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Acid Response of *Bifidobacterium longum* subsp. *longum* BBMN68 Is Accompanied by Modification of the Cell Membrane Fatty Acid Composition

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The acid response of *Bifidobacterium longum* subsp. *longum* BBMN68 has been studied in our previous study. The *fab* gene, which is supposed to be involved in membrane fatty acid biosynthesis, was demonstrated to be induced in acid response. In order to investigate the relationship between acid response and cell membrane fatty acid composition, the acid adaptation of BBMN68 was assessed and the membrane fatty acid composition at different adaptation conditions was identified. Indeed, the fatty acid composition was influenced by acid adaptation. Our results showed that the effective acid adaptations were accompanied with decrease in the unsaturated to saturated fatty acids ratio (UFA/SFA) and increase in cyclopropane fatty acid (CFA) content, which corresponded to previous studies. Moreover, both effective and non-effective acid adaptation conditions resulted in decrease in the $C_{18:1}$ *cis*-9/$C_{18:1}$ *trans*-9 ratio, indicating that the $C_{18:1}$ *cis*-9/$C_{18:1}$ *trans*-9 ratio is associated with acid tolerance response but not with acid adaptation response. Taken together, this study indicated that the UFA/SFA and CFA content of BBMN68 were involved in acid adaptation and the $C_{18:1}$ cis-9/$C_{18:1}$ trans-9 ratio was involved in acid tolerance response.

Keywords: *Bifidobacterium longum* subsp. *longum* BBMN68, acid adaptation, membrane fatty acid

Introduction

There is increasing evidence for the contribution of bifidobacteria to maintaining good health [22]. Fermented milk products are one of the most popular means of delivering bifidobacteria in foods. However, examination of existing products revealed low numbers of viable cells [11, 15, 23], which is mainly due to the generally high sensitivity of this genus to acidic environments [5, 11, 12, 15, 17]. Therefore, it is important to enhance the acid tolerance of bifidobacterial strains to obtain high numbers of viable cells in dairy products.

Exposure of bifidobacteria to a moderate acid environment can protect the microorganism in a hostile environment [6, 7, 16, 21], a phenomenon termed acid adaptation. The mechanisms involved in acid adaptation include discharging H^+ by H^+-ATPase, blocking H^+ by the cell membrane and cell wall, neutralizing H^+ by alkalinity products, intercellular communicating via quorum sensing, and other final transmission systems [3, 4, 13]. The cell membrane fatty acid composition has been correlated with the proton permeability of the cell membrane [14], which is supposed to be involved in the balancing of H^+. Changes in the membrane fatty acid composition have also been observed in acid adaptation responses in lactic acid bacteria and bifidobacteria [1, 9, 24]. In our previous study about acid adaptation of *Bifidobacterium longum* subsp. *longum* BBMN68, the *fab* gene, which is supposed to be involved in fatty acid biosynthesis, was demonstrated to be induced in acid adaptation [13]. In this paper, evidence is shown that the acid response of BBMN68 is accompanied by changes in the profile of membrane fatty acids.

In most studies on acid adaptation, inorganic acids instead of organic acids was used [24]. However, the acidic

environment created by the organic acids (mainly lactate) during fermentation and storage was not the same as that created by inorganic acid. The effect of organic acid on the cell was more negative than that of inorganic acid [3, 9, 18]. For instance, several strains belonging to *B. bifidum*, *B. infantis*, and *B. breve* showed good resistance to in vitro gastric conditions but had low survival rates in acidified skim milk [24]. To enhance the survival of bifidobacteria in fermented milk products, the first step is to increase the ability of the microorganism to survive in organic acid produced by the starter cultures. In this study, the membrane fatty acid composition of BBMN68 was assessed in correlation with its acid adaptation and acid tolerance response to both organic and inorganic acids.

Materials and Methods

Bacterial Growth

The strain used in this study was *B. longum* subsp. *longum* BBMN68. It was originally isolated from the feces of healthy centenarians. The strain was stored at –80°C in 12% (w/v) skim milk supplemented with 20% (v/v) glycerol as the cryoprotectant.

Fermentations were carried out in a 15 L fermenter (Bioengineering AG, CH-8638 Wald) containing 8 L of experimental medium. The inoculum was prepared as follows: strains were transferred from –80°C to MRS broth supplemented with 0.05% cysteine-HCl and incubated anaerobically at 37°C for 12 h. The strains were propagated twice and finally added to the fermenter at an inoculum concentration of 2% (v/v). Anaerobic conditions were ensured by continuously sparging the medium with pure nitrogen during fermentation. A constant fermentation temperature of 37°C and a constant pH value of 6.5 were maintained. The pH was automatically controlled using 25% (v/v) ammonia. The fermentations were performed in two different media—MRS broth (supplemented with 0.5 g/l cysteine-HCl and containing 10 g/l glucose instead of 20 g/l glucose) and an industrial medium (containing 55 g/l corn steep liquor, 10 g/l glucose, 1 ml/l Tween-80, 1 g/l K_2HPO_4, 1 g/l KH_2PO_4, and 0.5 g/l cysteine-HCl). This medium is used for the industrial production of BBMN68 [25], and for this reason we used it in our current study.

Throughout the fermentation, bacterial growth was monitored by cell plating. Samples were plated on MRS agar and incubated for 48 h in an anaerobic jar. Anaerobic conditions were achieved using GENbox (bioMérieux, France).

Acid Adaptation and Acid Challenge Conditions

Cells grown under the above-mentioned conditions were harvested in the late stationary phase by centrifugation at 6,000 ×*g* for 10 min and were then washed twice in PBS buffer (pH 6.8). The concentrated cells were resuspended to their initial volume. Prior to use, the resuspension buffer was adjusted to different pH

values (pH 5.0, 4.7, 4.5, and 4.2) using approximately 10 M lactic acid (LA) or 12 M HCl. The number of viable cells before and after culture was measured by plate counting, and the counts were expressed in terms of colony-forming units (CFU/ml). The acid adaptation conditions under which the CFU was not significantly affected were determined.

Cells were harvested and adaptation was carried out under the conditions described above. Cells treated at pH 6.5 in fresh medium were used as the control. For the acid challenge studies, adapted and non-adapted cells were collected by centrifugation and suspended to their initial volume in skim milk supplemented with 0.1 M lactic acid. They were then cultured at 37°C for 2 h. The cell numbers at 0 and 2 h were determined by plating on MRS and counting the colonies after incubation for 48 h. Strain survival was defined as the ratio of the CFU at 2 h versus the value at 0 h. The induction factor was calculated as follows:

Induction factor = survival rate after acid adaptation/survival rate of control

Analysis of the Fatty Acid Composition

The membrane fatty acid composition of the cells was determined by a previously reported method [20]. Cells (10 ml) were collected by centrifugation and washed twice with 0.05 M Tris buffer (pH 7). Methylation and extraction were performed simultaneously at 4°C by adding 1 ml of sodium methoxide (1 mol/l in methanol) (Sigma-Aldrich, Germany), shaking for 2 min. Fatty acid methyl esters (FAMEs) were extracted from the supernatants with 300 μl of hexane. After decanting for 10 min, the upper phase was removed and stored at –80°C until further analysis.

The analysis was performed by a gas chromatography system (HP 6890; Hewlett Packard, USA) equipped with a mass selective detector (Agilent 5973, Hewlett Packard). A capillary column (DB-23, 60 m × 0.25 mm × 0.25 μm; Agilent) was used for the analyses. Nitrogen was used as the carrier gas (1.5 ml/min), and the injection volume was 2 μl. The splitless mode was used for injection, and it was performed for 1 min. The oven temperature was increased as follows: 65°C for 1 min; 65°C to 180°C at 5°C/min, held 3 min; and 180°C to 230°C at 3°C/min, held 5 min at this temperature.

FAMEs were identified in the bacterial extracts by comparing their retention times with those of known standards (Sigma Cat. No. 4785-U). Unknown peaks were identified on the basis of their mass spectrum. The results are expressed as a relative percentage of each fatty acid, calculated as the ratio of the surface area of the specific peak to the total area of all peaks.

Statistical Analysis

The statistical significance of the difference in survival and membrane fatty acid composition of the acid-adapted and non-adapted cultures was analyzed by the *t*-test. A *p* value less than 0.05 was considered to be statistically significant.
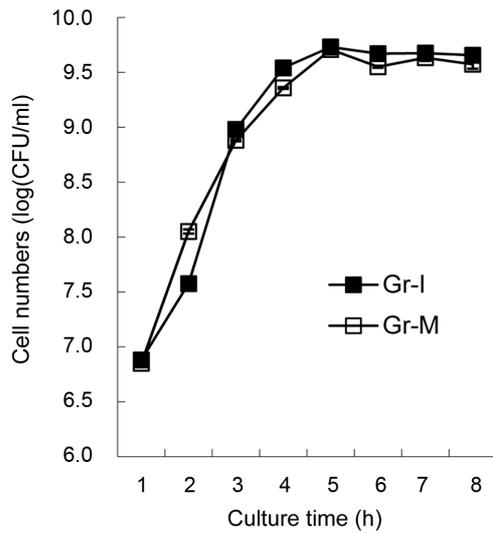
Fig. 1. Growth curves of BBMN68.

Gr-M: Growth curve in MRS; Gr-I: Growth curve in industrial medium. The values presented are means ± SD for three independent experiments.

Fig. 2. Acid resistance of BBMN68.

I-LA: Acid resistance of BBMN68 cultured in industrial medium to LA; I-HCl: Acid resistance of BBMN68 cultured in industrial medium to HCl; M-LA: Acid resistance of BBMN68 cultured in MRS to LA; and M-HCl: Acid resistance of BBMN68 cultured in MRS to HCl. The data are means of three to four independent experiments.

Results

Screening Acid Adaptation Conditions of Cells Cultured in Different Media

To precisely evaluate the effects of different acidification conditions on the acid tolerance of BBMN68, it was necessary to produce cells that were in a similar physiological state. In the first part of this study, we assessed and compared the kinetics and yields of this strain in two different media; namely, the industrial medium and MRS. As shown in Fig. 1, the growth curves of this strain in the two media fitted well, indicating that the growth in the industrial medium was unaffected. In both the cultures, the lag phase was very short and was followed by an exponential phase that lasted 9 h. The cells entered the stationary phase after 12 h of culture.

Results from the acid tolerance tests (Fig. 2) showed that in both media, the cells had the same survival rates at pH 5.0 and pH 4.7 ($p > 0.05$), and the decrease was less than 0.7 log, regardless of whether HCl or LA was used. However, under lethal conditions (pH 4.5 and pH 4.2), the tolerance of the cells grown in the industrial medium was better than that of the cells cultured in MRS. At the same pH values, LA was more lethal than HCl, which was in agreement with previously reported results [9]. Therefore, pH 5.0, 4.7 (for both industrial and MRS cultured cells), and 4.5 (for industrial medium cultured cells only) were selected as the
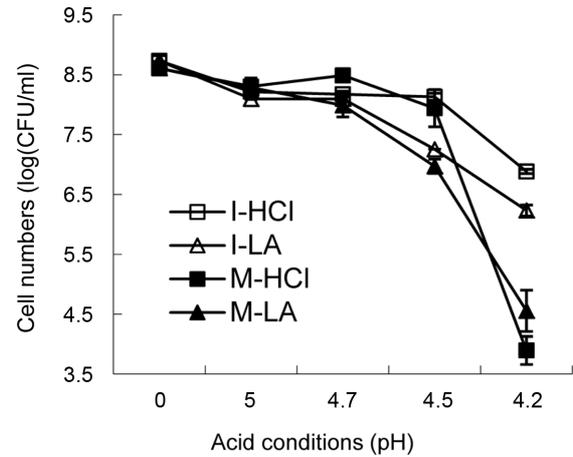
adaptation conditions to ensure a high CFU value prior to acid challenge.

Enhancement of the Acid Tolerance of BBMN68 after Acid Adaptation under Various Conditions

To study the effects of acidification on the acid tolerance of BBMN68, cells grown in the industrial medium (Table 1) or MRS (Table 2) were pretreated in fresh medium acidified according to the conditions determined above, and then challenged with acidified milk modified with 0.1 M LA. In almost all cases, the adapted cells showed higher survival rates than the control non-adapted ones. Under all conditions, the adaptation responses of cells grown in the industrial medium were more effective than those of cells cultured in MRS. HCl more effective than LA (Tables 1 and 2).

The effective adaptation conditions were pH 5.0_{HCl}, pH 5.0_{LA}, and pH 4.7_{HCl} for industrial medium and pH 5.0_{HCl} in MRS, on which the survival of adapted cells was elevated more than 10 times (1 log CFU/ml). The highest induction factor (110) was observed for cells that were cultured in the industrial medium and adapted at pH 5.0 by adjusting the pH with HCl. As the pH value decreased, the induction factor decreased. When the pH was adjusted to 4.5 with LA, the induction factor was less than 1, suggesting that the cells were more sensitive to the lethal effects of the acid after conditioning under these conditions.

However, when acid tolerance tests were conducted on

Table 1. Survival of BBMN68 cultured in industrial medium after acid challenge (skim milk acidified with 0.1 M LA) at various adapting conditions.

		Cell number (log$_{10}$CFU/ml)			
pH	Acidulant	Before acid challenge	After acid challenge	Survival rate (%)	Induction factor[d]
6.5		8.74 ± 0.071[a]	6.23 ± 0.085	0.31	
5	HCl[b]	8.22 ± 0.035	7.75 ± 0.042	33.88	110
	LA[c]	8.10 ± 0.007	6.99 ± 0.042	3.94	13
4.7	HCl	8.18 ± 0.049	7.17 ± 0.028	9.89	32
	LA	8.10 ± 0.057	6.16 ± 0.062	1.15	4
4.5	HCl	8.13 ± 0.057	6.62 ± 0.064	3.09	10
	LA	6.26 ± 0.00	<3.00		<1

[a]The values presented are means ± SD for three independent experiments.

[b]Medium acidified with hydrochloric acid.

[c]Medium acidified with lactic acid.

[d]Ratio of survival rate at considered conditions to that at control.

Table 2. Survival of BBMN68 cultured in MRS after acid challenge (skim milk acidified with 0.1 M LA) at various adapting conditions.

		Cell number (log$_{10}$CFU/ml)			
pH	Acidulant	Before acid challenge	After acid challenge	Survival rate (%)	Induction factor[d]
6.5		8.62 ± 0.094[a]	4.36 ± 0.19	0.0055	
5	HCl[b]	8.35 ± 0.014	5.09 ± 0.05	0.055	10
	LA[c]	8.45 ± 0.076	4.79 ± 0.16	0.0219	4
4.7	HCl	8.48 ± 0.031	4.41 ± 0.13	0.0085	2
	LA	7.93 ± 0.267	4.48 ± 0.20	0.0355	6

[a]The values presented are means ± SD for three independent experiments.

[b]Medium acidified with hydrochloric acid.

[c]Medium acidified with lactic acid.

[d]Ratio of survival rate at considered conditions to that at control.

the most efficiently adapted and non-adapted cells in acidified skim milk upon storage for 21 days at 4°C, there were no significant improvements (Fig. 3), suggesting that the improved acid tolerance was probably temporary.

Modification of the Membrane Fatty Acid Composition after Acid Adaptation

The membrane fatty acid composition of BBMN68 cultured under different growth conditions was determined by the gas chromatographic method. The unsaturated to saturated fatty acids ratio (UFA/SFA), and cyclopropane fatty acid (CFA) content, and C$_{18:1}$ *cis*-9/C$_{18:1}$ *trans*-9 ratios were used to determine the differences in the membrane fatty acid composition of BBMN68 under different adaptation conditions.

It should be noted that the modification in the membrane fatty acid composition observed in BBMN68 depended on the pH values, and differed between the two media used in this study (Table 3). What is most interesting is that the modification was correlated to the effect of adapting response. Effective acid-adapted cells showed higher CFA levels than non-acid-adapted control cells. Moreover, a significantly lower UFA/SFA was observed under effective adaptation conditions. Specifically, high induction factors corresponded to low UFA/SFA (Table 3), and the highest induction factor (110) was achieved at the lowest UFA/SFA ratio (0.36). The UFA/SFA ratio was unchanged in cells cultured in MRS under adaptation conditions at pH 5.0 and 4.7, at which the induction factor was lower than 10. Moreover, when cells cultured in MRS were challenged at pH 4.5 with LA-acidified medium, which is lethal to the cells, the UFA/SFA ratio abruptly increased to 0.91.

The C$_{18:1}$ *cis*-9/C$_{18:1}$ *trans*-9 ratio decreased from 5.6 to 2.9 for cells grown in the industrial medium and from 3.3 to 2.6
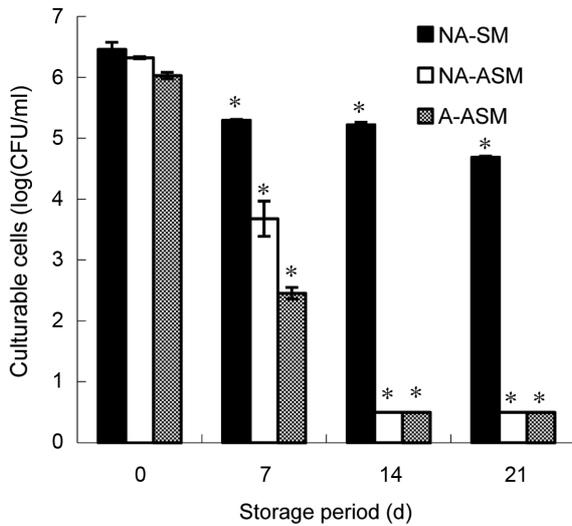
Fig. 3. Survial of BBMN68 during refrigerated storage (4°C). NA-SM: non-adapted BBMN68 in skim milk; NA-ASM: non-adapted BBMN68 in skim acidified milk; A-ASM: adapted BBMN68 in skim acidified milk. The acid adaptation was taken at the optimized condition (pH 5.0, cultured for 2 h at 37°C). *indicates that the value was significantly lower than the value in 0 day ($p < 0.05$).

Fig. 4. Effect of acid adaptation on the membrane in $C_{18:1}$ *cis*-9 /$C_{18:1}$ *trans*-9 ratio of BBMN68.

I-HC: Cells cultured in the studied industrial medium adapted in HCl acidified medium; I-LA: Cells cultured in the studied industrial medium adapted in LA acidified medium; M-HCl: Cells cultured in MRS adapted in HCl acidified medium; M-LA: Cells cultured in MRS adapted in LA acidified medium. The values present are means ± SD for three independent experiments. *indicates that the value was significantly lower than the value at pH 6.5 ($p < 0.05$).

for those grown in MRS (Fig. 4). It is speculated that as the pH value decreases, some proportion of $C_{18:1}$ *cis*-9 is converted to $C_{18:1}$ *trans*-9. Although cells cultured in both media showed the same trend in terms of change in this ratio, there was a significantly higher $C_{18:1}$ *trans*-9 content in cells cultured in MRS ($p < 0.05$). For cells grown in the industrial medium, acidification induced changes in the $C_{18:0}$ proportion, but such an effect was not observed for the cells cultured in MRS. The $C_{18:0}$ content was lower in non-acid-adapted cells and increased as the pH value decreased, reaching a maximum value at pH 4.5 (6.8%) (Table 3).

Discussion

It is generally assumed that bifidobacteria adapting to a moderate acid medium showed greater resistance to harsh environments [6, 7, 16, 21], but there was disagreement. Acid adaptation of a highly acid-sensitive strain of *B. longum* did not enhance its acid tolerance [16]. Morever, adaptation of *B. longum* E1884 at pH 3.5 for 1 h impaired its acid tolerance [21]. The adaptation conditions are considered to be crucial to the adaptation of a particular strain. In this study, adaptation results were acquired under different adapting conditions just as what have been reported in previous studies. We defined them effective adaptation
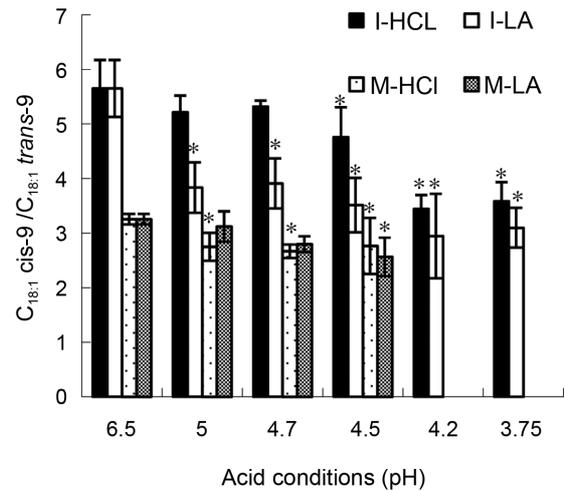
conditions (pH 5.0_{HCl}, pH 5.0_{LA}, pH 4.7_{HCl} for industrial medium and pH5.0_{HCl} in MRS) on which the survival of adapted cells was elevated more than 10 times (1 log CFU/ml).

Under effective adaptation conditions, *B. longum* BBMN68 cells showed regular modifications in their membrane fatty acid composition. In previous studies, bacteria such as *Lactobacillus sanfranciscensis* CB1 [9], *Lactobacillus delbrueckii* subsp. *bulgaricus* [24], and *Streptococcus thermophilus* [1] have been shown to alter the fatty acid composition of their cell membrane in response to acidification of their surrounding environment. The fatty acid composition is related to the cell membrane permeability [14], which is supposed to be related to maintaining internal pH [10] and further managing to survive better in acidic environments [2]. The greater tolerance of BBMN68 was supposed to be associated with a lower UFA/SFA ratio. When the industrial medium was used, the lowest UFA/SFA ratio was obtained at pH 5.0 (adjusted with HCl), and the highest UFA/SFA ratio was obtained at pH 4.5 (adjusted with LA), which corresponded to the highest and lowest acid tolerance respectively. A decrease in the UFA of adapted cells has been reported earlier [3], but other authors have reported an increase in the UFA/SFA ratio at low pH values in other strains [19]. The UFA/SFA ratio in the cell membrane is

Table 3. Relative fatty acid concentrations of BBMN68 cells recovered from MRS and the studied industrial medium without acid adaptation or with acid adapted at studied conditions.

Culture medium	Fatty acids	Relative concentrations (%)						
		Control	HCl$_{pH5.0}$[c]	LA$_{pH5.0}$[d]	HCl$_{pH4.7}$	LA$_{pH4.7}$	HCl$_{pH4.5}$	LA$_{pH4.5}$
Industrial	C$_{12:0}$	1.80 ± 0.52	0.78 ± 0.17	0.83 ± 0.18	0.79 ± 0.32	0.52 ± 0.16	1.09 ± 0.34	0.75 ± 0.08
	C$_{14:0}$	5.56 ± 0.63	6.72 ± 0.39	8.10 ± 1.00	6.48 ± 0.46	8.16 ± 2.97	7.33 ± 1.39	4.13 ± 0.42
	C$_{15:0}$	0.75 ± 0.19	0.58 ± 0.11	0.72 ± 0.24	0.87 ± 0.43	0.67 ± 0.04	0.92 ± 0.13	0.58 ± 0.11
	C$_{16:0}$	51.42 ± 2.59	58.47 ± 3.53	52.41 ± 2.39	52.64 ± 2.6	51.38 ± 3.34	50.07 ± 1.83	55.71 ± 0.62
	C$_{16:1}$	3.16 ± 0.25	1.74 ± 0.50	3.87 ± 0.21	1.77 ± 0.18	2.43 ± 0.39	2.19 ± 0.22	2.32 ± 0.25
	C$_{18:0}$	5.09 ± 0.28	5.22 ± 0.44	5.47 ± 0.49	6.76 ± 0.48	5.37 ± 0.84	6.57 ± 0.70	6.08 ± 0.40
	C$_{18:1t}$	3.46 ± 0.68	3.11 ± 0.40	3.85 ± 0.46	3.39 ± 0.44	4.20 ± 0.86	3.79 ± 0.37	4.42 ± 0.49
	C$_{18:1c}$	19.02 ± 1.38	14.66 ± 2.81	16.50 ± 0.68	16.08 ± 0.79	16.36 ± 3.07	14.24 ± 1.63	14.07 ± 0.43
	Cyc19	1.52 ± 0.07	2.44 ± 0.51	2.71 ± 0.15	2.78 ± 0.45	2.45 ± 0.82	2.63 ± 0.52	2.62 ± 0.09
	Others	7.47 ± 1.45	6.90 ± 1.13	6.71 ± 1.01	8.27 ± 0.60	8.57 ± 0.91	9.99 ± 1.07	9.16 ± 1.02
	U/S[b]	0.56 ± 0.02	0.36 ± 0.002[a]	0.45 ± 0.004[a]	0.48 ± 0.04[a]	0.59 ± 0.06	0.52 ± 0.04	0.48 ± 0.03
MRS	C$_{12:0}$	1.60 ± 0.38	1.08 ± 0.21	1.45 ± 0.46	1.31 ± 0.17	1.14 ± 0.36	1.01 ± 0.21	1.11 ± 0.30
	C$_{14:0}$	6.36 ± 0.14	7.65 ± 0.77	7.38 ± 0.35	8.42 ± 0.40	7.69 ± 0.52	7.47 ± 0.47	5.39 ± 0.52
	C$_{15:0}$	0.74 ± 0.14	0.81 ± 0.03	0.72 ± 0.12	0.65 ± 0.14	0.88 ± 0.35	0.68 ± 0.22	0.77 ± 0.08
	C$_{16:0}$	49.11 ± 0.64	48.53 ± 2.25	48.16 ± 0.76	49.30 ± 0.76	44.93 ± 1.95	45.77 ± 3.25	39.86 ± 1.42
	C$_{16:1}$	2.17 ± 0.17	2.16 ± 0.18	1.96 ± 0.28	1.77 ± 0.80	1.80 ± 0.54	2.09 ± 0.21	1.20 ± 0.61
	C$_{18:0}$	5.65 ± 0.59	5.80 ± 0.17	5.28 ± 0.34	5.74 ± 0.07	5.02 ± 0.74	5.77 ± 1.09	5.35 ± 0.29
	C$_{18:1t}$	6.11 ± 0.26	6.32 ± 0.85	6.59 ± 0.64	6.13 ± 0.12	7.16 ± 0.82	7.06 ± 1.03	8.73 ± 0.49
	C$_{18:1c}$	19.54 ± 0.44	17.33 ± 1.06	19.07 ± 0.52	16.94 ± 1.30	20.40 ± 2.25	19.25 ± 0.77	22.74 ± 1.23
	Cyc19	1.37 ± 0.05	1.53 ± 0.09[a]	1.44 ± 0.10	1.17 ± 0.07	1.38 ± 0.20	1.43 ± 0.07	1.56 ± 0.12
	Others	7.57 ± 0.79	8.32 ± 1.07	7.96 ± 0.72	8.57 ± 0.73	9.75 ± 0.86	9.07 ± 1.70	12.48 ± 1.25
	U/S	0.57 ± 0.02	0.56 ± 0.07	0.59 ± 0.02	0.53 ± 0.03	0.63 ± 0.13	0.64 ± 0.05	0.91 ± 0.04

The values present percentages of total fatty acids and are means ± standard deviations for three independent experiments.

[a]Statistically different ($p \leq 0.05$) from the percentage in non-adapted control cells.

C$_{12:0}$: dodecylic acid; C$_{14:0}$: tetradecanoic (myristic) acid; C$_{16:0}$: hexadecanoic (palmitic) acid; C$_{16:1}$: palmitoleic acid; C$_{18:0}$: stearic acid; C$_{18:1c}$: oleic acid; C$_{18:1t}$: elaidic acid; Cyc 19: methylenoctadecanoic acid (dihydrosterculic or *Lactobacillus*) acid.

[b]Unsaturated to saturated fatty acid ratio

[c]Acidified medium with hydrochloric acid at indicated pH.

[d]Acidified medium with lactic acid at indicated pH.

related to membrane fluidity. Some authors have reported a correlation between membrane fluidity and leakage [8]. Thus, the UFA/SFA ratio may influence cell leakage and thereby the maintenance of the internal pH. The change in UFA/SFA ratio and acid tolerance could be regarded as an indicator of the correlation between cell membrane fluidity and acid tolerance.

In this study, the decrease in the C$_{18:1}$ *cis*-9/C$_{18:1}$ *trans*-9 ratio of the cell membrane upon challenge in acidic environments also seems to be an important protective response. It is hypothesized that the isomerization of *cis*-UFA to *trans*-UFA is an emergency action undertaken by *Pseudomonas putida* to adapt its membrane fluidity to drastic changes in the environmental conditions. Hyperosmotic and hypoosmotic shocks led to alterations in the amounts of *trans*- and *cis*-isomers of monounsaturated fatty acids present in whole-cell fatty acid extracts of *Pseudomonas putida*, which contributed to changes in the membrane fluidity. A decrease in the C$_{18:1}$ *cis*-9/C$_{18:1}$ *trans*-9 ratio was observed when the pH decreased. The isomerization of *cis*- and *trans*-fatty acids seemed to contribut to acid tolerance in sublethal conditions other than that in lethal conditons, as there was no correlation between the C$_{18:1}$ *cis*-9/C$_{18:1}$ *trans*-9 ratio and the induction factors.

Previously, it has been suggested that CFA plays an important role in microbial acid adaptation responses. CFA

synthesis in the cell membrane during acid adaptation is important and protects the cell under adverse conditions [3]. It has been demonstrated that compared with control cells, *Escherichia coli* mutants with deletion of the CFA synthesis activity expressed approximately 10-fold lower survival at pH 3.0, whereas addition of exogenous CFA increased the acid tolerance of this strain [4] . In our study, the CFA content and acid tolerance were both higher in cells cultured in the industrial medium than in those cultured in MRS. The ingredients of corn steep liquor, which is one of the components of the industrial medium, may influence the physiological state of the cells, and the mechanism of this effect should be further investigated.

In preliminary experiments, it was found that the enhanced acid tolerance resulting from the most effective adaptation conditions was temporary; that is, the protective effect was no longer evident upon storage in acidified milk. It was speculated that during long-term acid challenge, the repair and defense systems developed to counter the adverse environment and became non-functional. Alternatively, the protection generated by the moderate acid challenge was not available for BBMN68 on a long-term basis when the microorganism encountered harsh environments in fermented milk.

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