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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 (Tzonuis 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.

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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 phenolic 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. Materials 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 SMT. 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 containing 0.05% (w/v) L-cysteine hydrochloride (cys-MRS) (Sigma, St. Louis, MO, U.S.A.) were inoculated with 2% (v/v) of each organism 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) and 0.05% (w/v) L-cysteine hydrochloride in double deionized water preheated to 50 °C. Upon reconstitution, SML was autoclaved at 120 °C for 15 min. Unlike commercial pasteurization, the advantage of autoclaving at high temperatures is to kill all existing bacteria or fungi, so the result of experiments should be the response of the testing bacteria only.

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–methanol–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
Concentrations of caffeine and tea phenolic compounds in soymilk supplemented with varying amounts of green tea (GT) or black tea (BT) extract.

	CF	GA	EGC	C	EGCG	EC	ECG	TF1	TF2	TF3	Total tea phenolics
GT1X	13.39	0.92	1.48	0.22	1.07	1.82	0.47	N.D.	N.D.	N.D.	5.98
GT2X	25.53	1.82	2.57	0.44	2.11	4.12	0.99	N.D.	N.D.	N.D.	12.05
GT3X	37.86	2.30	4.80	0.69	3.69	5.86	1.50	N.D.	N.D.	N.D.	18.85
GT4X	51.16	3.07	6.16	0.97	4.67	9.24	2.03	N.D.	N.D.	N.D.	26.15
BT1X	12.42	0.50	0.52	0.11	0.04	0.13	0.08	0.48	0.20	0.36	2.42
BT2X	23.11	1.20	1.02	0.22	0.14	0.26	0.17	0.91	0.41	0.67	5.01
BT3X	34.17	1.70	1.38	0.37	0.27	0.49	0.25	1.31	0.65	0.92	7.34
BT4X	47.09	2.23	1.89	0.53	0.35	0.78	0.37	1.75	0.81	1.16	9.87
BT5X	59.44	2.80	2.37	0.69	0.44	0.99	0.48	2.21	1.07	1.49	12.54

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: theaflavins; 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.