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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Relationship between acid tolerance and cell membrane in *Bifidobacterium*, revealed by comparative analysis of acid-resistant derivatives and their parental strains grown in medium with and without Tween 80

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Abstract The acid tolerance is particularly important for bifidobacteria to function as probiotics because they usually encounter acidic environments in food products and gastrointestinal tract passage. In this study, two acid-resistant derivatives *Bifidobacterium longum* JDY1017dpH and *Bifidobacterium breve* BB8dpH, which displayed a stable acid-resistant phenotype, were generated. The relationship between acid tolerance and cell membrane was investigated by comparing the two acid-resistant derivatives and their parental strains grown in medium with and without Tween 80. The fold increase in acid tolerance of the two acid-resistant derivatives relative to their parental strains was much higher when cells were grown in medium with Tween 80 (10^4~10^5-fold) than without Tween 80 (181- and 245-fold). Moreover, when cells were grown in medium with Tween 80, the two acid-resistant derivatives exhibited more C18:1 and cycC19:0, higher mean fatty acid chain length, lower membrane fluidity, and higher expression of *cfa* gene encoding cyclopropane fatty acid synthase than their parental strains. No significant differences in cell membrane were observed between the two acid-resistant derivatives and their parental strains when cells were grown in medium without Tween 80. The present study revealed that, when cells were grown in medium with Tween 80, the significant fold increase in acid tolerance of the two acid-resistant derivatives was mainly ascribed to the pronounced changes in cell membrane compared with their parental strains. Results presented here could provide a basis for developing new strategies of cell membrane modification to enhance acid tolerance in bifidobacteria.

Keywords Bifidobacteria · Acid tolerance · Cell membrane · Tween 80

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Introduction

Bifidobacteria are natural inhabitants of the human intestinal tract, constituting up to 4.4–6.9 % of the total gut microbiota in adults and 91 % in breast-fed infants (Harmsen et al. 2000; Langendijk et al. 1995; Lay et al. 2005; Russell et al. 2011). Application of certain species of bifidobacteria as probiotics can exert specific health benefits; therefore, they are commonly applied in probiotic products (FAO/WHO 2001). It is generally believed that they must survive passage through the gastrointestinal tract and reach the distal part of the intestine in sufficient numbers (10^6 to 10^8 CFU/g) to function as probiotics (Aureli et al. 2011; Marteau and Rambaud 1993). However, various acidic environments (e.g., the low pH of fermented dairy products in which bifidobacteria are added as probiotics and the low pH of the stomach) commonly reduce the survival of bifidobacteria, resulting in less than the recommended sufficient numbers reaching the intestine. Consequently, acid tolerance is recognized as a desirable property of potential probiotic bifidobacteria (Parvez et al. 2006). However, the acid tolerance of most bifidobacteria is weak (Matsumoto et al. 2004; Ruiz et al. 2011), limiting the application of bifidobacteria in probiotic products.

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To improve the acid tolerance of bifidobacteria, it is necessary to understand acid tolerance mechanisms. To survive in acidic environments, several responses are employed by bifidobacteria, including maintenance of pH homeostasis by H^+-ATPase, production of NH_3, regulation of global signaling systems, and general stress response (Jin et al. 2012; Sánchez et al. 2006, 2007; Ventura et al. 2004a; Waddington et al. 2010). These studies mainly focused on the self-response of bifidobacteria to acid stress or adaptation. However, it is not clear why different species or strains of bifidobacteria have different acid tolerance levels. Since the cell membrane is regarded as the most important natural defense for cells, cell membrane with different properties may result in different levels of environmental tolerance, including acid tolerance. Despite the high level of genetic conservation in the cell membrane, the role of the cell membrane in acid tolerance differed even among closely related bacteria (Broadbent et al. 2010; Revilla-Guarinos et al. 2014; Wu et al. 2012). Accordingly, the relationship between the cell membrane and acid tolerance has not been established in bifidobacteria.

The aim of the present study was to explore the differences in acid tolerance between acid-resistant derivatives and their corresponding parental strains at the cell membrane level. In this study, we compared the acid tolerance, membrane fatty acid composition, and membrane fluidity between two acid-resistant derivatives and their parental strains. In addition, the expression of gene encoding key synthase for cyclopropane fatty acids (CFA) was also compared between two acid-resistant derivatives and their parental strains.

Materials and methods

Bacterial strains and growth conditions

Four strains of *Bifidobacterium* were used in this study. *B. longum* JDY1017 (deposited in the China General Microbiological Culture Collection Center (CGMCC) under accession number CGMCC No. 9882) and *B. breve* BB8 (CGMCC No. 9881) were isolated from the human intestine. *B. longum* JDY1017dpH (CGMCC No. 8371) and *B. breve* BB8dpH (CGMCC No. 8370) were acid-resistant derivatives of strains *B. longum* JDY1017 and *B. breve* BB8, respectively. The "dpH" in the name of the acid-resistant derivatives stands for "derivatives isolated in low pH conditions." All strains were maintained by subculturing 1 % inocula in de Man Rogosa Sharpe (MRS) medium supplemented with 0.05 % (*w*/*v*) L-cysteine (MRSC) and incubating for 16 h at 37 °C under anaerobic conditions (AnaeroGen™, Oxoid Ltd, Basingstoke, UK). The cultures were stored at 4 °C between transfers and were subcultured three times prior to experimental use.

Isolation and molecular identification of acid-resistant derivatives

To isolate acid-resistant derivatives, *B. longum* JDY1017 and *B. breve* BB8 were cultured in MRSC medium anaerobically at 37 °C overnight. Cells of each culture were washed in phosphate-buffered saline with 0.05 % (*w*/*v*) L-cysteine (pH 7.4) and then transferred to fresh MRSC medium adjusted to low pH (pH 3.5 for *B. longum* JDY1017 and pH 3.2 for *B. breve* BB8) with 6 N HCl and incubated anaerobically at 37 °C overnight, after which aliquots were spread on MRSC agar (pH 6.5) and incubated at 37 °C for 3–4 days under anaerobic conditions to recover possible acid-resistant derivatives. After repeating the above processes 52 and 65 times respectively, two acid-resistant derivatives *B. longum* JDY1017dpH and *B. breve* BB8dpH were obtained.

For identification of strains, genomic DNA was extracted according to the method of Ausubel et al. (2002), which was slightly modified by adding a cell disruption step with a Fast prep instrument (Thermo Fisher Scientific, USA) prior to the extraction procedure. In order to obtain 16S rRNA partial gene (900-bp fragment length), universal oligonucleotides were used (5′-ATAATGCGGCCGCACGGGCGGTGTGTRC-3′ and 5′-TAATAGCGGCCGCAGCMGCCGCGGTAATWC-3′) as previously described (Vaugien et al. 2002). The PCR products were purified with TaKaRa DNA fragment Purification Kit (TaKaRa Biotechnology, Dalian, China), and then sequenced on an ABI-Prism 3730 automated sequencer (PE Applied Biosystems. USA). Molecular identification of strains was accomplished by analyzing the 16S rRNA gene sequences using RDP classifier software (version 2.2) in the GenBank database (http://www.ncbi.nlm.nih.gov/blast).

Evaluation of acid tolerance of bifidobacterial strains

Stationary growth phase cells grown in MRSC medium with Tween 80 (1 g/L) and without Tween 80 at 37 °C for 16 h anaerobically were collected by centrifugation (7600×*g*, 10 min), respectively, washed twice with phosphate-buffered saline with 0.05 % (*w*/*v*) L-cysteine (pH 7.4), resuspended in phosphate-buffered saline with 0.05 % (*w*/*v*) L-cysteine (adjusted to pH 3.5 for *B. longum* and pH 3.2 for *B. breve* with 6 N HCl, respectively), and then incubated at 37 °C under anaerobic conditions. After the acid challenge for 4 h, serial dilutions of cells were spread on MRSC agar. Plates were incubated at 37 °C for 3–4 days under anaerobic conditions, after which colony-forming units (CFU) were enumerated. Survival was calculated by dividing the number of CFU per mL after acid challenge by the number of CFU per mL at the time 0. The fold increase in survival of the acid-resistant derivatives versus their parental strains was then calculated as the survival of acid-resistant derivatives divided by the

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survival of their corresponding parental strains. The experiments were performed in triplicate.

Stability evaluation of acid-resistant phenotype of the two derivatives

The stability of acid-resistant phenotype of the two acid-resistant derivatives *B. longum* JDY1017dpH and *B. breve* BB8dpH was determined according to the following procedure. Briefly, cultures of each acid-resistant derivative were transferred daily for 20 consecutive days in fresh MRSC medium, with 1 % inocula at each transfer. The cultures after each transfer were grown at 37 °C for 16 h anaerobically, and then used to test their survival in acidic conditions (pH 3.5, 4 h for *B. longum* JDY1017dpH, and pH 3.2, 4 h for *B. breve* BB8dpH) as described above. The parental strains of the two acid-resistant derivatives were included as controls.

Extraction and analysis of membrane fatty acid

Membrane fatty acids of the bifidobacterial strains were extracted from stationary growth phase cells grown in MRSC medium with Tween 80 (1 g/L) and without Tween 80 at 37 °C for 16 h anaerobically, respectively, and then methylated as described by Wang et al. (2005). Methylated fatty acid esters were analyzed using a gas chromatographer (HP 6890, Hewlett Packard, USA) equipped with a capillary column (30 m×0.25 mm×0.25 μm, J&W HP-88, Agilent, USA). Sample of 1.0 μL was injected with the injector temperature at 250 °C. The oven temperature was held at 80 °C for 5 min and then increased to 100 °C at a rate of 2 °C/min and maintained for 2 min, then from 100 to 160 °C at a rate of 1 °C/min and maintained for 5 min, the detector temperature was maintained at 280 °C. Nitrogen was used as the carrier gas. The fatty acid methyl esters were identified by comparing their retention times with those of known standards. Results were expressed as relative percentages of each fatty acid, which were calculated as the ratio of the surface area of each considered peak to the total area of all peaks. The ratio of saturated fatty acids to unsaturated fatty acids (SFA/UFA) was determined without considering CFA. The mean fatty acid chain length was expressed by following equation according to Guerzoni (2001):

$$\text{Mean fatty acid chain length} = \sum (\text{FAP} \times \text{C}) / 100$$

where FAP is the percentage of fatty acid and C is the number of carbon atoms. The experiments were performed in triplicate.

Measurement of fluorescence anisotropy

Membrane fluidity of stationary growth phase bifidobacterial cells grown in MRSC medium with Tween 80 (1 g/L) and without Tween 80 at 37 °C for 16 h anaerobically was determined by measuring fluorescence anisotropy using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe, respectively, as previously described by Aricha et al. (2004). Steady-state fluorescence anisotropy was measured at 37 °C with a spectrofluorimeter (Photon Technology International Inc. USA) with excitation at 360 nm and emission 430 nm (6- and 6-nm slits, respectively). The intensities of parallel (I_{vv}) and perpendicular (I_{vh}) to the vertically polarized excitation beam were recorded. Fluorescence anisotropy values (r) were automatically calculated by the spectrofluorimeter according to Shinitzky (1984):

$$r = (I_{vv} - G\,I_{vh}) / (I_{vv} + 2G\,I_{vh})$$

where G is the correlation factor for instrument polarization and expressed as the ratio of vertical to horizontal measurements when the excitation light is polarized in the horizontal direction. Higher fluorescence anisotropy values indicate lower membrane fluidity. The experiments were performed in triplicate.

RNA extraction, cDNA synthesis, and real-time quantitative PCR

Total RNA of bifidobacterial cells grown in MRSC with Tween 80 (1 g/L) and without Tween 80 by 1 % inocula at 37 °C for 16 h anaerobically was extracted using Easy Pure RNA Kit (TransGen Biotech, Beijing, China) according to the manufacture's protocol, respectively. RNA quality and quantity were measured using NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and integrity was checked by agarose gel electrophoresis. The cDNA was synthesized from each RNA sample (500 ng) using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology, Dalian, China) according to the manufacture's protocol. Real-time quantitative PCR (RT-PCR) was performed using Mastercycler ep realplex system (Eppendorf, Hamburg, Germany) to evaluate the transcription levels of *cfa* gene which encodes CFA synthase. The primers of *cfa* gene used in this study are listed in Table 1. The amplification efficiency of each primer set was determined using the standard curve. The RT-PCR mixture with a total volume of 25 μL contained 12.5-μL 2× SYBR® *Premix Ex Taq*™ II (TaKaRa Biotechnology, Dalian, China), 0.6 μM of the forward and reverse primers, 2 μL of cDNA template, and nuclease-free water. Thermal cycling conditions comprised 1 cycle at 95 °C for 30 s and 40 cycles at 95 °C for 5 s and

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Table 1 Primers of *cfa* gene used for real-time quantitative RT-PCR

Organism	Primer sequence (F/R: 5′–3′)	Product size (bp)	Amplification efficiency
B. longum	ATGTCCAACGAGTTCTACG CAGCCGATGTCCAGTAAG	167	0.98
B. breve	GTATCCGCAGGAATACTCAG GGCATAGAACTCGTTAGACA	165	1.02

60 °C for 2.5 min. The *cfa* gene expression fold change of acid-resistant derivatives versus their parental strains was determined according to $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), using 16S rRNA as the reference gene as previously described (Gueimonde et al. 2004). Individual RT-PCR reactions were performed in triplicate.

Statistical analysis

Student's *t* test was employed to investigate statistical differences using SPSS version 19.0 (IBM, USA). Differences with *P* value less than 0.05 were considered statistically significant.

Results

Isolation and identification of acid-resistant derivatives

Two acid-resistant derivatives *B. longum* JDY1017dpH and *B. breve* BB8dpH were successfully isolated from *B. longum* JDY1017 and *B. breve* BB8 after repeating the isolation procedures many times, respectively. The partial 16S rRNA gene of the two acid-resistant derivatives was found to have 100 % identity with their corresponding parental strains (data not shown).

The standard MRSC medium, which included routinely Tween 80, was frequently used for cultivation of bifidobacteria in numerous studies (Collado and Sanz 2007; Jin et al. 2012; Sánchez et al. 2007; Waddington et al. 2010). We therefore investigated the acid tolerance of the two acid-resistant derivatives and their corresponding parental strains when cells were grown in standard MRSC medium containing Tween 80. As shown in Fig. 1, after exposed to acidic conditions (pH 3.5, 4 h for *B. longum*, and pH 3.2, 4 h for *B. breve*), the survival of *B. longum* JDY1017dpH and *B. breve* BB8dpH was approximately 10^4~10^5-fold higher than that of their corresponding parental strains, respectively. In addition, the two acid-resistant derivatives had a stable acid-resistant phenotype by daily cultivation for 20 consecutive days (Fig. 2). During the comparison analysis of acid tolerance of the two acid-resistant derivatives and their corresponding parental strains, a surprising finding was that when cells were grown in medium without Tween 80, the survival of *B. longum* JDY1017dpH and *B. breve* BB8dpH was only 181- and 245-fold higher than that of their corresponding parental strains, after exposed to acidic conditions (pH 3.5, 4 h for *B. longum*, and pH 3.2, 4 h for *B. breve*), respectively (Fig. 1).

These results indicated that the fold increase in acid tolerance of the acid-resistant derivatives relative to their corresponding parental strains was more significant

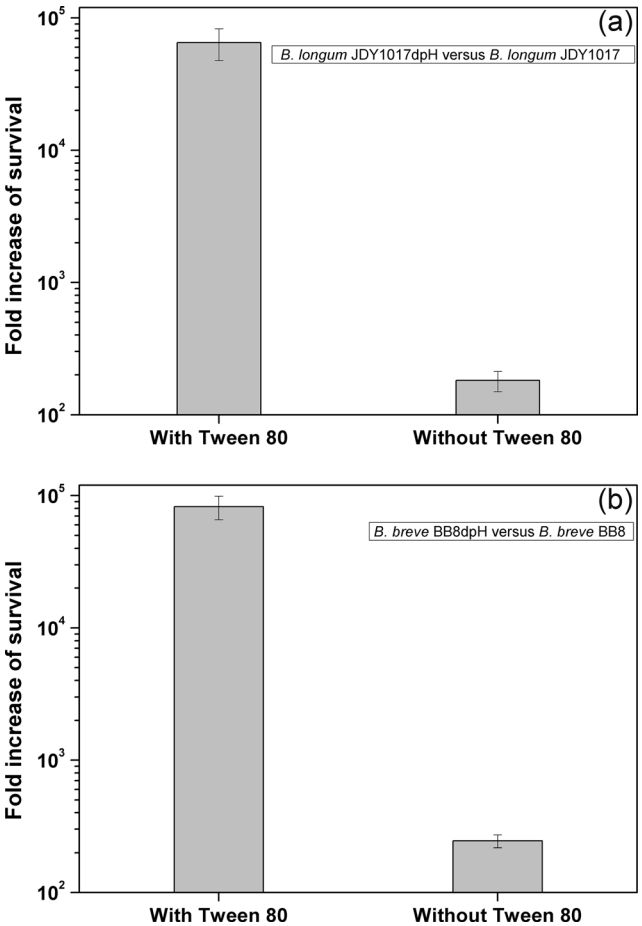

Fig. 1 Fold increase of survival of the acid-resistant derivatives versus their corresponding parental strains grown in medium with and without Tween 80, respectively. **a** Fold increase of survival of *B. longum* JDY1017dpH versus its parental strain JDY1017 after exposed to pH 3.5 for 4 h. **b** Fold increase of survival of *B. breve* BB8dpH versus its parental strain BB8 after exposed to pH 3.2 for 4 h. *Vertical lines on the bars* represent standard deviations

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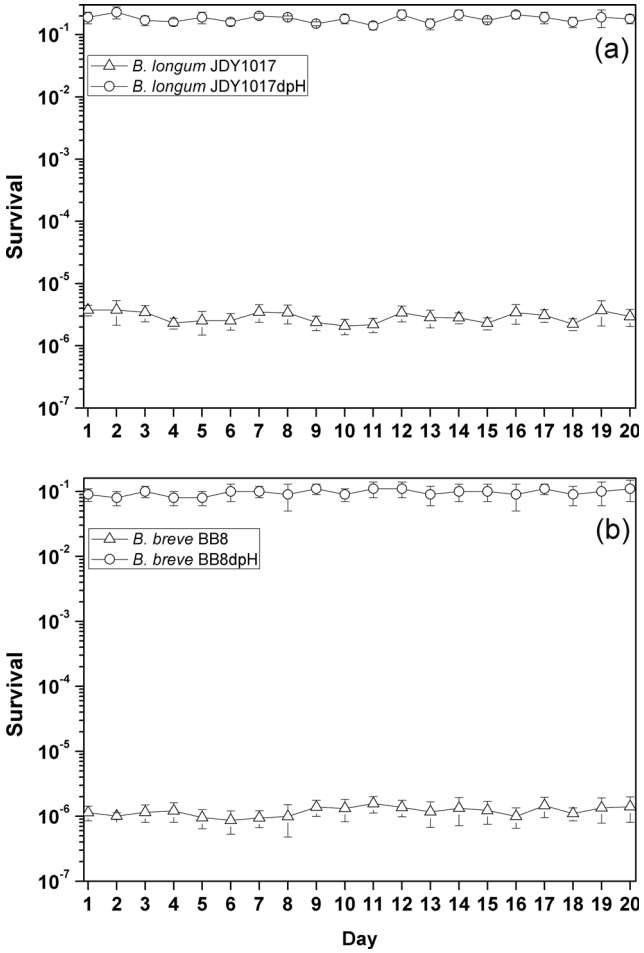

Fig. 2 Stability of acid-resistant phenotype of the two acid-resistant derivatives. Cultures of each acid-resistant derivative and its parental strain were transferred daily for 20 consecutive days in fresh MRSC medium, respectively. The daily cultures were used to evaluate the survival in acidic conditions (pH 3.5, 4 h for *B. longum* JDY1017dpH and JDY1017, and pH 3.2, 4 h for *B. breve* BB8dpH and BB8), respectively. **a** Survival of *B. longum* JDY1017 and *B. longum* JDY1017dpH cells after exposure to pH 3.5 for 4 h. **b** Survival of *B. breve* BB8 and *B. breve* BB8dpH cells after exposure to pH 3.2 for 4 h. The *error bars* represent the standard deviations calculated from three independent experiments

in growth medium with Tween 80 than without Tween 80. Previous studies reported that Tween 80 was a derivative of octadecenoic acid, which as the exogenous fatty acid could be incorporated into cell membrane directly, and thus was closely related to cell membrane fatty acid composition in some lactic acid bacteria (Broadbent et al. 2014; Johnsson et al. 1995). Moreover, in *B. animalis*, different strains showed the differences in ability to incorporation of octadecenoic acid from the growth medium with Tween 80 into cell membrane (Oberg et al. 2013). These encouraged us to further compare cell membrane of the acid-resistant derivatives and their parental strains grown in MRSC medium with and without Tween 80.

Membrane fatty acid composition of the two acid-resistant derivatives and their parental strains grown in medium with and without Tween 80

The membrane fatty acid composition of the two acid-resistant derivatives and their parental strains grown in medium with and without Tween 80 was determined (Fig. 3 and Table 2), respectively. Seven main fatty acids were observed among all strains, dodecanoic acid (C12:0), tetradecanoic (C14:0), hexadecanoic acid (C16:0), hexadecenoic acid (C16:1), octadecanoic acid (18:0), octadecenoic acid (C18:1), and CFA (cycC19:0).

When cells were grown in medium with Tween 80, the membrane fatty acid composition of the two acid-resistant derivatives differed significantly from that of their corresponding parental strains (Fig. 3 and Table 2). Specifically, the acid-resistant derivatives displayed more C18:1 and

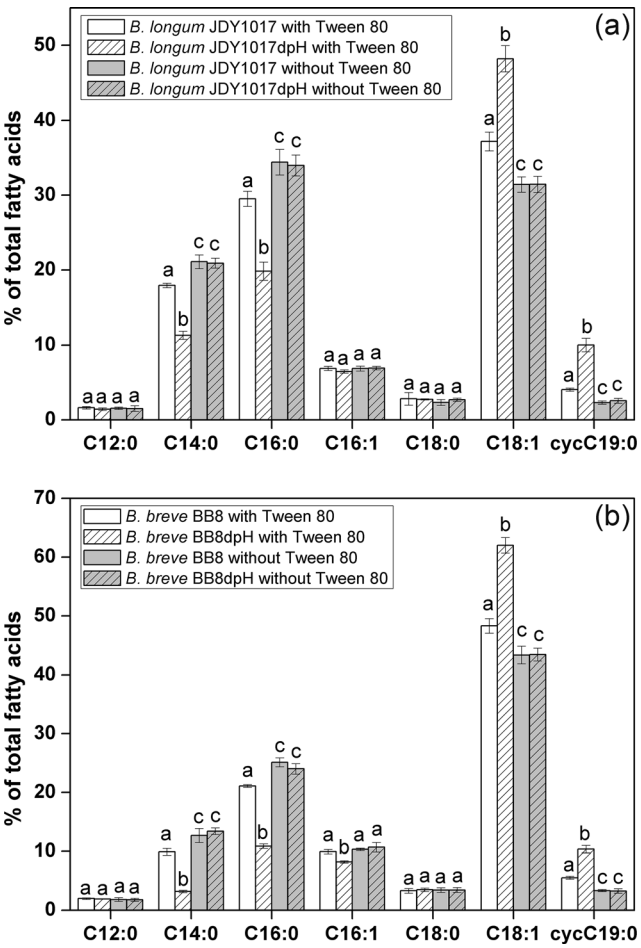

Fig. 3 Membrane fatty acid composition of the two acid-resistant derivatives and their corresponding parental strains grown in medium with and without Tween 80. **a** *B. longum* JDY1017 and *B. longum* JDY1017dpH. **b** *B. breve* BB8 and *B. breve* BB8dpH. *Error bars* correspond to the standard deviations of the mean, and *different letters within each lipid type* indicate that the means differ significantly ($P<0.05$)

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Table 2 Mean fatty acid chain length and ratio of saturated fatty acids to unsaturated fatty acids of the two acid-resistant derivatives and their parental strains grown in medium with and without Tween 80

	Medium with Tween 80				Medium without Tween 80			
	B. longum JDY1017	*B. longum* JDY1017dpH	*B. breve* BB8	*B. breve* BB8dpH	*B. longum* JDY1017	*B. longum* JDY1017dpH	*B. breve* BB8	*B. breve* BB8dpH
SFA/UFA	1.18±0.07	0.65±0.04*	0.62±0.03	0.28±0.01*	1.55±0.06	1.54±0.06	0.80±0.04	0.79±0.03
Mean fatty acid chain length	16.50±0.01	17.04±0.02*	16.92±0.02	17.48±0.01*	16.26±0.03	16.28±0.05	16.71±0.03	16.70±0.05

SFA total saturated fatty acids, *UFA* total unsaturated fatty acids, *SFA*/*UFA* the ratio of saturated fatty acids to unsaturated fatty acids without considering cyclopropane fatty acid (cycC19:0)

All values are means ± standard deviations. The *asterisk* represents significant difference between the two acid-resistant derivatives and their corresponding parental strains ($P<0.05$)

cycC19:0 content, less C14:0 and C16:0 content than their corresponding parental strains. These differences resulted in the lower SFA/UFA ratio and higher mean fatty acid chain length in the acid-resistant derivatives than those in their parental strains. The mean fatty acid chain length was 16.50 for *B. longum* JDY1017 and 17.04 for its acid-resistant derivative JDY1017dpH, and 16.92 for *B. breve* BB8, and 17.48 for its acid-resistant derivative BB8dpH. On the other hand, when cells were grown in medium without Tween 80, no significant differences in membrane fatty acid composition between the two acid-resistant derivatives and their corresponding parental strains were observed (Fig. 3 and Table 2). These results indicated that the addition of Tween 80 to the growth medium resulted in the significant differences in membrane fatty acid composition between the two acid-resistant derivatives and their corresponding parental strains.

Membrane fluidity of the two acid-resistant derivatives and their parental strains grown in medium with and without Tween 80

The membrane fluidity of the two acid-resistant derivatives and their parental strains grown in medium with and without Tween 80 was investigated by measuring the rotational diffusion of the fatty acyl chains. The steady-state fluorescence anisotropy values in stationary growth phase cells, which were estimated using DPH as a probe, are shown in Fig. 4. When cells were grown in medium with Tween 80, the two acid-resistant derivatives displayed higher anisotropy values than their corresponding parental strains ($P<0.05$), suggesting a lower membrane fluidity in the acid-resistant derivatives. Specifically, the anisotropy value was 0.126 for *B. longum* JDY1017, and 0.135 for its acid-resistant derivative JDY1017dpH, and 0.130 for *B. breve* BB8, and 0.138 for its acid-resistant derivative BB8dpH. However, when cells were grown in medium without Tween 80, no significant differences in anisotropy values were observed between the two acid-resistant derivatives and their parental strains.

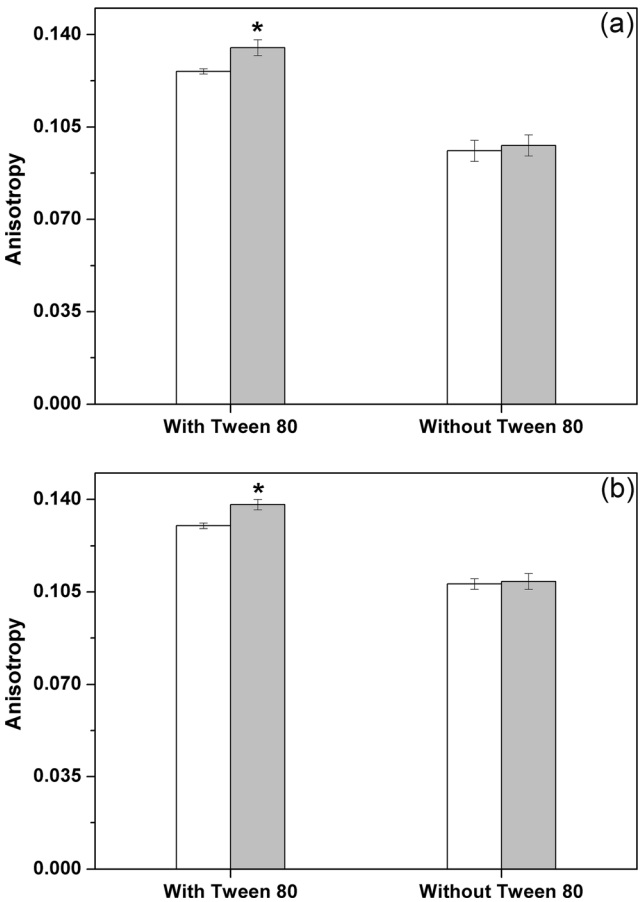

Fig. 4 Anisotropy values of the two acid-resistant derivatives and their corresponding parental strains grown in medium with and without Tween 80. **a** *B. longum* JDY1017 (*white bars*) and *B. longum* JDY1017dpH (*gray bars*). **b** *B. breve* BB8 (*white bars*) and *B. breve* BB8dpH (*gray bars*). Higher anisotropy values indicate lower membrane fluidity. The *bars followed by asterisk* indicate significant difference between the acid-resistant derivatives and their corresponding parental strains ($P<0.05$). *Vertical lines on the bars* represent standard deviations

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Transcriptional expression of *cfa* gene

Previous studies reported that CFA synthase encoded by *cfa* gene could catalyze the conversion of C18:1 to CFA, and CFA was generally regarded as a tool to reduce membrane fluidity in many bacteria (Grogan and Cronan 1997; To et al. 2011). We therefore investigated the *cfa* gene expression fold change of the two acid-resistant derivatives relative to their corresponding parental strains by RT-PCR according to $2^{-\Delta\Delta Ct}$ method (Fig. 5). When cells were grown in medium with Tween 80, the expression of *cfa* gene was 3.6-fold higher in *B. longum* JDY1017dpH than that in *B. longum* JDY1017 and was 2.2-fold higher in *B. breve* BB8dpH than that in *B. breve* BB8. When cells were grown in medium without Tween 80, the expression of *cfa* gene displayed almost the same level between the acid-resistant derivatives and their corresponding parental strains.

Discussion

As part of strategies to improve acid tolerance in bifidobacteria, it is necessary to understand the mechanisms involved in development of such tolerance. Molecular genetic tools available for analysis of bifidobacteria are rather limited, strongly restricting investigations of their acid tolerance mechanisms (Berger et al. 2010; Sánchez et al. 2006, 2007; Ventura et al. 2004b). Therefore, the present study compared the cell membrane of acid-resistant derivatives and their parental strains in order to reveal the relationship of cell membrane with acid tolerance in bifidobacteria.

Strategies based on short exposures to sublethal acidic conditions were applied to improve acid tolerance of bifidobacteria, but strains with stable acid-resistant phenotype were not always generated (Maus and Ingham 2003; Saarela et al. 2004). As an alternative strategy, wild-type strains were prolonged exposed to lethal acidic conditions for obtaining strains with stable acid-resistant phenotype (Collado and Sanz 2007). In the present study, two bifidobacterial acid-resistant derivatives were obtained by the method of prolonged exposure to lethal acidic conditions, exhibiting higher stable acid tolerance than their corresponding parental strains (Fig. 2). Additionally, the fold increase in acid tolerance of the acid-resistant derivatives relative to their corresponding parental strains when cells were grown in medium with Tween 80 (about 10^4~10^5-fold) was much higher than that when cells were grown in medium without Tween 80 (181- and 245-fold) (Fig. 1). This suggested that the acid-resistant derivatives had a trait that utilizing Tween 80 in medium to exhibit greatly increase in acid tolerance.

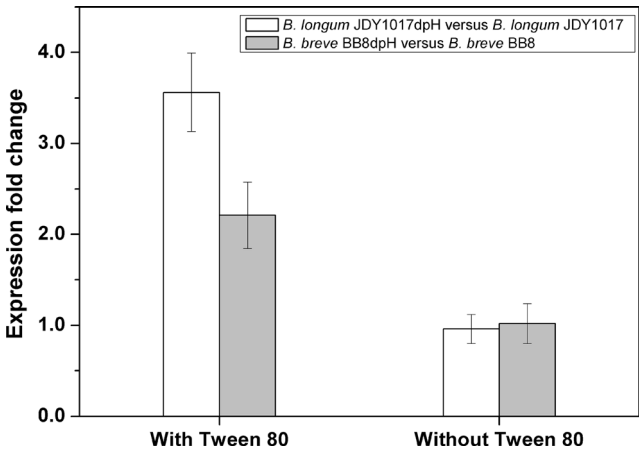

Fig. 5 The *cfa* gene expression fold change of the two acid-resistant derivatives relative to their corresponding parental strains by RT-PCR according to $2^{-\Delta\Delta Ct}$ method. Cells were grown in medium with and 'without Tween 80, respectively. *Vertical lines on the bars* represent the standard deviations

In the present study, the two acid-resistant derivatives exhibited more C18:1 content than their corresponding parental strains when cells were grown in medium with Tween 80 (Fig. 3). Previous studies reported that some lactic acid bacteria such as *Lactobacillus* and *Lactococcus* could incorporate the exogenous C18:1 from growth medium containing Tween 80 (a derivative of C18:1) into cell membrane directly, consequently changing their fatty acid composition (Broadbent et al. 2014; Johnsson et al. 1995). Oberg et al. (2013) reported that the growth medium with Tween 80 resulted in an increase in C18:1 content of *B. animalis*. In *Lactobacillus casei*, more C18:1 content was observed in an acid-resistant derivative compared with the wild type (Wu et al. 2012). Furthermore, Fozo et al. (2004) reported that the mutant of *Streptococcus mutans* defective in production of monounsaturated fatty acids including C18:1 was extremely sensitive to acidic environments compared with the wild type, and the acid-sensitive phenotype of the mutant could be relieved by growth in the presence of exogenous C18:1. Taken together, the present results suggested that the two acid-resistant derivatives had a stronger ability to incorporate Tween 80 from medium than their corresponding parental strains, which consequently led to much more C18:1 content in the acid-resistant derivatives than in the corresponding parental strains when cells were grown in medium with Tween 80. That could be one of the reasons for the higher acid tolerance of two acid-resistant derivatives when cells were grown in medium with Tween 80.

The C18:1 could be converted to CFA (cycC19:0) by the transfer of a methylene group from *S*-adenosyl-L-methionine to a double of unsaturated fatty acid chains of membrane phospholipids. This conversion was catalyzed by CFA synthase in many bacteria (Grogan and Cronan 1997; To et al. 2011). In the present study, the two acid-resistant derivatives displayed more cycC19:0 content and higher expression of *cfa* gene encoding CFA synthase than their corresponding parental strains when cells were grown in medium with Tween 80 (Figs. 3 and 5). More cycC19:0 content in the acid-resistant

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derivatives could be ascribed to their more C18:1 content and higher expression of *cfa* gene. Additionally, previous studies reported that *cfa* defective mutants of *E. coli* and *Salmonella enterica* serovar *typhimurium* were more acid-sensitive (Chang and Cronan 1999; Kim et al. 2005). The CFA was generally regarded as a tool to prevent the entry of undesirable molecules (e.g., H^+) into cells (Mykytczuk et al. 2007; Wu et al. 2012). It was also reported that the increased CFA content contributed to the enhancement of acid tolerance in *E. coli* (Brown et al. 1997), *Salmonella enteritidis* (Yang et al. 2014b), and *Lactobacillus casei* (Wu et al. 2012). Taken together, more CFA content could be one of the reasons for the higher acid tolerance of two acid-resistant derivatives when cells were grown in medium with Tween 80.

In the present study, when cells were grown in medium with Tween 80, the two acid-resistant derivatives exhibited higher mean fatty acid chain length (due to more C18:1 and cycC19:0 content) and lower SFA/UFA ratio than their corresponding parental strains (Table 2). The mean fatty acid chain length, cycC19:0, and SFA/UFA ratio are key factors affecting membrane fluidity (Mykytczuk et al. 2007). Higher mean fatty acid chain length could result in lower membrane fluidity since longer chains more easily span the width of the bilayer, contributing to acyl chain packing and making the membrane environment more gel-like (Cao-Hoang et al. 2008; Wu et al. 2012). Moreover, cycC19:0 is a cyclic fatty acid and generally regarded as a tool to reduce membrane fluidity in many bacteria (Grogan and Cronan 1997; To et al. 2011). However, it was reported that lower SFA/UFA ratio was associated with higher membrane fluidity in some bacteria (Álvarez-Ordóñez et al. 2009; Russell 1984). In fact, we found that the two acid-resistant derivatives displayed lower membrane fluidity (higher anisotropy values) than their corresponding parental strains when cells were grown in medium with Tween 80 (Fig. 4). Previous studies on *Lactobacillus casei* (Wu et al. 2012) and *Desulfovibrio piezophilus* (Khelaifia et al. 2011) reported that the higher mean fatty acid chain length was the major factor contributing to the decrease in membrane fluidity, in spite of the lower SFA/UFA ratio. Therefore, it is likely that the mean fatty acid chain length and cycC19:0 could play a more important role than the SFA/UFA ratio in affecting membrane fluidity of bifidobacteria. Furthermore, membrane fluidity is crucial for the tolerance of bacterial cells to various adverse environments including acidic environments (Da Silveira et al. 2003; Mykytczuk et al. 2007; Vitali et al. 2008; Wu et al. 2012). The acidic environments could cause the higher membrane disorganization reflected by higher membrane fluidity, consequently resulting in the high cell mortality (Chu-Ky et al. 2005). Previous studies demonstrated that the lower membrane fluidity (higher membrane rigidity) could prevent the influx of H^+ into cells, and thus help cells protect against acidic environments (Brown et al. 1997; Guerzoni et al. 2001; Kanjee and Houry 2013; Yang et al. 2014a). It was also reported that decreasing membrane fluidity is an important approach available to cells to enhance the acid tolerance in *Lactobacillus casei* (Broadbent et al. 2010), *Salmonella enteritidis* (Yang et al. 2014b), and *E. coli* (Yuk and Marshall 2004). The present results suggested that the higher mean fatty acid chain length and more cycC19:0 content resulted in the lower membrane fluidity, which would enhance the ability of cell membrane to prevent the entry of H^+ into cells. This could be an important reason for the higher acid tolerance of acid-resistant derivatives compared with their corresponding parental strains when cells were grown in medium with Tween 80.

It is worth noting that the two acid-resistant derivatives still displayed relatively higher acid tolerance than their corresponding parental strains when cells were grown in medium without Tween 80, although the fold increase in acid tolerance without Tween 80 (181- and 245-fold) was much lower than that with Tween 80 (10^4~10^5-fold) (Fig. 1). Moreover, no obvious differences in membrane fatty acid composition and membrane fluidity were observed between the two acid-resistant derivatives and their corresponding parental strains when cells were grown in medium without Tween 80 (Table 2, Figs. 3 and 4). It was reported that mechanisms underlying acid tolerance used by lactic acid bacteria were complex, including not only cell membrane changes but also the others (Corcoran et al. 2008; Cotter and Hill 2003; Wu et al. 2014). Therefore, there may be some other mechanisms that simultaneously play unignorable roles in affecting acid tolerance of the two acid-resistant derivatives besides cell membrane changes.

In conclusion, the present study revealed that the fold increase in acid tolerance of the two bifidobacterial acid-resistant derivatives mainly resulted from the pronounced changes in cell membrane compared with their corresponding parental strains when cells were grown in medium with Tween 80. Although the other unknown mechanisms affecting the acid tolerance of acid-resistant derivatives are less significant than the changes in cell membrane, it is still meaningful to further clarify these unknown mechanisms in the future by use of other methods (e.g., RNA sequencing). The results of this study could provide a basis for developing new strategies of cell membrane modification to enhance acid tolerance of potential probiotic bifidobacteria.

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