Ann Microbiol (2013) 63:1047–1055
DOI 10.1007/s13213-012-0561-1

ORIGINAL ARTICLE

Dermal bioactives from lactobacilli and bifidobacteria

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Received: 15 May 2012 / Accepted: 3 October 2012 / Published online: 17 October 2012

Abstract Lactobacilli and bifidobacteria are the most common genera of probiotics with documented potentials on gut health. Recent studies have suggested that such potentials can be extended beyond gut well-being, such as that of dermal health. Our present study aimed to evaluate the production of bioactives that are essential for skin defense, such as lipoteichoic acid, peptidoglycan, hyaluronic acid, sphingomyelinase, lactic acid, acetic acid, and diacetyl, from lactobacilli and bifidobacteria grown in milk. All strains studied showed the presence of LTA in the cell wall fraction, with higher amounts from *Lactobacillus rhamnosus* FTDC 8313 and *Bifidobacterium longum* BL 8643 than other strains studied. Meanwhile, all strains studied showed equal concentrations of cell wall peptidoglycan. Our results showed that all strains studied were capable of producing hyaluronic acid, with higher production by lactobacilli than bifidobacteria. Production of diacetyl was more prevalent from strains of lactobacilli, while bifidobacteria produced higher amounts of acetic acid. Strains of lactobacilli and bifidobacteria studied also produced acid and neutral sphingomyelinase, an enzyme that generates ceramides and subsequent development of physical barriers in the stratum corneum. Our current findings show that bioactive and inhibitive extracts are produced from the fermentation of lactobacilli and bifidobacteria in milk, with potentials for dermal applications.

Keywords Bioactives · Lactobacilli · Bifidobacteria · Dermal · Skin

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Introduction

Lactobacilli and bifidobacteria are the most common genera of probiotics, and have been intensively reported for the treatment or prevention of gastrointestinal disorders. However, emerging clinical studies suggest that numerous strains of probiotic have great potentials beyond gut well-being, including dermal health. Increasing demand for natural formulations for skin care in the market indicate that there is an emerging new potential for probiotics in dermatology. It is estimated that the global probiotics market will grow at a compound annual growth rate of 13 % from 2009–2014 (Koncept Analytics 2010). In general, non-intestinal applications of lactobacilli and bifidobacteria are few and there is little information available on the use of microorganisms generally recognized as safe (GRAS) for the production of bioactive metabolites for skin applications. Natural cell components and metabolites may be the preferred choice in cases where safety and side effects are of concern. Moreover, cell components and metabolites are more stable than viable cells at room temperature, and are thus more suitable for various product developments.

Lactobacilli and bifidobacteria are known to exert various health benefits via the production of antimicrobial compounds that are able to inhibit growth of some pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Tham et al. 2011). They are also capable of producing compounds that are beneficial to the skin such as acetic acid, lactic acid, and diacetyl which could inhibit the invasion of various dermal pathogens (Pasricha et al. 1979; Nagoba et al. 2008; Lanciotti et al. 2003). Production of lactic acid and acetic acid by lactobacilli and bifidobacteria is one of the most important properties contributing to their antimicrobial activities. It has been reported that acetic acid showed excellent bactericidal effect even at low concentration, especially on Gram-negative bacteria, and thus may inhibit opportunistic dermal pathogens. The inhibitory effect of acetic and lactic acids is mainly attributed to interference with essential metabolic

functions, dissipation of cell membrane permeability, and reduction of intracellular pH (Suskovic et al. 2010). Moreover, lactic acid, as one of the α-hydroxy acids, has potential in skin applications, attributed to its ability to improve the stratum corneum barrier function and enhance the production of ceramides by keratinocytes (Rawlings et al. 1996). Diacetyl on the other hand is a product of citrate metabolism and is one of the identified antimicrobial compounds produced by lactobacilli and bifidobacteria during fermentation. It plays a role in controlling the growth of Gram-negative skin bacteria such as *Escherichia coli* by inhibiting arginine utilization (Vandergh 1993).

In addition, lactobacilli and bifidobacteria are also capable of producing bioactive compounds such as sphingomyelinase, hyaluronic acid, peptidoglycan, and lipoteichoic acid which could enhance skin homeostasis, barrier and immune systems (Di Marzio et al. 1999; Chong et al. 2005; Sullivan et al. 2009; Nell et al. 2004). Sphingomyelinase is an enzyme which generates a family of ceramides and phosphorylcholine from glucosylceramide and sphingomyelin precursors for the development of extracellular lipid bilayers, which is important for the physical barrier of the stratum corneum (Jensen et al. 2005). Meanwhile, hyaluronic acid (HA) is a naturally occurring biopolymer in bacteria and in tissues of higher animals. It consists of a basic unit of two sugars, glucuronic acid and N-acetylglucosamine, polymerized into large macromolecules of over 30,000 repeating units. The importance of HA in biological functions of human has been extensively reviewed and has been used in a number of cosmetic applications since the 1960s (Chong et al. 2005). The application of exogeneous HA has been reported to enhance keratinocyte proliferation and aid in wound healing (Price et al. 2007). HA also plays an important role in morphogenesis and tissue repair as well as in the homeostatic turnover of epithelial surfaces (Chong et al. 2005). Lipoteichoic acids (LTA) on the other hand are membrane-anchored molecules in the cell envelopes of Gram-positive bacteria that contribute to the homeostasis of physiochemical surface properties (Fedtke et al. 2007). It has been demonstrated that LTA isolated from non-pathogenic Gram-positive bacteria such as *Lactobacillus plantarum* has anti-inflammatory properties and is less inflammatory than LTA from pathogenic bacteria (Jang et al. 2011). It has also been suggested that LTA might be the stimulatory component responsible for eliciting beta-defensin and LL-37 responses in skin, one of the most common types of antimicrobial peptides participating in the host response against bacterial infections (Sullivan et al. 2009). Peptidoglycan, an essential component of the cell wall of Gram-positive bacteria, has also been suggested to play an important role in skin defence against pathogens by stimulating the innate immunity system via Toll-like receptor-2 (TLR2), leading to secretion of a variety of cytokines and chemokines that are involved in immune responses (Niebuhr et al 2010). Sullivan et al. (2009) has postulated that peptidoglycan might also be one of the stimulatory components responsible for eliciting beta-defensin responses in skin cells, leading to activation of host innate immunity.

The potential benefits of lactobacilli and bifidobacteria are, however, dependent on the selection of strains. We hypothesize that certain strains of both lactobacilli and bifidobacteria could exert dermal benefits via the production of inhibitive and bioactive compounds. To our knowledge, little emphasis has been given on such properties of lactobacilli and bifidobacteria. Thus, the objective of the current study was to evaluate the potential of lactobacilli and bifidobacteria in producing bioactives that are essential for skin defense and dermal health.

Materials and methods

Bacterial cultures

Strains of lactobacilli and bifidobacteria such as *Lactobacillus casei* FTDC 0442, *L. casei* BT 1268, *L. gasseri* CHO 220, *L. acidophilus* FTDC 2333, *L. fermentum* BT 8219, *L. fermentum* FTDC 8312, *L. bulgaricus* FTDC 8611, *L. bulgaricus* FTDC 0411, *L. rhamnosus* FTDC 8313, *L. gasseri* FTDC 8131, *Bifidobacterium longum* BL 8643, *B. longum* BB 8843, *B. bifidum* BB 12, *Bifidobacterium* BB 2142 and *Bifidobacterium* BB 8943 were obtained from the culture collection of School of Industrial Technology, Universiti Sains Malaysia (Penang, Malaysia). The strains were propagated in sterile de Mann, Sharpe (MRS) broth (Hi-Media, Mumbai, India) for three successive times using 10 % (v/v) inoculum and incubated for 24 h at 37 °C prior to use. The sterile MRS broth was supplemented with 0.15 % (w/v) filter-sterilized (0.45 μm) L-cysteine hydrochloride (Hi-Media). Stock cultures were stored at −20 °C in 40 % (v/v) sterile glycerol.

Preparation of extracellular, intracellular and cell wall fractions

Reconstituted skimmed milk (RSM; 8 %w/v) was inoculated with 1 % (v/v) inoculum and fermented for 20 h at 37 °C with continuous shaking at 100 rpm. Fermented RSM was then centrifuged at 10,000 *g* for 15 min at 4 °C. The supernatant (extracellular extract) were filtered (pore size, 0.22 μm) and stored at −20 °C prior to analysis. The sediment was suspended in sterile phosphate buffer saline (PBS), sonicated (2 rounds of 15 min each, duty cycle 50 %, on ice) and recentrifuged at 10,000 *g* for 15 min at 4 °C. The supernatant (intracellular extract) were filtered (pore size, 0.22 μm) and stored at −20 °C prior to analysis. The cell wall-containing sediment was resuspended into sterile PBS. Samples were stored at −20 °C prior to analysis.

Microbial analysis

RSM (8 %, w/v) supplemented with filter-sterilized 0.15 % (w/v) L-cysteine hydrochloride were inoculated with 1 % (v/v) inoculum and fermented at 37 °C, 100 rpm. Growth and viability of lactobacilli and bifidobacteria were determined every 4 h for 24 h using the pour-plate method. MRS agar was supplemented with filter-sterilized 0.15 % (w/v) L-cysteine hydrochloride. Plates for the enumeration of bifidobacteria were incubated in anaerobic jars containing gas generation sachets at 37 °C for 48 h.

Chemical analyses

Lipoteichoic acid (LTA) A 1:1 ratio of n-butanol was added to cell wall fractions and left to stand for 30 min at 25 °C. The mixture was then centrifuged at 10,000 *g* for 15 min at 4 °C to remove phospholipids and amphiphilic substances. The aqueous phase was used for LTA determination. An LTA enzyme-linked immunosorbent assay (ELISA) was used where mouse immunoglobulin G3 (IgG3) monoclonal antibody was directed against the glycerol phosphate moiety of LTA molecule (van Langevelde et al. 1998). Purified LTA of *S. aureus* (Sigma-Aldrich, Steinheim, Germany) were used to generate a standard curve at concentrations of 0–500 ng/mL of PBS. Standard and samples were incubated for 24 h at 25 °C in a 96-well plate. After three washes with 200 μL of PBST (PBS containing 0.05 % Tween 20), the plate was blocked with 150 μL of 0.5 % (w/v) bovine serum albumin (Sigma-Aldrich) in PBST at 37 °C for 1 h. After three times washing, 1.2 μg/mL of mouse IgG3 anti-LTA (Genway, San Diego, CA, USA) was added and incubated for 1 h at 37 °C. The plate was washed three times and incubated with a 4,000-fold-diluted goat anti-mouse IgG-HRP conjugate (Southern Biotech, Birmingham, AL, USA) at 37 °C for 90 min. After three washes, 1 mg/mL of 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich) in 0.1 M sodium acetate buffer (pH6.0) containing 0.006 % (v/v) H_2O_2 were added. The reaction was stopped after 5 min by the addition of 4 N H_2SO_4. The concentration of LTA was determined at 450 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyville, CA, USA).

Peptidoglycan Concentrations of peptidoglycan were assayed using a commercial human peptidoglycan ELISA kit (Novatein Biosciences, Cambridge, MA, USA). Briefly, purified human peptidoglycan antibody was used to coat a 96-well plate. Fifty microliters of diluted samples and standards containing peptidoglycan were added to the wells and incubated for 30 min at 37 °C. After washing five times, 50 μL HRP-conjugate reagent was added and incubated for 30 min at 37 °C to produce an antibody–antigen–enzyme–antibody complex. After washing again five times, 100 μL of TMB substrate solution was added and incubated at 37 °C for 15 min. The reaction was terminated by the addition of 50 μL H_2SO_4 (2 N) solution. Peptidoglycan was determined at 450 nm (SpectraMax; Molecular Devices).

Hyaluronic acid (HA) Hyaluronic acid content in intracellular and extracellular extract was measured using the cetyltrimethylammonium bromide (CTAB) turbidimetric method. Briefly, samples were mixed with 2.5 volumes of absolute ethanol and rested at 4 °C for 1 h. After centrifuging at 10,000 *g* for 15 min at 4 °C, the sediment was dissolved in five volumes of distilled deionized water. CTAB reagent was prepared by dissolving 2.5 g CTAB (Sigma-Aldrich) in 100 mL of 0.2 mol/L NaCl solution. One mililiter of HA standards and samples were mixed gently with 2.0 mL of CTAB reagent. Subsequently, the solutions were left for 10 min and read at 400 nm (Shimadzu, Kyoto, Japan).

Diacetyl Concentrations of diacetyl in intracellular and extracellular extract were determined based on the colorimetric reaction method, using creatine and α-naphthol in an alkaline medium. Briefly, 525 μL of sample was mixed with 325 μL of saturated creatine (Sigma-Aldrich) solution and 150 μL of a solution containing 3 % NaOH and 3.5 % α-naphthol (Merck, Darmstadt, Germany) . After standing at 25 °C for 1 h with light preservation, the absorbance was measured at 525 nm (Shimadzu). A standard curve was established using freshly prepared diacetyl (Sigma-Aldrich) at concentrations of 0–10 ng/mL. The diacetyl and the solution containing 3 % NaOH and 3.5 % α-naphthol were both protected from light.

Lactic and acetic acid Concentrations of lactic and acetic acid were measured using high performance liquid chromatography (HPLC). Samples were filtered through a 0.22-μm MCE syringe filter. The HPLC system (Shimadzu) consisted of a Luna C18 (2) column (150×4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA) and the temperature of the column was maintained at 40 °C. Filtered samples (20 μL) were injected into the HPLC equipped with a UV absorbance detector (Shimadzu) set at 220 nm. Degassed mobile phase (25 mM KH_2PO_4, pH 2.5: methanol, 97:3) was used at a flow rate of 0.3 mL/min. HPLC-grade acetic and lactic acid (Sigma-Aldrich) were used as standards.

Acid and neutral sphingomyelinase Sphingomyelinase activity was determined using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red reagent), a sensitive fluorogenic probe for H_2O_2. A working solution of 100 μM Amplex® Red reagent containing 2 U/mL horseradish peroxidase, 0.2 U/mL choline oxidase, 8 U/mL of alkaline phosphatase, and 0.5 mM sphingomyelin was prepared freshly for detection of neutral sphingomyelinase. Next, 100 μL of the

working solution was added to 100 μL of diluted samples in 96-well plates. Samples were diluted in buffer containing 0.1 M Tris-HCl and 10 mM $MgCl_2$ at pH7.4. Plates were protected from light during incubation at 37 °C for 1 h. Fluorescence was measured using a fluorescence microplate reader (Varian, Santa Clara, CA, USA) at an excitation of 540 nm and emission of 590 nm. Sphingomyelinase from *Bacillus cereus* was used for the generation of a standard curve.

A working solution was prepared freshly for detection of acid sphingomyelinase as mentioned above without addition of 0.5 mM sphingomyelin. Samples were diluted using 50 mM sodium acetate at pH 5.0. Next, 100 μL of diluted samples was added with 10 μL of 5 mM sphingomyelin solution and incubated at 37 °C for 1 h. After incubation, 100 μL of working solution was added and incubated again with light protection for 1 h at 37 °C. Sphingomyelinase from *Bacillus cereus* was used as standard and fluorescence was measured at an excitation of 540 nm and emission of 590 nm.

Statistical analysis

Data analysis was performed using SPSS software (v.14.0; Chicago, IL, USA). One-way ANOVA was used to study the significant differences between sample means, with the significance level at $\alpha=0.05$. Mean comparisons were assessed by Tukey's test. All data presented are mean values obtained from three separate runs ($n=3$), unless stated otherwise.

Results

Growth of bifidobacteria and lactobacilli in RSM

Preliminary growth studies of 15 strains of bifidobacteria and lactobacilli in 8 % reconstituted skimmed milk at 37 °C showed that all strains were able to grow with maximum viable counts ranging between 7.93 and 10.19 $\log_{10}$ CFU/mL (Table 1). All strains reached maximum viable counts upon fermentation for 8–16 h and reached the stationary phase after 20 h. The increase in cell counts for strains of lactobacilli was lower as compared to strains of bifidobacteria. Also, the total cell increase was less than 1 log for certain strains of lactobacilli such as *L. casei* FTDC 0442, *L. acidophilus* FTDC 2333, *L. fermentum* FTDC 8312 and *L. bulgaricus* FTDC 8611. However, strains of lactobacilli showed less decrease of viability throughout the incubation period, whereas bifidobacteria showed a rapid decrease in cell viability after reaching maximum viable counts at 12 h. In this current study, *Lactobacillus casei* BT 1268, *L. rhamnosus* FTDC 8313, *L. gasseri* FTDC 8131, *B. animalis* subsp. *lactis* BB 12 and *B. longum* BL 8643 showed higher growths among the 15 strains studied ($P<0.05$), and were thus selected for further evaluation.

Lipoteichoic acids (LTA)

In this study, concentrations of LTA in extracellular, intracellular and cell wall fractions of bifidobacteria and lactobacilli

Table 1 Growth of bifidobacteria and lactobacilli ($\log_{10}$ CFU/mL)[a] in 8 % (w/v) reconstituted skimmed milk at 37 °C

Strains	Time (h)						
	0	4	8	12	16	20	24
L. casei FTDC 0442 f	7.17±0.02	7.69±0.14	7.93±0.09	7.68±0.11	7.85±0.07	7.84±0.06	7.78±0.03
L. casei BT 1268 abc	7.48±0.08	7.95±0.04	8.11±0.04	8.95±1.27	7.91±0.05	7.85±0.08	8.31±0.05
L. gasseri CHO 220 bcd	7.35±0.03	7.93±0.07	8.49±0.29	8.20±0.19	7.96±0.03	8.04±0.10	8.01±0.17
L. acidophilus FTDC 2333 cd	7.31±0.20	7.92±0.06	8.16±0.06	7.94±0.02	7.90±0.06	7.96±0.05	8.25±0.40
L. fermentum BT 8219 de	7.11±0.07	7.52±0.12	7.98±0.10	8.23±0.09	8.16±0.07	8.05±0.09	8.26±0.32
L. fermentum FTDC 8312 f	7.21±0.16	7.36±0.11	7.42±0.14	8.13±0.25	7.84±0.14	7.71±0.11	7.54±0.16
L. bulgaricus FTDC 8611 f	7.08±0.02	7.08±0.06	7.74±0.07	8.02±0.07	7.91±0.02	7.94±0.18	7.30±0.16
L. bulgaricus FTDC 0411 f	7.17±0.03	6.93±0.15	7.95±0.23	8.66±0.06	7.91±0.24	7.70±0.06	7.61±0.13
L. rhamnosus FTDC 8313 a	7.32±0.09	7.86±0.06	8.18±0.02	9.24±0.14	8.15±0.04	8.59±0.02	8.12±0.05
L. gasseri FTDC 8131 abcd	7.57±0.01	7.81±0.07	8.05±0.05	7.89±0.04	8.38±0.13	8.68±0.14	7.99±0.05
B. longum BL 8643 ab	7.39±0.01	7.61±0.06	7.68±0.10	9.51±0.05	8.24±0.05	8.07±0.04	8.36±0.34
B. longum BB 8843 de	6.90±0.03	7.07±0.12	7.59±0.10	10.19±0.08	7.91±0.06	7.89±0.11	7.73±0.06
B. animalis subsp. *lactis* BB 12 ab	7.34±0.03	7.80±0.06	7.75±0.02	9.77±0.24	7.88±0.04	7.85±0.18	8.53±0.28
Bifidobacterium BB 2142 ef	6.49±0.15	6.89±0.04	7.51±0.09	9.46±0.03	7.98±0.46	7.58±0.28	8.21±0.36
Bifidobacterium BB 8943 bcd	7.09±0.06	7.19±0.12	7.87±0.30	10.09±0.10	7.87±0.02	7.93±0.27	7.94±0.50

[a] Results are expressed as mean±standard deviation. Values are means of duplicates from three separate runs ($n=3$)

Means of strains with different lowercase letters are significantly different ($P<0.05$)

were evaluated (Table 2). Results showed that concentrations of LTA in cell wall fraction were higher ($P<0.05$) as compared to the intracellular and extracellular fractions. Meanwhile, *L. rhamnosus* FTDC 8313 and *B. longum* BL 8643 showed significantly higher ($P<0.05$) amounts of LTA in the cell wall fraction as compared to the other strains studied, while all strains produced similar amounts of LTA in the extracellular extracts. Also, our data showed that the intracellular concentration of LTA of *L. gasseri* FTDC 8131 was significantly higher ($P<0.05$) than othe ther strains studied.

Peptidoglycan

Peptidoglycan content in extracellular, intracellular and cell wall fractions of bifidobacteria and lactobacilli are shown in Table 2. Peptidoglycan content in the cell wall fraction was higher ($P<0.05$) as compared to the intracellular and extracellular fractions. Our current study also showed that there was no significant difference ($P>0.05$) in the concentrations of cell wall peptidoglycan among the strains studied. However, the amounts of peptidoglycan detected in both intracellular and extracellular extracts varied among the strains studied. The results from this study also showed that concentrations of peptidoglycan in both intracellular and extracellular fractions were more prevalent ($P<0.05$) in strains of bifidobacteria compared to lactobacilli studied.

Hyaluronic acid

Data from our present study showed that all strains were able to produce varying concentrations of HA (Table 3), with higher ($P<0.05$) concentrations in the extracellular fraction as compared to the intracellular fraction, except for *L. casei* BT 1268 which showed a nonsignificant difference in concentrations of HA in both fractions. The results from this study also showed that concentrations of HA were higher in lactobacilli compared to bifidobacteria, where *L. gasseri* FTDC 8131 and *L. casei* BT 1268 showed higher ($P<0.05$) production of HA intracellularly, while *L. gasseri* FTDC 8131 contained a higher ($P<0.05$) amount of HA extracellularly, as compared to other strains studied.

Diacetyl

Our current data showed that all strains studied produced diacetyl (Table 3), with higher ($P<0.05$) concentrations in the extracellular extracts compared to the intracellular fractions, except for *L. rhamnosus* FTDC 8313. Data from our present study also showed that diacetyl production was higher ($P<0.05$) in lactobacilli compared to bifidobacteria. When comparing the strains studied, *Lactobacillus casei* BT 1268 contained significantly higher ($P<0.05$) amounts of diacetyl in both intracellular and extracellular extracts while *B. animalis* subsp. *lactis* BB 12 and *B. longum* BL 8643 showed lower ($P<0.05$) concentrations in both fractions. Our results showed that *B. animalis* subsp. *lactis* BB 12 and *B. longum* BL 8643 produced detectable amounts of diacetyl but at lower concentrations compared to lactobacilli.

Lactic and acetic acid

Our data showed that all strains produced lactic and acetic acids, with varying amounts in the intracellular

Table 2 Concentrations of lipoteichoic acid and peptidoglycan in extracellular and intracellular extracts and cell wall fraction of bifidobacteria and lactobacilli cultured in 8 % (w/v) reconstituted skimmed milk (RSM) for 20 h at 37 °C

Strains	Intracellular	Extracellular	Cell wall
Concentrations of lipoteichoic acid (ng/mL of RSM)			
L. casei BT 1268	18.08±22.14 bB	18.98±22.70 aB	188.25±131.60 cA
B. animalis subsp. *lactis* BB 12	29.93±9.13 abB	13.25±15.68 aB	387.75±34.89 bcA
B. longum BL 8643	42.43±5.09 abB	32.10±7.38 aB	523.50±98.73 abA
L. rhamnosus FTDC 8313	43.68±7.04 abB	24.75±17.55 aB	639.75±102.04 aA
L. gasseri FTDC 8131	48.33±9.36 aB	35.33±8.75 aB	413.00±85.45 bA
Concentrations of peptidoglycan (ng/mL of RSM)			
L. casei BT 1268	12.90±1.83 bB	11.69±0.71 abB	399.70±60.70 aA
B. animalis subsp. *lactis* BB 12	16.77±0.41 aB	15.29±2.06 aB	367.30±103.10 aA
B. longum BL 8643	18.30±1.67 aB	16.27±4.14 aB	390.60±119.80 aA
L. rhamnosus FTDC 8313	6.72±1.30 cB	10.88±1.54 abB	223.50±64.90 aA
L. gasseri FTDC 8131	12.04±1.01 bB	6.83±1.25 bB	399.00±116.20 aA

Results are expressed as mean±standard deviation. Values are means of duplicates from three separate runs ($n=3$)

Means in the same column with different lowercase letters are significantly different ($P<0.05$)

Means in the same row with different uppercase letters are significantly different ($P<0.05$)

Table 3 Concentrations of hyaluronic acid and diacetyl in extracellular and intracellular extracts of bifidobacteria and lactobacilli cultured in 8 % (w/v) reconstituted skimmed milk (RSM) for 20 h at 37 °C

Strains	Intracellular	Extracellular
Concentrations of hyaluronic acid (mg/mL of RSM)		
L. casei BT 1268	0.28±0.05 abA	0.36±0.10 cA
B. animalis subsp. *lactis* BB 12	0.06±0.01 cB	0.39±0.08 cA
B. longum BL 8643	0.09±0.03 cB	0.39±0.07 cA
L. rhamnosus FTDC 8313	0.23±0.02 bB	0.99±0.16 bA
L. gasseri FTDC 8131	0.35±0.06 aB	1.42±0.15 aA
Concentrations of diacetyl (mg/mL of RSM)		
L. casei BT 1268	13.51±1.54 abB	17.80±0.62 aA
B. animalis subsp. *lactis* BB 12	2.07±0.53 cB	4.63±0.73 dA
B. longum BL 8643	2.22±0.53 cB	4.48±0.64 dA
L. rhamnosus FTDC 8313	15.04±1.51 aA	8.13±0.52 cB
L. gasseri FTDC 8131	11.12±1.02 bA	14.02±2.81 bA

Results are expressed as mean±standard deviation. Values are means of duplicates from three separate runs (n=3)

Means in the same column with different lowercase letters are significantly different (P<0.05)

Means in the same row with different uppercase letters are significantly different (P<0.05)

and extracellular fractions (Table 4). Concentrations of lactic and acetic acids in extracellular fraction were higher (P<0.05) than the intracellular fraction. There was no significant difference in the concentrations of intracellular lactic acid among the strains studied, whereas, for extracellular lactic acid, *L. rhamnosus* FTDC 8313 showed significant higher (P<0.05) concentrations as compared to the other strains studied. The results from the current study also showed that *B. longum* BL 8643 produced higher (P<0.05) concentrations of intracellular and extracellular acetic acid followed by *B. animalis* subsp. *lactis* BB 12. Also, data from current study showed that lactobacilli produced higher (p<0.05) concentrations of lactic acid than acetic acid.

Acid and neutral sphingomyelinase

Our results showed that all strains studied produce acid and neutral sphingomyelinase, and are detected in both extracellular and intracellular extracts (Table 5). However, enzyme activity of neutral sphingomyelinase was higher (P<0.05) than that of acid sphingomyelinase. Data from our current study indicated that *L. gasseri* FTDC 8131 showed higher extracellular acid sphingomyelinase activity compared to the other strains studied (P<0.05), while all strains studied contained equal activity of acid sphingomyelinase intracellularly. Our results demonstrated that lactobacilli showed higher (P<0.05) neutral sphingomyelinase activity extracellularly, while bifidobacteria showed higher (P<0.05) neutral sphingomyelinase activity intracellularly.

Discussion

Production of bioactive metabolites by microorganisms are often growth-associated, and it has been reported that most bioactive production takes place upon cessation of exponential growth (Carvalho et al. 2010). In this study, *Lactobacillus casei* BT 1268, *L. rhamnosus* FTDC 8313, *L. gasseri* FTDC 8131, *B. animalis* subsp. *lactis* BB 12 and *B. longum* BL 8643 showed the highest growth among the 15 strains studied, and thus were selected for evaluation on bioactive and inhibitive dermal compounds during growth in the stationary phase.

Lipoteichoic acids are part of the microbial membrane component; thus, a higher amount is normally detected from the cell wall fraction. Despite being a membrane component, LTA were also detected in the intracellular and extracellular fractions. It has been reported that LTA are released spontaneously into the culture medium during growth in the stationary phase (van Langevelde et al. 1998). The concentrations of cellular LTA have also been reported to be strain-dependent and growth-associated. In addition, adaptation of different strains to growth conditions would lead to important variations of the cellular envelope, and subsequently cause variations in the amount of LTA produced (Machado et al. 2004). Our current study demonstrated that all strains contain a total LTA amount ranging from 225.31 to 708.18 ng/mL. It

Table 4 Intracellular organic acid content and concentrations of organic acids in 8 % (w/v) reconstituted skimmed milk (RSM) fermented by bifidobacteria and lactobacilli for 20 h at 37 °C

Strains	Concentration of organic acids (mg/mL of RSM)	
	Intracellular	Extracellular
Lactic acid		
L. casei BT 1268	0.61±0.17 aB	2.95±0.57 bA
B. animalis subsp. *lactis* BB 12	0.75±0.24 aB	3.92±0.40 abA
B. longum BL 8643	0.60±0.12 aB	2.90±0.30 bA
L. rhamnosus FTDC 8313	0.51±0.18 aB	4.24±0.27 aA
L. gasseri FTDC 8131	0.59±0.19 aB	3.52±0.36 abA
Acetic acid		
L. casei BT 1268	0.38±0.12 bB	1.56±0.25 cA
B. animalis subsp. *lactis* BB 12	0.38±0.15 bB	2.29±0.17 abA
B. longum BL 8643	0.82±0.12 aB	2.50±0.19 aA
L. rhamnosus FTDC 8313	0.18±0.08 bB	1.82±0.10 bcA
L. gasseri FTDC 8131	0.13±0.04 bB	1.71±0.26 cA

Results are expressed as mean±standard deviation. Values are means of duplicates from three separate runs (n=3)

Means in the same column with different lowercase letters are significantly different (P<0.05)

Means in the same row with different uppercase letters are significantly different (P<0.05)

Table 5 Acid and neutral sphingomyelinases activity in extracellular and intracellular extracts of bifidobacteria and lactobacilli cultured in 8 % (w/v) reconstituted skimmed milk (RSM) for 20 h at 37 °C

Strains	Sphingomyelinase activity (mU/mL of RSM)	
	Intracellular	Extracellular
Acid sphingomyelinase		
L. casei BT 1268	0.22±0.02 aB	0.55±0.08 bA
B. animalis subsp. *lactis* BB 12	0.19±0.03 aB	0.50±0.13 bA
B. longum BL 8643	0.25±0.09 aB	0.48±0.11 bA
L. rhamnosus FTDC 8313	0.28±0.06 aB	0.64±0.15 bA
L. gasseri FTDC 8131	0.27±0.04 aB	1.25±0.17 aA
Neutral sphingomyelinase		
L. casei BT 1268	1.16±0.05 aB	2.40±0.63 aA
B. animalis subsp. *lactis* BB 12	1.29±0.40 aA	0.66±0.12 bB
B. longum BL 8643	0.92±0.17 aA	0.94±0.21 bA
L. rhamnosus FTDC 8313	0.35±0.14 bB	1.10±0.12 bA
L. gasseri FTDC 8131	1.31±0.25 aB	2.57±0.61 aA

Results are expressed as mean±standard deviation. Values are means of duplicates from three separate runs (n=3)

Means in the same column with different lowercase letters are significantly different (P<0.05)

Means in the same row with different uppercase letters are significantly different (P<0.05)

is demonstrated that exposure of LTA from non-pathogenic bacteria at the epithelial surface increases skin mast cell antimicrobial activity via the activation of TLR2 (Wang et al. 2012). It has also been reported that LTA at a concentration of 100 ng/mL or higher significantly increased the expression of antimicrobial peptides in epithelial tissue (Nell et al. 2004), suggesting that our current strains of lactobacilli and bifidobacteria contained sufficient amounts of LTA to increase dermal cellular defence against bacterial infection. Thus, it is postulated that LTA isolated from our current strains of lactobacilli and bifidobacteria could be potentially used as bioactive ingredients in cosmeceutical application.

Peptidoglycan is also part of the membrane component; hence, a higher amount is normally detected from the cell wall fraction compared to the intracellular and extracellular fractions. While peptidoglycan is especially abundant in the cell wall of Gram-positive bacteria, our results showed that it was also present in the extracellular and intracellular fractions of lactobacilli and bifidobacteria. The lower amounts of intracellular and extracellular peptidoglycan detected could be due to fragments of peptidoglycan being released into the medium during cell growth and elongation. It has been previously stated that peptidoglycan is steadily broken down by peptidoglycan-cleaving enzymes during cell growth, especially during the exponential phase, where a cell wall turnover process occurs, resulting in the release of peptidoglycan fragments from the wall (Reith et al. 2011). It has also been reported that the turnover rate and the amount of cell wall material vary among species and even strains. Reith et al. (2011) reported that the magnitude of cell wall turnover is also dependent on the growth conditions. In addition, the capability of microorganisms in the recycling of cell wall material also affected the amount of peptidoglycan detected in the extracellular and intracellular extracts (Litzinger et al. 2010). Our current study demonstrated that all strains contain a total peptidoglycan amount ranging from 0.241 to 0.425 μg/mL. Although larger amounts of peptidoglycan, in the range of 10–100 μg/mL are necessary to stimulate cellular responses, peptidoglycan has also been reported to be effective at lower concentrations, via synergism with LTA (Yang et al. 2001). It is postulated that peptidoglycan at concentrations as low as 0.24 μg/mL in the presence of LTA is sufficient to induce production of antimicrobial peptides in keratinocytes.

HA is synthesized intracellularly according to the proposed biosynthetic pathway by Matsubara et al. (1991) and transported out across the cellular membrane upon synthesis. Thus, the varying concentrations of HA in the intracellular and extracellular extracts among strains studied would be dependent on the efficiency of transport across the membrane. In addition, it has been suggested that biosynthesis of HA was dependent on the glucose uptake of the microorganism (Cooney et al. 2008). Our current study showed that lactobacilli were able to produce higher amounts of HA. Parche et al. (2006) revealed that, unlike many other bacteria, glucose utilization of some bifidobacteria was impaired in the presence of glucose and lactose such as in milk, and thus may have resulted in the lower production of HA. In our current study, strains of lactobacilli and bifidobacteria were able to produce HA at concentrations ranging from 0.4 to 1.4 mg/mL. Hyaluronic acid has been used as an ideal bio-component in dermatological and pharmaceutical products, mainly due to its remarkable rheological, hygroscopic and viscoelastic properties which are relevant for dermal tissue function. It is worth noting that, in this current study, *L. rhamnosus* FTDC 8313 and *L. gasseri* FTDC 8131 produced HA at concentrations of more than 1 mg/mL. Kobayashi and Terao (1997) reported that HA at concentration of 1 mg/mL enhanced the release of interleukin-1β, which is responsible for the secretion of RNase 7, which is an antimicrobial peptide that enhances the innate immune defense system of keratinocytes.

Citrate utilization and production of diacetyl among lactobacilli has been extensively studied over the past few decades, and these studies showed that homofermentative species produced diacetyl more readily and in larger volumes than the heterofermenters (Christensen and Pederson 1958). Interestingly, our results showed that the heterofermenter, *L. casei* BT 1268 strains produced higher amounts of diacetyl than the homofermenter, *L. gasseri* FTDC 8131.

Østlie et al. (2003) reported that the production of diacetyl through citrate metabolism was dependent on the genus of bacteria and the growth conditions, while Christensen and Pederson (1958) has reported that the production of diacetyl was dependent on the different strains within the same species. The difference in concentrations of diacetyl in extracellular and intracellular extracts has been attributed to different efficiencies of transport across the cellular membrane. It has been reported that the limiting step for the utilization of citrate is the requirement for specific transporters, which facilitate its intake into the cells prior to production of diacetyl (Quintans et al. 2008). Also, this limiting step and transport efficiency vary between microorganisms, which support the varying concentrations among the strains studied, for both intracellular and extracellular extracts. Our results showed that all strains were able to produce diacetyl at total concentrations ranging from 6.70 to 31.31 mg/mL. It has been reported that diacetyl exhibited antimicrobial activity against Gram-positive skin pathogens such as *Staphylococcus aureus* at concentrations as low as 3 mg/mL (Lanciotti et al. 2003), indicating that the amount of diacetyl produced by our current strains of lactobacilli and bifidobacteria have the potential to exhibit dermal antimicrobial activities.

Lactic and acetic acids are generated inside the cell via carbohydrate catabolism and exported out through the membrane by citrate transporter to maintain cell homeostasis (Magni et al. 1999). Lactobacilli metabolize carbohydrates either homofermentatively or heterofermentatively to produce lactic acid and acetic acid as predominant end-products, with at least 50–85 % lactic acid. Bifidobacteria, on the other hand, metabolize carbohydrates via the bifidus pathway to produce more acetic acid than lactic acid (Østlie et al. 2003). However, our results showed that bifidobacteria produced more lactic acid than acetic acid. Bifidobacteria have been reported to alter their metabolic pathways, depending on the availability of carbon sources, and thus may result in higher production of lactic acid than acetic acid (Palframan et al. 2003). In this study, all strains of lactobacilli and bifidobacteria were able to produce lactic acid at total concentrations ranging from 3.50 to 4.75 mg/mL. Beneficial effects of lactic acid on skin such as improving skin barrier function, hydration and lightening have been extensively reported. It is also reported that topical application of lactic acid at low concentrations of 0.1–1 % could exhibit antibacterial activity against most dermal pathogenic bacteria (Pasricha et al. 1979). Our current study also showed that strains of lactobacilli and bifidobacteria were able to produce acetic acid at total concentrations ranging from 1.84 to 3.32 mg/mL. Nagoba et al. (2008) reported that topical application of acetic acid at a low concentration of 0.5 % successfully eliminated *Pseudomonas aeruginosa* from burns and soft tissue wounds of 14 out of 16 patients within 2 weeks treatment. Low concentrations of acetic acid in the range of 0.25–0.5 % were also shown to exert bactericidal effects against Gram-positive and Gram-negative microorganisms proliferating at sites of skin infections (Landis 2008).

Neutral sphingomyelinase are cell membrane-associated and are important for cell signaling during permeability barrier repair by enhancing the accumulation of ceramide (Jensen et al. 2005). It is also detected in bacteria, yeast and mammalian cells, with great variations in sphingomyelinase activity among different bacterial strains (Di Marzio et al. 1999). In an in vitro study, ceramide reportedly increased in keratinocytes that were co-cultured with sonicated cells of *Streptococcus thermophilus* (Di Marzio et al. 1999). The results from our current study showed that strains of lactobacilli and bifidobacteria were able to produce acid and neutral sphingomyelinase with enzyme activities ranging from 0.7 to 1.5 mU/mL and 1.4 to 3.9 mU/mL, respectively. It has been suggested that the increased level of ceramide was attributed to sphingomyelinase (>0.1 mU/mL) obtained from sonicated cells of *Streptococcus thermophilus* (Di Marzio et al. 1999). Thus, it is postulated that the concentration of sphingomyelinase in our strains of lactobacilli and bifidobacteria may be sufficient to also promote ceramide production in skin cells, thus improving skin barrier properties.

In conclusion, our current findings showed that bioactive and inhibitive extracts are produced from the fermentation of lactobacilli and bifidobacteria in milk, with potential in dermal applications. The results from our study expanded the potential of lactobacilli and bifidobacteria beyond gut health and beyond the need of viable cells, where topical applications of cellular extracts without viable cells may also present beneficial dermal effects.

Acknowledgements This work was supported by the FRGS grant (203/PTEKIND/6711239) provided by Ministry of Higher Education, Malaysia and USM Fellowship provided by Universiti Sains Malaysia.

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