Influence of tea extract supplementation on bifidobacteria during soymilk fermentation

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Abstract
In this study, the influence of tea extract (TE) supplementation on the viability and membrane lipid compositions of Bifidobacterium was investigated. Fermented soymilk-tea (SMT) was produced by culturing selected bifidobacteria in soymilk supplemented with green or black TE. Culturability of four bacteria in the presence of various concentrations of TE was examined by plate count method. Bifidobacterium longum CSCC 5089 (BL5089) and B. longum CSCC 5022 (BL5022) were selected for further study based on their sensitivity to TE. The effect of TE supplementation on bacterial cell viability and integrity was assessed by flow cytometry in combination with fluorescence probes. Total lipids of bacterial cell were extracted using an enzyme-assistant extraction method. Fatty acids (FAs) were determined and quantified by GC-MS. Phospholipids (PLs) were separated by high performance thin-layer chromatography (HPTLC) and their relative abundances were determined by densitometry. Total tea phenolic content (TTP) in SMTs with varying concentrations of TE was quantified by HPLC. Among the four Bifidobacterium monitored, TE only significantly inhibited BL5089 (p < 0.01) in a dose-dependent manner, with minimum inhibition concentrations (MICs) determined to be 15.45 mg/mL TTP for green TE and 7.34 mg/mL TTP for black TE. Flow cytometric analysis revealed different staining patterns of cell populations and compromise in cell integrity upon exposure to high concentrations of TE. Results from GC–MS showed that unsaturated to saturated FA ratios significantly decreased (p < 0.01) in the membrane of BL5089 cells upon TE exposure. Separation of PLs by HPTLC showed dramatic alterations in phosphatidylcholine and phosphatidylglycerol contents due to TE treatment.

1. Introduction

Bifidobacterium is one generally-recognized genre of probiotics for their capacity to restore the balance of gut microbiota and to deliver other health benefits to consumers (Picard et al., 2005). According to FAO/WHO (2002), probiotics are “live microorganisms which, when consumed in adequate amounts, confer a health effect on the host”. In addition to the “adequate amounts” of intake, probiotic viability at the point of consumption is also essential for efficacy consideration (Sanders, 2000).

Nowadays, mounting evidence of the health-protective effect of dietary consumption of phenolic compounds attracts more consumers’ attention. However, when probiotics interact with dietary phenolic compounds, their viability and physiological characteristics may be affected (Parkar et al., 2008). Tea is known for its high amount of polyphenols, with catechins and theaflavins being the major phenolic components in green tea (GT) and black tea (BT), respectively (Li et al., 2013). Besides its special taste and aroma, the potential of tea as antioxidant, anti-carcinogenic and anti-inflammatory agents (Khan and Mukhtar, 2007) makes it one of the world’s most popular beverages. In addition, it has been found that tea polyphenols (TPs) are able to promote the growth of commensal bacteria, such as bifidobacteria and yogurt microflora (Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus) (Ankolekar et al., 2011; Jaziri et al., 2009; Najgebauer-Lejko et al., 2011), function as prebiotics (Tzonouis et al., 2008) or redox potential reducing agents (Gaudreau et al., 2013). TPs have also been reported to act as inhibitors to food-borne pathogens and viruses (Lee et al., 2006; Perumalla and Hettiarachchy, 2011; Yi et al., 2010). Tea phenolics exert bactericidal effects mainly through damaging cytoplasmic membrane (Shimamura et al., 2007; Sivarooban et al., 2008), binding to or altering cell wall-anchored proteins (Nakayama et al., 2012) and lipid bilayer (Hashimoto et al., 1999; Ikigai et al., 1993), and altering membrane fluidity and permeability (Yi et al., 2010).

Soymilk has received intense attention and popularity worldwide as a healthy dairy substitute and it is rich in isoflavone. As we reported recently (Zhao and Shah, 2014), S. thermophilus ASCC 1275, L. delbrueckii ssp. bulgaricus ASCC 859 and Bifidobacterium longum CSCC 5089 (BL5089) were able to grow well in soymilk. The addition of TE significantly inhibited (p < 0.05) the growth of BL5089 compared with the control while it had no harmful effect on yogurt starters.
Inhibition of bifidobacteria by polyphenols was also reported by Tabasco et al. (2011). Hence, it is worth examining whether some other bifidobacteria will be affected by tea components and the bactericidal mechanism. According to Puupponen-Pimiä et al. (2001) and Tabasco et al. (2011), higher resistance to polyphenolic compounds in some lactic acid bacteria (LAB) than other Gram-positive species, such as Bifidobacterium, is attributed to metabolism of polyphenols into monomeric phenolics. However, to our knowledge, no data is available on such metabolic activity in Bifidobacterium. Moreover, there is limited information on the mechanism of how tea phenolic compounds influence the growth and its effect on cell structures of bifidobacteria. In this study, four Bifidobacterium strains were exposed to varying concentrations of tea extract (TE) in soymilk. The influence of TE supplementation on the viability of bacteria was examined by both the conventional plate count method and flow cytometric analysis. Compositions of cell membrane fatty acids and phospholipids were also monitored. As far as we know, this is the first mechanistic study on the inhibitory effect of Bifidobacterium during fermentation of a soymilk-tea (SMT) beverage.

2. Microorganisms and methods

2.1. Microorganisms and culture conditions

B. longum CSCC 5022 (BL5022), B. longum CSCC 5089 (BL5089), Bifidobacterium bifidum CSCC 5286 (BB5286) and B. bifidum BB-12 (BB12) were used for the production of fermented SML. Each organism was previously stored at −80 °C. Multiple transfers were performed to produce active cultures since storage at −80 °C would result in bacteria with longer lag phase. For activation, 10 mL aliquots of sterile MRS broth, the activated organisms were transferred into sterile soymilk or soymilk-tea. The activated cultures (3%, v/v) were transferred into 10 mL of fermented SML or SMT were referred to as FS or FST, respectively. Samples were incubated at 37 °C for 24 h and incubated at 37 °C for 18 h. After the second transfer in MRS broth, the activated organisms were transferred into sterile soymilk for another two transfers at 10% (v/v). Activated cultures were used for fermentation.

2.2. Preparation of soymilk

Soymilk was prepared as per the method of Donkor and Shah (2007) with some modifications. In brief, soymilk containing lactose (SML) was made by dissolving 4% (w/v) soy protein isolate (DuPont, Shanghai, China), 1% (w/v) α-lactose (Sigma Chemical Co., St. Louis, MO, U.S.A.) were inoculated with 2% (v/v) of each organism and incubated at 37 °C for 24 h. Soymilk-containing lactose (SML) was used as a control and 2 mL of a control sample was used for normal concentration (1×) of TE. Double (2×), triple (3×), quadruple (4×) and quintuple (5×) concentrations of TE supplemented to SML were prepared by dissolving TE in 1/2, 1/3, 1/4 and 1/5 SML volume that was used for normal concentration, respectively.

2.3. Preparation of tea extract (TE)

Green tea (Zhuyeqing tea, Sichuan Province, China) and black tea (Dianhong, Yunnan Province, China) were purchased from local tea retailers. TE was prepared as per the method of Zhao and Shah (2014) with minor modifications. In brief, tea leaf powder (2%, w/v, corresponding to the strength of a normal cup of tea) according to Yam et al. (1997) was infused in boiling deionized water for 10 min. Green or black TE were produced by first suction filtered through triple-layered Whatman #1 filter paper twice and then filter-sterilized with 0.22-μm membrane (Millipore, Bedford, MA, U.S.A.). The sterilized filtrates were collected in 50 mL sterile tubes and frozen at −80 °C before freeze-drying using a Virtis freeze mobile (Virtis Co., Gardiner, U.S.A.). The freeze-dried TE powder was stored at −20 °C for further use. TE power was dissolved in the same volume of SML as that before freeze-drying to produce SMT containing “normal concentration (1×)” of TE. Double (2×), triple (3×), quadruple (4×) and quintuple (5×) concentrations of TE supplemented to SML were prepared by dissolving TE in 1/2, 1/3, 1/4 and 1/5 SML volume that was used for normal concentration, respectively.

2.4. Preparation of fermented soy-milk tea and enumeration of viable cell by plate count method

The activated cultures (3%, v/v) were transferred into 10 mL of SML or SMT with green TE and black TEs both at normal and double concentrations. Samples were incubated at 37 °C for 24 h and fermented SML or SMT were referred to as FS or FST, respectively. Upon completion of fermentation, viable cells were enumerated using cys-MRS agar by pour plate method. Plates were incubated at 37 °C for 24 h in an anaerobic jar (BD GasPak™, Sparks, MD, U.S.A.).

2.5. Extraction and HPLC quantification of tea polyphenols

A 2 mL aliquot of FS or FST with varying concentrations of TE was mixed with 4 mL 80% methanol, vortexed for 1 min and centrifuged at 5000 × g for 10 min at 20 °C. The non-inoculated SMT or SML incubated at 37 °C for 24 h was used as a control and 2 mL of a control sample was mixed with 4 mL of water–methylene–acetic acid (20:78:2, v/v). The precipitate was further extracted twice with 2 mL of 80% methanol and the supernatants were combined. All solvents were of HPLC-grade (Fisher Scientific, Pittsburgh, PA, U.S.A.) and pooled supernatants were filtered through 0.45-μm hydrophilic filter (Corning, N.Y., U.S.A.) before loaded onto a HPLC column.

Table 1

<table>
<thead>
<tr>
<th>CF</th>
<th>GA</th>
<th>EGC</th>
<th>C</th>
<th>EGCg</th>
<th>EC</th>
<th>ECG</th>
<th>TF1</th>
<th>TF2</th>
<th>TF3</th>
<th>Total tea phenolics</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT1X</td>
<td>13.39</td>
<td>0.92</td>
<td>1.48</td>
<td>0.22</td>
<td>1.07</td>
<td>1.82</td>
<td>0.47</td>
<td>N.D.</td>
<td>N.D.</td>
<td>9.98</td>
</tr>
<tr>
<td>GT2X</td>
<td>25.53</td>
<td>1.82</td>
<td>2.57</td>
<td>0.44</td>
<td>2.11</td>
<td>4.12</td>
<td>0.99</td>
<td>N.D.</td>
<td>N.D.</td>
<td>12.05</td>
</tr>
<tr>
<td>GT3X</td>
<td>37.86</td>
<td>2.30</td>
<td>4.80</td>
<td>0.69</td>
<td>3.69</td>
<td>5.86</td>
<td>1.50</td>
<td>N.D.</td>
<td>N.D.</td>
<td>18.85</td>
</tr>
<tr>
<td>GT4X</td>
<td>51.16</td>
<td>3.07</td>
<td>6.16</td>
<td>0.97</td>
<td>4.67</td>
<td>9.24</td>
<td>2.03</td>
<td>N.D.</td>
<td>N.D.</td>
<td>26.15</td>
</tr>
<tr>
<td>BT1X</td>
<td>12.42</td>
<td>0.50</td>
<td>0.52</td>
<td>0.11</td>
<td>0.04</td>
<td>0.13</td>
<td>0.08</td>
<td>0.48</td>
<td>0.20</td>
<td>0.36</td>
</tr>
<tr>
<td>BT2X</td>
<td>23.11</td>
<td>1.20</td>
<td>1.02</td>
<td>0.22</td>
<td>0.14</td>
<td>0.26</td>
<td>0.17</td>
<td>0.91</td>
<td>0.41</td>
<td>0.67</td>
</tr>
<tr>
<td>BT3X</td>
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<td>1.38</td>
<td>0.37</td>
<td>0.27</td>
<td>0.49</td>
<td>0.25</td>
<td>1.31</td>
<td>0.65</td>
<td>0.92</td>
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<tr>
<td>BT4X</td>
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<td>1.89</td>
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<td>0.37</td>
<td>1.75</td>
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</tr>
<tr>
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<td>2.37</td>
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<td>0.44</td>
<td>0.99</td>
<td>0.48</td>
<td>2.21</td>
<td>1.07</td>
<td>1.49</td>
</tr>
</tbody>
</table>

Data is presented as mean (mg/mL) ± SEM of three independent fermentation products.

Abbreviations of phenolic compound: GA: gallic acid; EGC: (-)-epigallocatechin; C: catechin; EGCg: (-)-epigallocatechin gallate; EC: (-)-epicatechin; ECG: (-)-epicatechin gallate; TF-1: theaflavin; TF-2: theaflavin-3-gallate; TF-3: theaflavin-3,3′-digallate.

1/2/3/4/5× indicates normal/double/triple/quadruple/quintuple concentration of the tea extract supplemented to soymilk to produce soymilk-tea. Normal concentration refers to tea extract prepared from tea leaf powder (2%, w/v) brewed in boiling water.

N.D. = not detected.
HPLC separation of nine phenolic compounds including gallic acid (GA), catechin (C), (−)-epigallocatechin gallate (EGCG), theaflavin (TF-1), theaflavin-3-gallate (TF-2) and theaflavin-3,3′-digallate (TF-3) (Sigma Chemical Co., St. Louis, MO, U.S.A.); (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-epicatechin gallate (EGC) and caffeine (CF) (Wako, Osaka, Japan) was achieved as previously described (Zhao and Shah, 2014). Quantification result is shown in Table 1.

2.6. Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of TE against BL5089 in SMT

Cell growth of BL5089 at 37 °C for 24 h in soymilk-tea (SMT) at varying concentrations of TE was recorded by plate count method. BL5022 was used as a phenolic-resistant strain for comparison. MIC is defined as the lowest total tea phenolic content (TTP) of green or black TE that completely inhibited the bacterial growth as compared with the initial cell population. MBC is defined as the lowest TTP in TE that killed >99.9% of the initial inoculum (ca. log CFU/mL < 4) (Dong et al., 2012). For determining MIC and MBC, cells were plated on cys-MRS agar plate and incubated aerobically at 37 °C for 24 h. TTP were determined by adding up all major phenolic compounds in TE as quantified by the HPLC method described above.

2.7. Fluorescent staining of bacterial cells and flow cytometry (FCM)

The impact of TE supplementation on cell viability and cell integrity of BL5089 and BL5022 was investigated by fluorometric FCM. Cells were cultured in 10 mL cys-MRS containing varying concentrations of TE at 37 °C for 24 h. Cells grown in MRS without TE was used as a control. After incubation, cells were harvested by centrifugation (4000 × g, 8 min, 4 °C) and washed twice with filter-sterilized PBS buffer (pH 7.4, 4 °C). Cell pellets were resuspended in 10 mL of buffer and filtered through 1.0-μm hydrophilic membrane (Costar, Nuclepore, Charlotte, NC, U.S.A.). Cell density of filtrates was adjusted to OD600 = 0.5–0.6 before staining. Fluorescence staining with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) (Invitrogen, Karlsruhe, Germany) was performed as per the method of Amor et al. (2002).

Samples were analyzed in a BD FACsAria III flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.) equipped with an air-cooled laser emitting blue light at 488 nm. Four parameters were recorded using BD FACSDiva software: forward scatter (FSC), side scatter (SSC), green fluorescence (cFDA) and red fluorescence (PI). All registered signals were logarithmically amplified and 10,000 events were recorded per sample. Different cell populations were separated and gated according to their fluorescence staining pattern and intensity of the premixed sample of 50% freshly harvested and 50% heat-killed (90 °C for 30 min) bacterial cells in both single and double-staining assays. Data was analyzed in Flowjo alias software (version 7.6, TreeStar, Ashland, OR, U.S.A.).

2.8. Extraction and profiling of membrane fatty acids (FAs)

2.8.1. Isolation of bacterial cells and enzymatic digestion of protein matrix

Enzymatic digestion of protein was performed in order to free cells from the FS or FST matrices and to “shave off” bacterial surface proteins for more efficient extraction of membrane lipids. Five milliliters of FS or FST was homogenized with 40 mL of PBS buffer (pH 7.4, 4 °C) in a 50-mL centrifugal tube. The mixture was centrifuged at 20 × g for 10 min, followed by 150 × g for 5 min at 4 °C. The supernatant was transferred to another tube and centrifuged at 4000 × g for 10 min at 4 °C. The supernatant was discarded and the pellet was washed with 40 mL of chilled PBS for twice and re-collected by centrifugation at 4000 × g for 10 min at 4 °C. The cell pellet was resuspended in 0.5 mL of chilled PBS for the same buffer. Protease K (25 μg/mL, Sigma) was added to the cell suspension in the presence of 5 mM dithiothreitol (DTT) and incubated for 30 min at 37 °C. An aliquot (100 μL) of protease inhibitor cocktail solution was then added to stop enzymatic reaction. The cocktail solution was prepared by dissolving one tablet of EDTA-free Protease Inhibitor Cocktail (complete, Roche Applied Science, Penzberg, Germany) in 10 volumes (w/v) of PBS (pH 7.4). The digested mixture before and after incubation was serially diluted with PBS to assess bacterial viability (data not shown). The subsequent mixture was centrifuged at 4000 × g for 10 min at 4 °C and cell pellets were collected.

2.8.2. Extraction of cellular fatty acids

Membrane FAs were extracted from ca. 0.5 g cell pellets (wet weight) and then converted to fatty acid methyl ester (FAME) according to the Microbial Identification System (MIS) protocol (1990). Decanoic acid (C10:0) was used as the internal standard and was added to cell isolates before extraction and methylation. The extracts were evaporated under nitrogen flow and re-dissolved in GC-grade hexane (Fisher Scientific, Pittsburgh, PA, U.S.A.) for injection.

2.8.3. Separation and identification of fatty acids by gas chromatography–mass spectroscopy (GC–MS)

Profiles of bacterial FAME were obtained using an Agilent 6590N-5973N GC–MS system (Agilent, Atlanta, GA, U.S.A.) equipped with an Agilent 7694E auto-sampler and a capillary DB-wax column (30 m × 0.25 mm id, 0.25 μm film thickness; J&W Scientific, Folsom, CA, U.S.A.), with an injection volume of 1 μL (splitless mode). The injector and the detector were both held at 250 °C. The temperature was programmed from 100 °C (held for 1 min) to 190 °C at a rate of 4 °C/min, elevated to 235 °C at a rate of 10 °C/min, and finally to 250 °C at a rate of 4 °C/min (held for 4 min). The carrier gas was helium at a rate of 1 mL/min. Results were expressed as relative molar percentages (mol%) for each FA. The ratios of unsaturated to saturated FA (U/S) were calculated accordingly.

2.9. Extraction and profiling of membrane phospholipids (PLs)

2.9.1. Enzymatic digestion of cell wall and cell surface proteins

Approximately 1 g of cell pellets (wet weight) was suspended in 1 mL PBS (pH 6.2, the optimal pH with ionic strength at ca. 0.18 M for lysozyme according to Dickman and Proctor, 1952) and incubated with 1 mg/mL lysozyme (ca. 50,000 units/mg, Sigma–Aldrich, St. Quentin-Fallavier, France) at 37 °C for 1 h, washed once with PBS (pH 7.4), followed by digestion with protease K as described in Section 2.8.1. After digestion, cell pellets were collected and the extraction process was performed under N2 to minimize oxidation.

2.9.2. Neutral and acidic extraction of membrane phospholipids (PLs)

For efficient extraction of major PLs from bacterial cells, a two-step extraction method was employed (Özbali et al., 2013). For neutral extraction, cell pellets were resuspended in 1800 μL chloroform/methanol (1:2, v/v) and rotatively agitated for 60 min at room temperature (RT), while vortexing for 1 min every 10 min. Samples were centrifuged at 10,000 × g for 5 min at 4 °C. The supernatant was transferred to a 5-mL centrifugal tube, followed by adding 600 μL chloroform and 1 mL 0.8% NaCl solution. The supernatant was vortexed for 1 min and centrifuged at 5500 × g for 5 min at 4 °C. The lower organic phase was collected in another glass tube. After neutral extraction, cell pellets were processed with acidic extraction by resuspending in 1 mL chloroform/methanol/37% HCl (40:80:1, v/v) and rotatively agitated for 30 min at RT, while vortexing for 30 s every 5 min. The tube was transferred to ice, followed by adding 250 μL cold chloroform and 450 μL cold 0.1 N HCl. The mixture was vortexed for 1 min before centrifugation (5500 × g, 5 min at 4 °C). The chloroform-rich phase from both neutral and acidic extraction was collected in a glass tube and dried under N2 stream. The weight of dried lipids was determined and stored in chloroform/methanol (90:10, v/v, 50 mg/mL) at −20 °C until analysis.
2.9.3. Separation of phospholipids by high performance thin layer chromatography (HPTLC)

Silica gel 60 thin-layer chromatographic plates (Merck, Darmstadt, Germany) were developed with solvent prior to spotting in order to remove impurities from the adsorbent layer. The plates were air-dried for 10 min and oven activated for 20 min at 100 °C before use. A filter paper was placed in the developing chamber and the developing solvent added. The chamber was allowed to saturate for 10 min before chromatography. For identification, six standard PLs (Sigma), cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylcholine (PC), and lyso-phosphatidylcholine (lyso-PC) were dissolved in chloroform:methanol (90:10, v/v). The same volume of PL extract and PL standards was spotted on the plates using spotting capillaries.

Phospholipids were separated on TLC plate using chloroform/methanol/acetonic acid/water (71:20:6:2.5, v/v) as mobile phase. For visualization of lipid bands, plates were sprayed with the following reagents: a) 0.5% (w/v) vanillin in ethanol/concentrated sulphuric acid (97:3, v/v) solution, for all polar lipids (Rakhuba et al., 2009); b) ammonium molybdate/perchloric acid reagent, for phospholipids (Nzai and Proctor, 1998). Since the bands detected with vanillin solution were much sharper in color, bands on plates treated with vanillin solution were scanned in a Molecular Imager XR+ System (Bio-Rad, Hercules, CA, U.S.A.) and analyzed in Image Lab (version 4.0, Bio-Rad). Identification of major PLs was achieved by comparing relative mobility (Rf) values of PL standards and the lipid bands. Relative quantity of individual PLs was determined according to the density of detected bands.

2.10. Statistical analysis

For each assay, three or more independently incubated samples were prepared for conducting plate count, FCM assay, bacterial FA and PL profiling. The data was subjected to one-way analysis of variance (ANOVA) (Turkey and Games–Howell tests) by SPSS 20.0 (IBM SPSS Statistics, IBM Corp, Somers, NY). Results with p < 0.01 or p < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Impact of tea extract supplementation on culturability and cellular properties of bifidobacteria

3.1.1. Cell culturability

Cell culturability of four bifidobacteria was assessed by viable cell count on agar plates and results are displayed in Table 2. All strains showed ca. 2 log CFU/mL increase during fermentation of SML, with a fold change at 165 for BL5022, followed by BL5089 (44 fold), BB12 (42 fold) and BBS286 (41 fold). When cells were exposed to TE at normal and double concentrations, TE promoted the growth of BLS022, BBS286 and BB12. In particular, GTX (5.98 mg/mL TTP) favored the growth of BLS022 and BTX (2.42 mg/mL TTP) promoted BBS286 significantly (p < 0.05) by increasing 2.6% and 4.3% of their cell population in SML, respectively. Studies have revealed that the reducing nature of tea polyphenols in TE might protect cells from oxidative stress and create a more anaerobic environment (Gaudreau et al., 2013), and thus promoted the growth of bifidobacteria. Similar to our previous study (Zhao and Shah, 2014), BL5089 was the only strain whose proliferation was significantly limited in SMT compared with that in SML, without significant difference among tea types (p > 0.05). It appears that TEs, mainly tea polyphenols, acted as antibacterial agents against BL5089.

To confirm the deleterious effect of TE on the growth of BL5089, bacteria were treated with higher TE concentrations in soymilk and the phenolic-resistant strain, BL5022, was used as a positive control. Fig. 1 depicts the alteration in viable cell count of BL5089 and BL5022 and there is a pronounced trend of inhibition against BL5089 by TE in a dose-dependent manner. GT exhibited higher inhibitory effects against BL5089 than BT, which was significant at 3× and 4× TE concentrations (p < 0.01). The MIC was determined to be 2.5× (15.45 mg/mL TTP) for GT and 3× (7.34 mg/mL TTP) for BT, while MBC was 4× (26.15 mg/mL TTP) and 4.5× (11.21 mg/mL TTP) for GT and BT, respectively. Our result is in agreement with that reported by Chan et al. (2011), which indicated that BT had higher MIC values than GT against the pathogens tested. TE is a complex system containing a variety of components and constituent analysis of green and black TE by HPLC helped to explain

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culturability (cell forming unit/mL) of Bifidobacterium longum CSCC 5022 (BL5022), B. longum CSCC 5089 (BLS022), B. bifidum CSCC 5286 (BBS286) and B. bifidum BB-12 (BB12) in soymilk supplemented with normal (1×) or double (2×) concentration of green tea (GT) or black tea (BT) extract in soymilk.</td>
</tr>
<tr>
<td>BL5022</td>
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<td>---</td>
</tr>
<tr>
<td>Initial</td>
</tr>
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</tr>
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<td>BT2X</td>
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</table>

Data is presented as the mean log CFU/mL ± SEM of three independent fermentation products. Asterisks in the same column indicate significant difference at a level of p < 0.05 compared with SML. 1/2/3/4/5× indicates normal/double/triple/quadruple/quintuple concentration of the tea extract added to soymilk (refer to Table 1).
the discrepancy (Table 1). As detected by HPLC, the major components in GT extract are catechins, gallic acid and caffeine, while for BT, theaflavins, gallic acid and caffeine contribute the majority. The difference in phenolic profiles can be the main reason and this was also suggested by Chan et al. (2011). Regarding BL5022, although a slight decrease in CFU was found at higher TE concentrations, it obtained a log CFU/mL value of 8.67 at 26.15 mg/mL TTP (GT5X) and 8.31 at 12.54 mg/mL TTP (BT5X), which demonstrated its high phenolic tolerance.

Although cell viability determination by counting bacterial colonies on agar plates has been employed as the standard practice in microbiological research and also in food processing industry, it actually determines the culturability of cells adapted to a certain environment on agar plates (Ananta et al., 2004). Mounting evidence demonstrates that cells grown under stress may stay in a non-culturable but viable or sub-lethally injured state (Mizunoe et al., 2000). On the contrary, some injured cells may resuscitate and recover their ability to replicate on agar plates (Wu, 2008). For more accurate detection of cell populations in different physiological status, flow cytometric analysis was performed.

3.1.2. Cell viability and integrity assessment by flow cytometry

Fig. 2 depicts the evolution of BL5089 cell populations in different physiological status. Cells with cellular esterase activity convert cFDA, a membrane permeant fluorescence precursor, into carboxyfluorescein (cF), a membrane impermeant fluorescent compound with an emission at 530 nm. PI is non-effective towards cells with intact membrane, but can enter cells with compromised membranes. It binds to DNA to form a fluorescent DNA-complex that emits red fluorescence at 635 nm (Ananta et al., 2004). Thus, cell viability can be indicated by the retention of cF in cell, while membrane integrity can be indicated by both the exclusion of PI and the retention of cF.

Since FCM analyzes the quantity, size and physiological characteristics at single cell level (Joux and Lebaron, 2000), clear characterization of individual cells is required. Doherty et al. (2010) suggested using two-step proteinase digestion–filtration method to free cells from complicated protein matrices such as our FS and FST products. Our preliminary results revealed that individual cells were effectively released and background signal decreased by 48% compared with samples without enzymatic treatment in FCM. However, ca. 80% cells from the control FS sample appeared in gate Q2, indicating that they were injured and were thus stained with both dyes. Thus, isolation of bacteria from the complex tea-protein matrices while maintaining the original status of bacteria was difficult to achieve. In order to assess the effect of TE supplementation on the selected bifidobacteria while minimizing the damage to bacterial cells during sample preparation, cells were grown in MRS-TE, instead of SMT. Pure populations of bacteria harvested from MRS broth were easily detected as fluorescent individual particles, but poor detection was found for cells encapsulated within MRS-TE media. Thus, a filtration step was employed to get rid of the excess interfering particles, which may be formed due to complicated interaction between tea compounds and MRS constituents, so that the detection of stained cells in the flow stream will not be affected.

As shown in Fig. 2, the majority of stained BL5089 cells (>95%) of “MRS control” only produced green fluorescence in gate Q3, showing their state of being alive and having intact membrane. With respect to cells cultivated in MRS-TE media, populations began to shift from lower left corner to upper left and right corners, implying that cell viability and membrane integrity were markedly altered by tea compounds. At lower concentrations (GT1X, BT1X & BT2X), cells were either viable (the majority, gate Q3) or dead (the minority, gate Q1), with only very few cells appeared in gate Q2. With higher concentrations of TE (GT2X, GT3X & BT3X), tendency of bacterial cells entering intermediate stages, i.e. sublethally-injured status, became pronounced, which is revealed by the elevated dual-stained cell density in gate Q2. Loss of membrane integrity makes a cell susceptible to the deleterious materials in the environment. At the highest tested TE concentration (4×), cells exposed to GT gave 90% dead and 7% injured of total stained cells and those grown in BT media showed more than 60% dead cells and 27% injured cells (Fig. 3). As displayed in Fig. 3, high concentrations of BT triggered membrane damaged to BL5089 cells more seriously than GT, leading to the higher percentages of double-stained cells in gate Q2. It can be suspected that major constituents of TE posed stress on BL5089 cells and caused cell damage and finally death. It has been documented that catechins from GT are the most potent antibacterial phenolic components against a number of pathogenic bacteria (Chan et al., 2011; Nakayama et al., 2012). Theaflavins from black TE also exhibited bactericidal activity against some bacteria but possessed lower potency.

![Fig. 2. Multiparameter dot plots of cFDA and PI double-stained B. longum C5089 cells treated with varying concentrations of green tea (GT) or black tea (BT) extract. Fluorescence intensities indicate densities of cells in different physiological status, with Q1: stained dead bacteria (PI+/cFDA−); Q2: stained injured bacteria (PI+/cFDA+); Q3: stained live bacteria (PI−/cFDA−); Q4: unstained cells (PI−/cFDA−). Plot of cells grown in cys-MRS media without tea is referred to MRS control. 1/2/3/4× indicates normal/double/triple/quadruple concentration of the tea extract added to cys-MRS media (Table 1).](image-url)
3.2. Impact of tea extract supplementation on cell membrane lipids

Bacterial lipid metabolism is closely related to stress responses. Environmental fluctuations may induce alteration in cell membrane lipid compositions, mostly FAs and polar lipid species to maintain an ideal physiological state of cell membrane (Guerzoni et al., 2001; Stepinkus et al., 1995). Tea polyphenols and other phenolic acids have been documented for their high affinity to bind to lipid bilayer and the ability to damage membrane structures (Ikigai et al., 1993; Kumazawa et al., 2004). In order to explain bacterial cell responses to phenolic stress induced by TE, membrane FAs and PLs of two bifidobacteria were extracted and quantified.

3.2.1. Membrane fatty acid composition

The relative percentages of major FAs (contributing a mol% higher than 1) and the unsaturated to saturated FA ratios (U/S) are presented in Fig. 4, with results for BL5089 in the upper panel and BL5022 in the lower panel. A total of 6 fatty acids were observed in BL5089 membranes, and an additional saturated FA, dodecanoic acid (C12:0), was detected in BL5022 membranes, regardless of the culture media. For BL5089, TE substantially altered relative percentages of individual FAs in a dose-dependent manner. Particularly, relative abundances of C14:0, C18:1, C18:2 and C18:3 were significantly reduced (p < 0.01), while those of C16:0 and C18:0, both saturated FAs, were increased. This lead to gradual decrease in U/S ratios and finally obtained values as low as 0.32 (GT4X) and 0.45 (BT4X). It is noteworthy that green TE caused a sharp decrease in the proportion of C18:2 by 2.1 folds and an increase in C16:0 by 1.6 folds when its concentration was increased from 2× to 3×. Black tea exposure decreased the U/S ratio steadily, from 0.88 for cells grown in control SML to 0.45 (BT4X). In addition, fluctuations in FA composition of cells grown in GT media, particularly at high TE concentrations (3× and 4×), were more significant as compared with their BT counterparts (p < 0.01). Regarding the phenolic-resistant strain, BL5022, some decrease in unsaturated FA abundance was also detected but it was much less than that for BL5089. The variations of individual FAs in cells exposed to TE were not significant (p < 0.05), except for those of C16:0 and C18:2, compared with that of the control.

The distribution of saturated and unsaturated FAs and the fatty acyl chain conformation are among the major factors that affect cell membrane fluidity, and it is necessary to maintain a suitable fluidity for membrane to function properly (Denich et al., 2003; Murga et al., 1999). In many studies related to the successful adaptation of LAB or bifidobacteria cells to various environmental stresses, such as cold shock (Wang et al., 2005), bile stress (Kimoto-Nira et al., 2009) and acid stress (Wu et al., 2012), increases in U/S ratio were observed. In our study, however, relative unsaturated FA contents in both bifidobacteria were reduced, implying that TE, or more specific, TPs, impeded the synthesis of unsaturated FAs in cell membrane and increased cell rigidity, as pointed out by Tsuchiya (1999). As determined by Murga et al. (1999), L. acidophilus cells grown at 25 °C showed considerably more C18:2 and C16:0 than cells grown at 37 °C, and such alteration in lipid content enhanced the lipid membrane stability. Similar results were also obtained in a study by Kimoto-Nira et al. (2009). It was also found that increase in the degree of FA unsaturation or decrease in the average chain length was a common adaptation response in many stress-adapted bacterial strains (Aricia et al., 2004; Guerezoni et al., 2001). However, these were not found in the phenolic-sensitive BL5089, which may partly explain the loss of cell viability and integrity for BL5089 (Figs. 2 and 3), particularly at high TE concentrations.

3.2.2. Membrane phospholipid composition

The PLs in the cell membrane of BL5089 and BL5022 were also characterized to investigate the influence of TE supplementation on bacterial growth. As shown in Fig. 5, CL, PE, PG, PI, PC, lyso-PC and an unknown polar lipid (mobility R f value: 0.790–0.823), were found to be the major lipid components in the membrane of BL5089 and BL5022 cells. Although the Folch et al. method (1957), involving the use of a single-phase mixture of chloroform/methanol to extract lipids from homogenized whole cells, has been widely applied...
For total lipid extraction, the recovery of some charged lipids and minor lipid components was low (Özbaliç et al., 2013). In addition, as suggested by Filgueiras and den Kamp (1980), CL was poorly extracted from Gram-positive bacteria due to the presence of cell wall, possibly due to the strong interaction between CL and some intracellular constituents. Thus, our study first utilized an enzymatic digestion process with lysozyme and protease K, followed by a two-step extraction using both a neutral chloroform/methanol solution and a HCl-acidified mixture (Limonet et al., 2007). Novik et al. (2006) isolated and analyzed the major PLs in the representative species of *Bifidobacterium* and their findings substantially agreed with our results. However, the relative amount of individual PLs varies from strain to strain, and also from medium to medium for the same strain, as demonstrated by the profiles of PL extracted from BLS089 and BLS022 cultured in various media.

For BLS089, the majority of PLs was contributed by PE (24.2%), followed by PI (23.0%), PC (21.3%) and PG (16.4%) in cells grown in SML. When TE was added, the abundance of PC was considerably reduced regardless of TE type, and PG was significantly increased (p < 0.01) and relative percentages of the rest PLs were little changed. Relative percentage of PG increased by nearly 50% in cells grown in GT4X and BT4X while that of PC dropped to 3% of total detected PLs. In contrast, for the phenolic-resistant strain BLS022, relative percentages of PL of cells grown in different media did not substantially alter upon exposure to TE, rather than the phenolic-resistant strain. The net charge of the lipid bilayer was more negative due to the significantly higher (p < 0.01) amount of PG (anionic) but lower PC (zwitterionic). This indicated a shift in the membrane lipid metabolism towards the synthesis of anionic PLs, which may result in greater electrostatic repulsion between the lipid bilayer and TPs (Lewis and McElhaney, 2000). As found by Ikigai et al. (1993), tea catechins damaged membrane integrity and had high affinity to neutral or positively charged components in the lipid bilayer, rather than negatively charged components. Thus, increase in synthesis of anionic PLs can be a self-protective response to phenolic stress. However, additional negative charge on the membrane alters the balance of electrostatic and hydrophobic interactions with external environment. As a result, the binding of cationic compounds and elevated level of hydration may lead to conformational changes in the structure of transmembrane α-helices of membrane proteins and produce the subsequent changes in helix packings (Lopez et al., 2006). Since there is strict cross-regulation between the different membrane components, dramatic alteration in individual constituents may cause malfunction of cell membrane and finally lead to death.

Referring to our findings on membrane lipid compositions, the membrane of BLS089 cells was found to be disturbed in an unusual and extreme manner due to TE supplementation. TE, particularly TPs has been reported to induce irreversible changes to cell membrane and cause substantial damage to bacteria (Ikigai et al., 1993). Severe damage in membrane is also related to compromise in cell integrity, which is demonstrated in the FCM assays (Figs. 2 and 3). As in a micro-biosystem, any dramatic variation in individual components may lead to lethal damage to the whole organism,
significant modification of cell membrane lipid constituents due to TE supplementation may account for the intolerance of BLS089 cells in high-phenolic media.

4. Conclusion

In this study, a novel soymilk-tea beverage was developed for cultivation of selected bifidobacteria. Bifidobacteria exhibited different responses to tea phenolics and different strains of the same species showed distinct sensitivity to tea phenolic compounds. Cell membrane was one of the targets of tea components as the integrity of BLS089 cell and the composition of its cell membrane lipids were greatly altered upon 24 h exposure to varying concentrations of TE. This phenolic concentration-dependent alteration in membrane lipid compositions and compromise in cell integrity were the possible mechanism for the bactericidal effect of TE on BLS089. The combination of dietary phenolic concentrate and probiotics may open a new field of functional food development, while the influence of phenolic compounds at certain concentrations on those bacteria should be evaluated in order to prevent the undesired bactericidal effects.

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References


Fig. 5. Distribution of individual phospholipids extracted from bacterial cells treated with varying concentrations of tea extract. Phospholipids: CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine. Unknown, unknown polar lipids detected. 1/2/3/4× indicates normal/double/triple/quadruple concentration of the tea extract added to soymilk (Table 1). PLs extracted from cells grown in soymilk containing lactose (SML) was used as a control.