the control *S. aureus* was able to produce a biofilm, but the biofilm took approximately 24 hours to establish itself prior to reaching a state of dynamic equilibrium (Figure 2). The highest absorbance measurement (OD_{596nm}) recorded for the biofilm of the control was 1.0 at 24 hours prior to when the biofilm degraded to OD 0.06 at 36 hours and 48 hours. After 48 hours, an increase in absorbance suggested that the staphylococcal biofilm was reformed.

The growth of the biofilm in the control throughout the 60 hours was significantly higher ($p < 0.05$) than that in the sample. The sample where *S. aureus* was incubated with the extracellular extracts of *L. bulgaricus* FTDC 8611 (A30) showed relatively no growth of the biofilm, in which the absorbance (OD) measured was near to 0.01 for up to 48 hours. After 48 hours, an increase in OD to 0.3 was observed, indicating that the biofilm started to grow (Figure 2). This phenomenon suggested that the extracellular extract used was able to inhibit the formation of the biofilm for up to 48 hours. For a clearer illustration, the crystal violet bounded to the biofilm of the control and the sample in the microplate wells is shown in Figure 3.

Discussion

LAB has been receiving significant attention lately due to their ability to exhibit an antagonistic effect on closely related bacteria and many other food-borne and dermatological pathogens.[7,22] In this study, the extracellular extracts (CFS) of 90 strains of LAB and

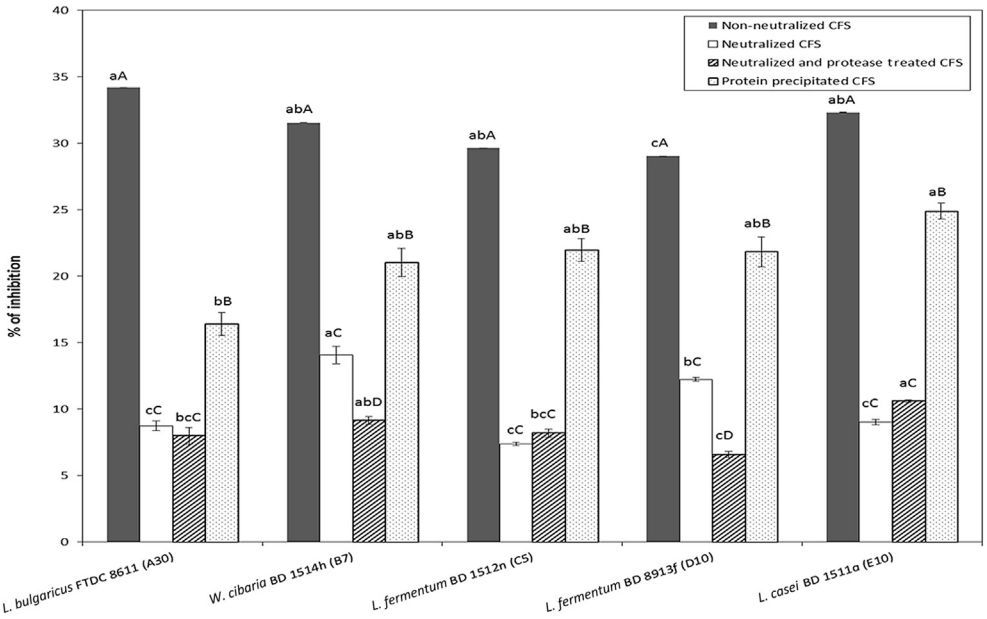

Figure 1 Effects of non-neutralized CFS, neutralized CFS, neutralized and protease-treated CFS, and neutralized and protein-precipitated CFS on the growth inhibition of *Staphylococcus aureus*. Values are expressed in percentage of growth inhibition in relation to the control. Error bars represent standard errors of means ($n = 3$). $^{a-c}$ Means in the CFS of same treatment from five tested strains, with different superscripted lowercase letters are significantly different ($p < 0.05$). $^{A-D}$ Means in the same strain among CFS of different treatment, with different superscripted uppercase letters are significantly different ($p < 0.05$). CFS = cell-free supernatant.

bifidobacteria were used for antimicrobial activity evaluation, as it was proven in a previous study that the extracellular fraction contained more inhibitives than the intracellular extracts.[7]

Five LAB strains, which showed a higher percentage of growth inhibition in screening, namely, *L. bulgaricus* FTDC 8611 (A30), *W. cibaria* BD 1514h (B7), *L. fermentum* BD 1512n (C5), *L. fermentum* BD 8913f (D10), and *L. casei* BD 1511a (E10), were selected for detailed studies. The primary antimicrobial effect exerted by LAB is due to the production of organic acids, which reduces the pH of the immediate environment, rendering it unsuitable for the growth of a broad range of Gram-positive bacteria.[13] From the results (Figure 1) obtained, the deleterious effect of LAB on *S. aureus* was mainly due to the production of organic acids, as neutralization of CFS resulted in more than 50% drop in the percentage of inhibition from the initial level. Upon fermentation, the pH of the CFS was reduced to almost 4.0 for all strains (Table 2), which was unfavorable for the growth of *S. aureus*, as its survival requires the pH to be in the range 4.5–9.3.[23] In addition, the lipophilic and undissociated nature of lactic and acetic acids allow the molecules to exhibit antibacterial action through the penetration of bacterial membrane. The higher pH environment in the bacterial cytoplasm will cause the acids to dissociate and interrupt the transport process in the cells by disrupting the proton motive force.[24]

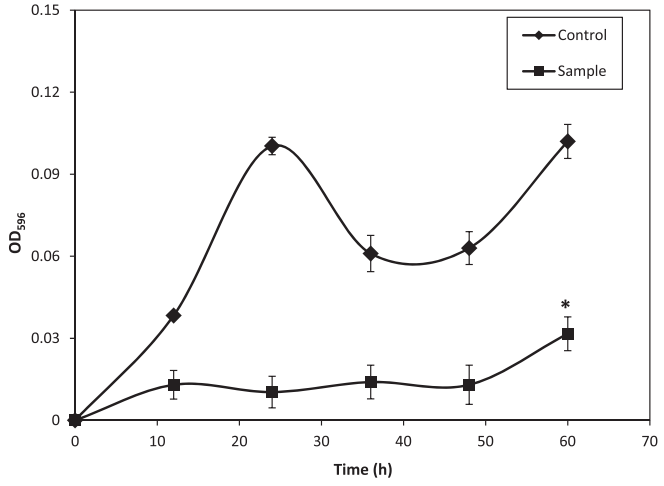

Figure 2 Absorbance measurement (OD_{596}) of the *Staphylococcus aureus* biofilm growth in the presence of *Lactobacillus bulgaricus* FTDC 8611 extracellular extracts (sample) and nonfermented MRS broth (control) up to 60 hours, with 12 hours of interval. Results are expressed as mean ± standard error ($n = 3$). Growth of the biofilm of the control is significantly different from that of the sample, indicated by an asterisk ($p < 0.05$). OD = optical density; MRS = De Man–Rogosa–Sharpe.

Bioactive peptides and/or bacteriocins are important antimicrobial metabolites produced by LAB, which are proteinaceous in nature. Such compounds inhibit specific microorganisms, particularly Gram-positive bacteria.[8] In order to investigate whether the antimicrobial compounds that inhibited the growth of *S. aureus* were proteinaceous, the CFS of selected LAB was neutralized to eliminate the effects of acids and treated with protease. Proteolytic enzymes that degrade proteins present in the CFS would therefore render the proteinaceous antimicrobial compounds ineffective in exerting their bactericidal effect.[22] Among the selected strains, the CFS of two strains, *W. cibaria* BD 1514h (B7) and *L. fermentum* BD 8913f (D10), showed a significant reduction ($p < 0.05$) in the inhibitive action compared to the neutralized CFS (Figure 1). The presence of proteinaceous antimicrobial compounds in these two strains was confirmed. In addition, the inhibitive action of the precipitated protein fractions of all selected strains was postulated to be a result of the concentration of bioactive peptide-like compounds through the salting out method. Many bioactive peptides of LAB were produced in small amounts; therefore, to effectively evaluate the antimicrobial activities of such peptides, one crucial step was to concentrate the CCFS with the ammonium sulfate precipitation method, which was employed in this study.[25] Bioactive peptides including bacteriocins can easily form pores on the cytoplasmic membrane of sensitive cells and disrupt nucleic acids, subsequently leading to ion leakage, loss of proton motive force, and ultimately cell death.[26]

Table 2 Concentration of organic acids produced by LAB in extracellular extracts and changes in pH of MRS broth prior to and upon fermentation for 20 hours at 37°C.

Strain	Organic acid concentration (mmol/mL)			pH	
	Lactic acid	Acetic acid	Total acid	0 h	20 h
Lactobacillus bulgaricus FTDC 8611 (A30)	0.032 ± 0.001^{cA}	0.014 ± 0.001^{bB}	0.046 ± 0.002^{c}	5.33 ± 0.03^{aA}	4.09 ± 0.04^{aB}
Weissella cibaria BD 1514h (B7)	0.035 ± 0.001^{abA}	0.014 ± 0.001^{bB}	0.049 ± 0.002^{a}	5.25 ± 0.01^{bA}	4.09 ± 0.02^{aB}
Lactobacillus fermentum BD 1512n (C5)	0.036 ± 0.001^{aA}	0.013 ± 0.001^{bB}	0.049 ± 0.002^{ab}	5.08 ± 0.02^{dA}	4.00 ± 0.01^{cB}
Lactobacillus fermentum BD 8913f (D10)	0.034 ± 0.001^{abcA}	0.015 ± 0.001^{aB}	0.049 ± 0.002^{a}	5.19 ± 0.01^{cA}	4.02 ± 0.01^{bcB}
Lactobacillus casei BD 1511a (E10)	0.033 ± 0.001^{bcA}	0.014 ± 0.001^{bB}	0.047 ± 0.002^{bc}	5.31 ± 0.02^{aA}	4.06 ± 0.01^{abB}

Results are expressed as mean ± standard deviation ($n = 3$).
[a–d] Different superscripted lowercase letters indicate that the quantities in the same column are significantly different ($p < 0.05$).
[A,B] Different superscripted uppercase letters indicate that the quantities in the same row are significantly different ($p < 0.05$).
LAB = lactic acid bacteria; MRS = De Man–Rogosa–Sharpe.

Table 3 Concentrations of hydrogen peroxide, diacetyl, and protein in the extracellular extracts of lactic acid bacteria cultured in MRS broth for 20 hours at 37°C.

Strain	Concentration		
	Hydrogen peroxide (mg/mL)	Diacetyl (ng/mL)	Protein (mg/mL)
Lactobacillus bulgaricus FTDC 8611 (A30)	0.006 ± 0.001^{ab}	0.171 ± 0.071^{c}	0.203 ± 0.012^{ab}
Weissella cibaria BD 1514h (B7)	0.007 ± 0.001^{a}	4.419 ± 0.444^{a}	0.194 ± 0.008^{abc}
Lactobacillus fermentum BD 1512n (C5)	0.003 ± 0.001^{c}	1.039 ± 0.330^{c}	0.198 ± 0.022^{ab}
Lactobacillus fermentum BD 8913f (D10)	0.004 ± 0.001^{bc}	4.217 ± 0.512^{ab}	0.119 ± 0.001^{d}
Lactobacillus casei BD 1511a (E10)	0.006 ± 0.001^{a}	1.349 ± 0.731^{c}	0.219 ± 0.005^{a}

Results are expressed as mean ± standard deviation ($n = 3$).
[a–d] Different superscripted lowercase letters indicate that the quantities in the same column are significantly different ($p < 0.05$).
MRS = De Man–Rogosa–Sharpe.

The characterization of CFS showed that the antimicrobial compounds produced by LAB, namely organic acids, hydrogen peroxide, and diacetyl, were strain dependent (Tables 2 and 3). *L. bulgaricus* FTDC 8611 (A30), the strongest of all these strains, produced a large amount ($p < 0.05$) of acetic acid and total acids compared to other strains (Table 2). Such data correlated well with the high percentage of inhibition exerted by this strain (Figure 1). The effect of acetic acid (pKa 4.74), although present in a small amount, is more lethal than that of lactic acid because the concentration of undissociated acetic acid is two to four times that of lactic acid at pH 4.0–4.6.[27] The higher percentage ($p < 0.05$) of inhibition exhibited by *L. bulgaricus* FTDC 8611 (A30) was postulated to be a result of the synergistic effect between the overall antimicrobial compounds produced. Two or more inhibitory factors would result in an inhibitory action greater than either of the different factors (synergism).[28] The antagonistic activity of diacetyl occurs through the blocking of enzyme's catalytic site responsible for arginine utilization, rendering the cells incapable of synthesizing essential proteins.[29] Hydrogen peroxide oxidizes targeted bacterial membrane via peroxidation, leaving the cells with increased membrane permeability and denatured metabolic enzymes.[9] The inhibition effect of bacteriocins was also found to increase efficiently via synergism between organic acids and bacteriocins.[7]

Biofilm formation is one of the ways *S. aureus* expresses its pathogenesis through the colonization of infection sites.[3] In the current study, results (Figure 2) showed that *S. aureus* (control) was able to form a biofilm throughout the 60 hours of incubation. Growth pattern of the biofilm was in good agreement with the results of a previous study, which suggested that the biofilm established itself in the first 24 hours of incubation prior to reaching a state of dynamic equilibrium.[30] After 24 hours of incubation, cyclical maturation and dispersal of the biofilm took place as the biofilm reached a critical mass, eventually unable to sustain the bacteria encased within the matrix. Bacteria on the outermost layers of the biofilm would then dissociate from it, leading to better

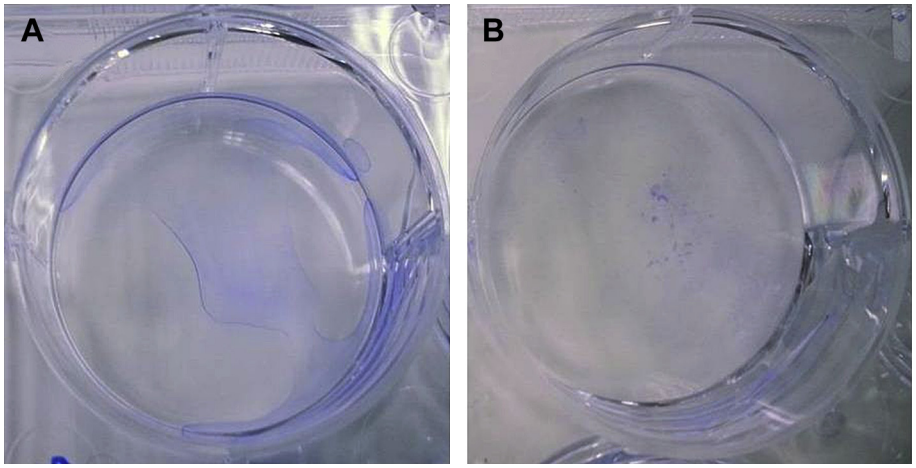

Figure 3 Growth of the (A) biofilm of the control and (B) biofilm of the sample in the polystyrene microplate at 24 hours. The purple color stain on the well was due to crystal violet bound to the biofilm of *Staphylococcus aureus* prior to being solubilized in acetic acid. The intensity of purple color was higher in (A) than in (B), indicating that the growth of biofilm was better in the control (unfermented MRS broth) than in the sample (CFS of strain *Lactobacillus bulgaricus* FTDC 8611). CFS = cell-free supernatant; MRS = De Man–Rogosa–Sharpe.

availability of nutrients to stimulate further growth until the condition became favorable for the bacteria to form the biofilm again, which occurred at 60 hours in this study.

Results of this study (Figure 2) successfully proved that LAB extracts can inhibit the biofilm formation of *S. aureus*; the growth of biofilm in the presence of *L. bulgaricus* FTDC 8611 (A30) CFS was significantly lower ($p < 0.05$) than that in the presence of a control. Antimicrobial compounds in the CFS were believed to work synergistically to halt the growth of the pathogen and even cause death in the cells, rendering the aggregation of cells to form the biofilm unsuccessful. Bacteriocins produced by LAB may alter the physical and chemical conditions of the culture conditions, and thus development of the biofilm becomes unfavourable.[31]

In conclusion, results of the present study illustrated that LAB have the ability to synthesize antimicrobial compounds that can inhibit the growth of the pathogen *S. aureus*. The biofilm formed by *S. aureus* was successfully inhibited by the extracellular extracts of LAB, proving that LAB can serve as a potential alternative in dermatological applications.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dsi.2014.03.001.

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