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Lactobacillus casei combats acid stress by maintaining cell membrane functionality

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Abstract *Lactobacillus casei* strains have traditionally been recognized as probiotics and frequently used as adjunct culture in fermented dairy products where lactic acid stress is a frequently encountered environmental condition. We have investigated the effect of lactic acid stress on the cell membrane of *L. casei* Zhang [wild type (WT)] and its acid-resistant mutant Lbz-2. Both strains were grown under glucose-limiting conditions in chemostats; following challenge by low pH, the cell membrane stress responses were investigated. In response to acid stress, cell membrane fluidity decreased and its fatty acid composition changed to reduce the damage caused by lactic acid. Compared with the WT, the acid-resistant mutant exhibited numerous survival advantages, such as higher membrane fluidity, higher proportions of unsaturated fatty acids, and higher mean chain length. In addition, cell integrity analysis showed that the mutant maintained a more intact cellular structure and lower membrane permeability after environmental acidification. These results indicate that alteration in membrane fluidity, fatty acid distribution, and cell integrity are common mechanisms utilized by *L. casei* to withstand severe acidification and to reduce the deleterious effect of lactic acid on the cell membrane. This detailed comparison of cell membrane responses between the WT and mutant add to our knowledge of the acid stress adaptation and thus enable new strategies to be developed aimed at improving the industrial performance of this species under acid stress.

Keywords Acid stress · *Lactobacillus casei* · Membrane fluidity · Membrane fatty acids

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Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group of bacteria that are used to produce fermented foods and which are generally recognized as safe (GRAS). They are normal inhabitants of the oral cavity and digestive tract in human [10, 17]. *Lactobacillus casei* has traditionally been recognized as a probiotic and used in commercial products for its health-promoting and nutritional properties [19, 32]. However, *L. casei*, similar to other lactic acid bacteria, encounters various stress conditions during industrial processes and in the gastrointestinal tract, among which acid stress is one of the important challenges to its survival. Consequently, acid tolerance is recognized as one of the desirable properties used to select potential probiotics [21]. Lactate, an organic acid, is especially toxic to many microorganisms. Undissociated lactate entering the

cytoplasm will dissociate and disrupt the pH_I and the anion pool of the cytoplasm, which in turn affects the integrity of purine bases and results in the denaturation of essential enzymes inside the cell [26, 28].

As the first barrier between the external environment and the intracellular medium, the cell membrane plays an important role in cellular growth, metabolism, energy transduction, and maintenance of a constant intracellular environment [31]. The membrane regulates the movement of substances entering or exiting the cells and catalyzes exchange reactions [22]. However, it is also a primary target for damage induced by environmental stresses. Although stress conditions result in changes in the organization, dynamic structure, and function of membrane lipids, the integrity and fluidity of the cytoplasmic membrane are key factors in maintaining the viability of cells and their metabolic activities [20, 22]. The results from a number of earlier studies suggest that the regulation of membrane status impacts cell survival under stress conditions. For example, *Lactobacillus bulgaricus* CFL1 reduced the ratio of both unsaturated to saturated fatty acids (UFA:SFA) and cyclic fatty acids to SFA in the membrane during acidification [25]. Wang et al. [27] reported that the high cryotolerance of *Lactobacillus acidophilus* RD758 during frozen storage was related to a high UFA:SFA ratio, a low C18:0 content, and high levels of C16:0 and cyclic C19:0. The cellular membrane responses involved in acid tolerance are not yet fully understood. The purpose of this study was, therefore, to conduct a more detailed analysis of the cell membrane response by comparing the wildtype (WT) *L. casei* Zhang and its acid-resistant mutant in order to identify the acid tolerance mechanisms utilized by *L. casei*.

Materials and methods

Strains and growth conditions

The bacterial strains used in this study were *L. casei* Zhang and its acid-resistant derivative Lbz-2 (mutant). The mutant was obtained by adaptive evolution, which consisted of serially transferring the WT cells into MRS broth (Oxoid, Basingstoke, Hampshire, UK) at pH 4.3 for 70 days (unpublished data). For both strains, inocula were transferred from −80°C frozen stock to MRS broth (Oxoid), then incubated statically at 37°C for 12 h as precultures. To investigate the growth performance of *L. casei* Zhang and Lbz-2, 2% (v/v) of the precultures were inoculated into 50 ml of MRS broth (initial pH of 4.3 adjusted with lactic acid) and incubated at 37°C for 64 h. Growth was monitored spectrophotometrically at 600 nm.

To investigate the cell membrane responses of *L. casei* Zhang and Lbz-2 to acid shock, the precultures were then inoculated with inoculum (2%, v/v) into a BIOFLO 110 chemostat (New Brunswick Scientific, Edison, NJ) with a working volume of 600 ml in fermentation medium [(in g l^{-1}) glucose, 8; soy peptone, 10; yeast extract, 5; K_2HPO_4, 2.6; NaAc, 5; $MnSO_4 \cdot H_2O$, 0.19; $MgSO_4$, 0.28; sodium citrate, 2]. The dilution rate of the culture was 0.1 h^{-1}, and the pH of the medium was maintained at 6.5 through the addition of 2 mol l^{-1} NaOH. Cells collected from a steady-state chemostat held at pH 6.5 were subjected to further physiological analyses.

Acid challenge and acid tolerance analyses

For the acid challenge experiments, steady-state chemostat cells were centrifuged at 10,000 *g* for 5 min, washed twice with saline, and then resuspended in modified MRS broth (adjusted to pH 3.0 or 3.5 with 6 mol l^{-1} hydrochloric acid or 25% lactic acid). After acid challenge for various times, 1 ml portion of cell suspension was withdrawn, centrifuged at 10,000 *g* for 2 min, and washed with saline to remove the residual acidified medium. Cells were then serially diluted, plated onto MRS agar plates, and incubated at 37°C for 48 h. Cell viability was expressed as the percentage of viable cells (CFU per milliliter) at time zero.

Artificial gastric juice treatment

The tolerance of cells to gastric acidity was evaluated by suspending cells in simulated gastric juice [3 g l^{-1} pepsin, 0.5% (w/v) NaCl] at pH 2.5 for 0, 0.5, 1, 1.5, and 2 h. At each time-point, cells were withdrawn and treated as in the acid challenge assay to determine viability.

Measurement of membrane lateral diffusion

The lateral diffusion of fatty acid chains within the membrane were determined by measuring intermolecular pyrene excimerization according to the method of Aricha et al. [3]. Measurements were made at 37°C with a spectrofluorimeter (model 650-60; Hitachi, Tokyo, Japan) with excitation at 335 nm and emission at 373 nm (for the monomer) and 470 nm (for the excimer) (5- and 5-nm slits, respectively). The excimer-to-monomer ratio increased linearly with the pyrene concentration, and the probe's lateral diffusion could be derived from the slope of the curve [3].

Measurement of fluorescence anisotropy

Measurement of fluorescence anisotropy is used for studying rotational diffusion of fatty acyl chains in the membrane interior. DPH (1,6-diphenyl-1,3,5-hexatriene)

was used to monitor changes in membrane dynamics, as previously described [29]. Steady-state fluorescence anisotropy was measured at 37°C with a spectrofluorimeter (model 650-60; Hitachi) with excitation at 360 nm and emission at 430 nm (5- and 5-nm slits, respectively). Fluorescence anisotropy was calculated as described by Ansari et al. [2]. The degree of fluorescence polarization (p) was calculated as follows:

$$p = \frac{I_{VV} - I_{VH}(I_{HV}/I_{HH})}{I_{VV} + I_{VH}(I_{HV}/I_{HH})},$$

where I_V is the corrected fluorescence and subscripts V and H indicate the values obtained with vertical or horizontal orientation, respectively, of the excitation and analyzer polarizer (in that order). Anisotropy was calculated as:

$$r = \frac{2p}{3 - p}$$

In these experiments, decreases in the degree of fluorescence anisotropy reflect increases in the fluidity of the lipid bilayer, which controls or alters the mobility of DHP in the membrane.

Fatty acid extraction and analysis

The extraction of bacterial lipids and preparation of fatty acid methyl esters (FAMEs) were carried out according to the method of Zhang et al. [30]. Briefly, cells were collected by centrifugation (7,500 g at 4°C for 20 min). The cell pellets were washed twice with cold saline (0.85% NaCl, w/v), then mixed with 1 ml NaOH in a methanol-distilled water solution (3:10:10, w/v/v) and heated at 100°C for 10 min. After rapid cooling in an ice bath, FAMEs were extracted with 1.25 ml methyl tertiary butyl ether–hexane (1:1, v/v) for 10 min and washed with 3 ml 0.33 mol l^{-1} NaOH. The top-layer organic phase (0.8 ml) was transferred to a 2 ml Silyler vial, evaporated under nitrogen flow, and then made up to 10 ml with hexane.

A 1-µl sample of the concentrated extract was separated on a 30-m fused silica capillary polar column [BPX70 (70% biscyanopropyl polysiloxane), inner diameter 0.22 mm, film thickness 0.25 µm; SGE, Austin, TX] using a Shimadzu GC-17A chromatograph system coupled with a QP-5000 mass spectrometer (Shimadzu, Kyoto, Japan). The carrier gas was helium at a flow rate of 29.6 ml min^{-1}, and the column pressure was 63.4 kPa with a flow of 0.5 ml min^{-1}. The temperatures for the injection port and detector were 260 and 280°C, respectively. The temperature program was 100°C isothermally for 1 min, followed by an increase of 4°C min^{-1} to 250°C, and then 250°C isothermally for 5 min. FAMEs were identified by their mass spectra compared against a spectrum database. The relative amount of FAMEs was calculated from peak areas. The degree of unsaturation (mol% UFA/mol% SFA; U/S ratio) and the mean chain length were assayed. All experiments were carried out in triplicate.

Transmission electron microscopy

Samples for transmission electron microscopy (TEM) were fixed in 2.5% (v/v) glutaraldehyde for 30 min. Cell pellets were harvested by centrifugation (5,000 g, 5 min) and mixed with 1.25% (w/v) water agar. The agar was then cut into approximately 1-mm pieces and fixed in phosphate-buffered 2.5% (v/v) glutaraldehyde for an additional 30 min. The agar pieces were rinsed with 0.02 mol l^{-1} phosphate buffer (pH 6.8) three times, postfixed in phosphate buffer containing 1% (w/v) osmium tetroxide for 1 h, then rinsed with water and fixed for 1 h in 1% (w/v) aqueous uranyl acetate. All fixations were carried out at room temperature. After dehydration in a graded series of ethyl alcohol and two washes in propylene oxide, the agar pieces containing cells were embedded in Epon 812 (Spi Supplies, New Chester, PA). Thin sections stained with uranyl acetate and lead citrate were examined in a JEM 1200 EX electron microscope (JEOL, Tokyo, Japan).

Measurement of inner membrane permeability

Permeability of the inner membrane was assessed by measuring the access of *o*-nitrophenyl-*b*-D-galactopyranoside (ONPG) to the cytoplasm essentially as described previously [18]. Cells were rinsed once by centrifugation (3,000 g, 15 min) and resuspended in 10 mmol l^{-1} sodium phosphate buffer (pH 7.4) to an OD_{600} of 1.0. ONPG was added to a final concentration of 100 µg ml^{-1} into quartz cuvettes containing 2 ml of cell suspension, and substrate cleavage by β-galactosidase was monitored by light absorption measurement at 420 nm in a spectrophotometer (UV-2450; Shimadzu).

Statistical analysis

Student's t test was employed to investigate statistical differences. Differences between samples with p values of ≤ 0.05 were considered to be statistically significant.

Results

Acid tolerance comparison of *L. casei* Zhang and its acid-resistant mutant

To address the acid tolerance of *L. casei* Zhang and its acid-resistant derivative mutant, we harvested steady-state chemostat cells at pH 6.5 and then shocked these with

hydrochloric acid (pH 3.0) (Fig. 1a), simulated gastric juice (pH 2.5) (Fig. 1b), or lactic acid (pH 3.5) (Fig. 1c). Under both hydrochloric acid and simulated gastric juice stresses, the survival rate of the mutant was higher than that of the WT (3.4- and 2.8-fold higher, respectively, after a 2 h shock; Fig. 1). Interestingly, the survival differences between the WT and the mutant were especially significant following prolonged exposure to lactic acid. After 2.5 h of lactic acid shock, the survival rates of both strains decreased sharply, and only 0.001% of the WT survived compared with more than 0.1% for the mutant, which is a 100-fold difference. The survival difference between hydrochloric acid and lactic acid may be ascribed to their attack mechanisms. In addition, the mutant was obtained from lactic acid-stressed conditions, which have resulted in it having a higher tolerance to lactic acid.

Growth performance, which is a property particularly relevant to industrial production, of both strains was tested in medium containing lactic acid (pH 4.3). When grown at low pH, the mutant exhibited an increase of more than 57% (maximum at OD_{600} values of 1.99 and 3.13 for WT and mutant, respectively) in biomass over the WT, suggesting again that the mutant was more tolerant of acid stress than the WT (Fig. 2).

These data demonstrate that the acid tolerance of the mutant was significantly higher than that of the WT when the strains were grown at low pH or challenged with severe acid stress. To investigate the mechanisms involved in the acid tolerance of *L. casei*, the physiological status of the cells, especially the cell membrane, which is considered to be the first line of defense against undesirable stresses, was then studied under acid-stressed conditions.

Changes in membrane fluidity in *L. casei* under acid stress

The lateral diffusion of pyrene and rotational diffusion of DPH were monitored as these processes are directly related to the fluidity of the cell membrane. Figure 3a shows the pyrene excimerization coefficient (K_a) in the WT and the mutant under acid stress. The lateral diffusion of pyrene decreased when cells finished the steady-state chemostat at pH 6.5 and then were challenged at pH 5.0 or pH 3.5 for 1 h. After being shocked at pH 5.0 for 1 h, the K_a of both the WT and the mutant decreased by 44.7 and 35.0%, respectively, compared to the K_a at pH 6.5. As the lateral diffusion of pyrene is directly related to the membrane viscosity [3], the decreased K_a was mainly due to the reduced lateral diffusion of the probe, indicating increased viscosity of the membrane in both strains under acid stress conditions. Interestingly, the mutant maintained a higher K_a under various pH values compared with the WT, indicating a lower membrane viscosity under acid stress.

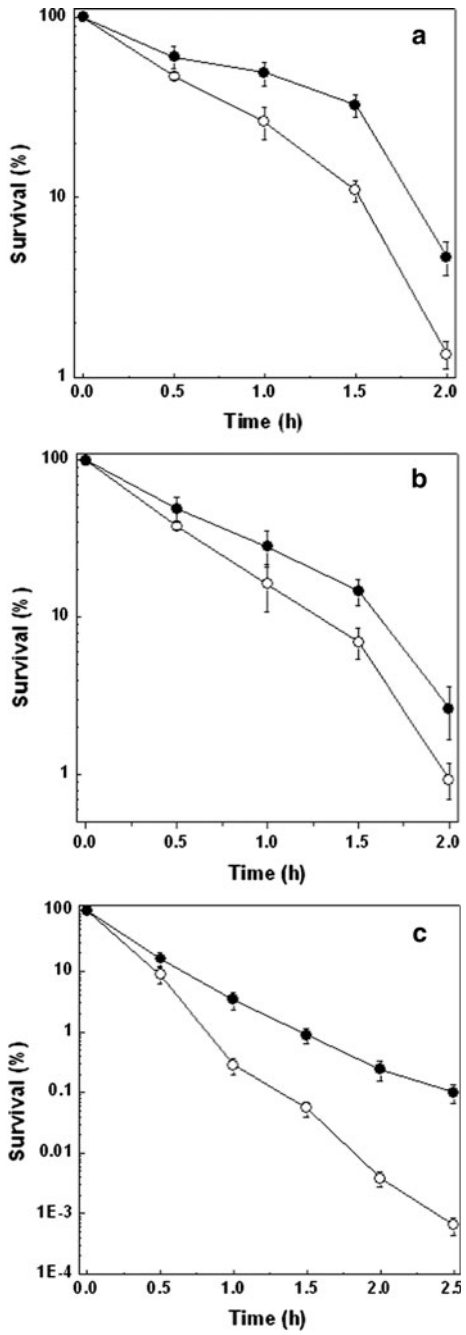

Fig. 1 Tolerance analysis of *Lactobacillus casei* cells to acid stress. **a** Cells were stressed in MRS broth acidified with hydrochloric acid to pH 3.0. **b** *L. casei* cells were stressed in simulated gastric juice adjusted to pH 2.5. **c** *L. casei* cells were stressed in MRS broth acidified with lactic acid to pH 3.5. *Open circle* Wildtype (WT), *filled circle* mutant. *Error bars*: Standard deviation (SD) ($n = 3$)

Figure 3b shows the changes in membrane micro-viscosity under acid stress, as determined by measuring the rotational diffusion of the fatty acyl chains in the membrane interior; fluorescence anisotropy was estimated by using DPH as a probe. Similarly, the WT displayed higher anisotropy at various pH, suggesting a higher micro-viscosity in

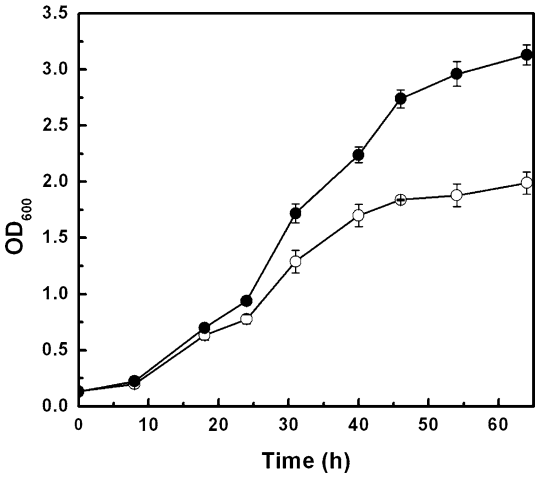

Fig. 2 Growth comparison of *L. casei* WT and mutant cells in the presence of lactic acid. Both cell types were cultivated in MRS at an initial pH of 4.3 and growth performance measured. *Open circle* WT, *filled circle* mutant. *Error bars*: SD ($n = 3$)

the WT than in the acid-resistant mutant. These results indicate that differences in membrane fluidity between the WT and acid-resistant mutant played an important role in improving the survival of the mutant cells at low pH.

Lactic acid-induced membrane fatty acid alterations in *L. casei*

The relationship between changes in membrane fatty acids and resistance to acid stress was established for *L. casei* Zhang and its acid-resistant mutant Lbz-2. As shown in Fig. 4, the composition of SFA and UFA changed in both WT and mutant strains in response to acid shock. Interestingly, the most significant change observed in both strains was a large decrease in the content of SFA and an increase in UFA content, reflecting marked changes in the UFA:SFA ratio (U/S ratio) under stress conditions (Fig. 5a). The U/S ratio generally increased with extended exposure time, and the acid-resistant mutant displayed higher U/S ratios than the WT under acid stress.

Although the mean chain length of the membrane fatty acids increased under acidic conditions, the mutant contained shorter fatty acid chains than the WT (Fig. 5b). It is worth noting that the proportion of the cyclopropane fatty acid (CFA) cyclopropaneoctanoic acid $C_{19:0}$-cyc) increased with acid stress and that the mutant contained a higher amount of CFA than the WT (Fig. 6). A requirement for CFA under conditions of low pH has previously been reported for the survival of *Escherichia coli* and *L. casei* [8]. These results indicate that in response to acid shock and to increase its chance of survival at low pH, *L. casei* increased the proportion of long-chained UFA in its membrane with a concomitant decrease in the proportion of short-chained SFA. These changes are similar to those reported in a number of other bacteria [12, 15].

Changes in cell integrity upon exposure to acid stress

To investigate the effect of lactic acid stress on the cell integrity of *L. casei*, the surface morphology of the WT and the mutant were compared by TEM. As shown in Fig. 7a, b, integral cell membranes were observed in both the WT and mutant cells prior to acid treatment. However, with prolonged exposure to lactic acid (pH 3.5), the cell wall of the WT cells became thinner and coarser relative to those of the mutant cells (Fig. 7c, d). After 1 h of lactic acid shock, many of the WT cells cracked and their cytoplasm effused, while the mutant cells retained cell integrity under acid stress (Fig. 7e, f).

Cell integrity during acid stress was further studied by evaluating the permeability of the inner membrane permeability using the β-galactosidase substrate ONPG as a probe. When ONPG passes through the inner membrane, it can be cleaved by β-galactosidase, resulting in the

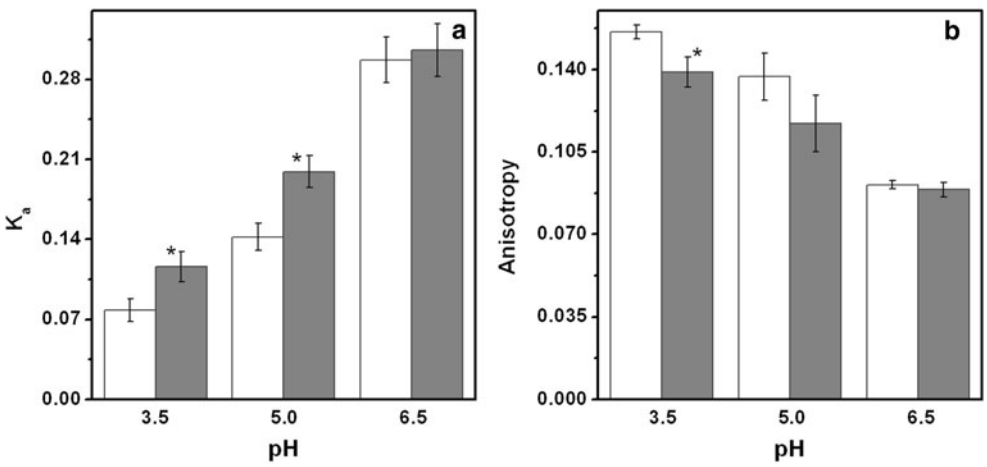

Fig. 3 Changes in membrane fluidity in *L. casei* WT and mutant cells under acid stress. Steady-state chemostat cells were shocked with lactic acid at various pH. *White bars*, *gray bars* WT and mutant, respectively. *Error bars*: SD ($n = 3$). Statistically significant differences ($p < 0.05$) were determined by Student's *t* test and are indicated with *asterisks*. K_a Pyrene excimerization coefficient

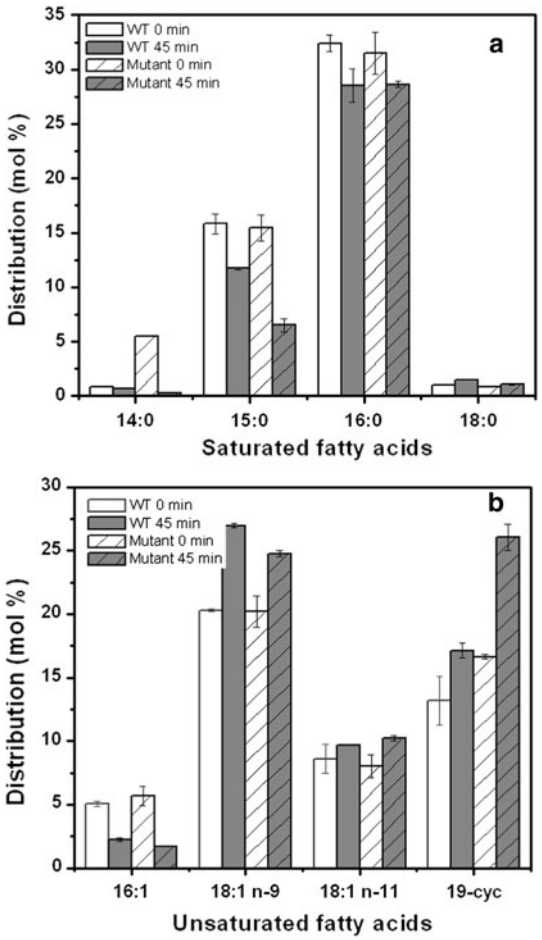

Fig. 4 Alteration in the distribution of saturated fatty acids (**a**) and unsaturated fatty acids (**b**) in the membrane of *L. casei* WT (*white bars*) and mutant (*gray bars*) cells upon acid stress. Cells were exposed to the acid stress (pH 3.5 for 45 min) in MRS medium. *Error bars*: SD ($n = 3$)

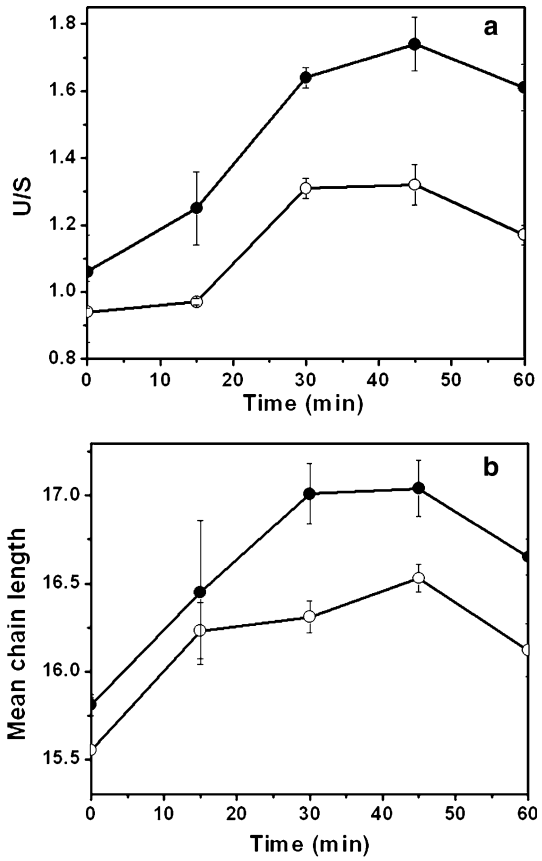

Fig. 5 Profiles of the unsaturated fatty acid:saturated fatty acid ratio (*U/S*) (**a**) and mean chain length (**b**) in *L. casei* WT and mutant cells upon acid treatment at pH 3.5. *Open circle* WT, *filled circle* mutant. *Error bars*: SD ($n = 3$)

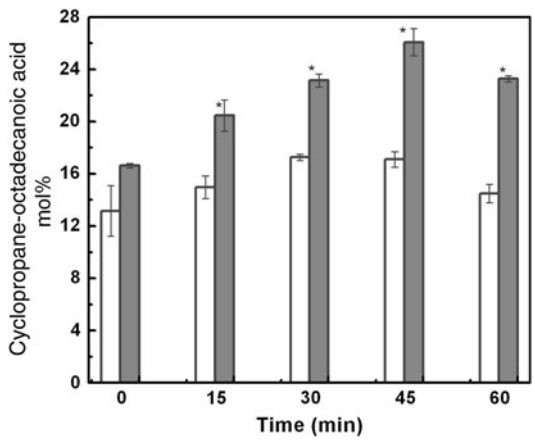

Fig. 6 Time-course of cyclopropane-octadecanoic acid (C19-cyc) content upon acid stress at pH 3.5. *White bars*, *gray bars* Fatty acid composition of C19-cyc in the *L. casei* WT and mutant, respectively. *Error bars*: SD ($n = 3$). Statistically significant differences ($p < 0.05$) were determined by Student's *t* test and are indicated with *asterisks*

appearance of yellow color. Thus, absorbency at 420 nm and the increased rate of ONPG passage indicates inner permeability [18]. As shown in Fig. 8, the permeability of the membrane of both strains was almost the same and relatively low prior to being exposed to acid stress. However, after being shocked at pH 3.5 for 1 h, the permeability of the inner membrane increased, with the WT exhibiting a higher level of membrane permeability. These results suggest that the mutant maintained cell integrity during acid stress.

Discussion

To investigate the protective mechanisms employed by the *L. casei* acid-resistant derivative Lbz-2 (mutant) against acid stress, we used a physiological approach to compare the stress responses of the WT and mutant strain at the membrane level under conditions of acid stress.

Membrane fluidity is a complex reflection of the effects of fatty acyl chain conformation, lateral diffusion, rotational diffusion, and transbilayer diffusion ("flip flop") and

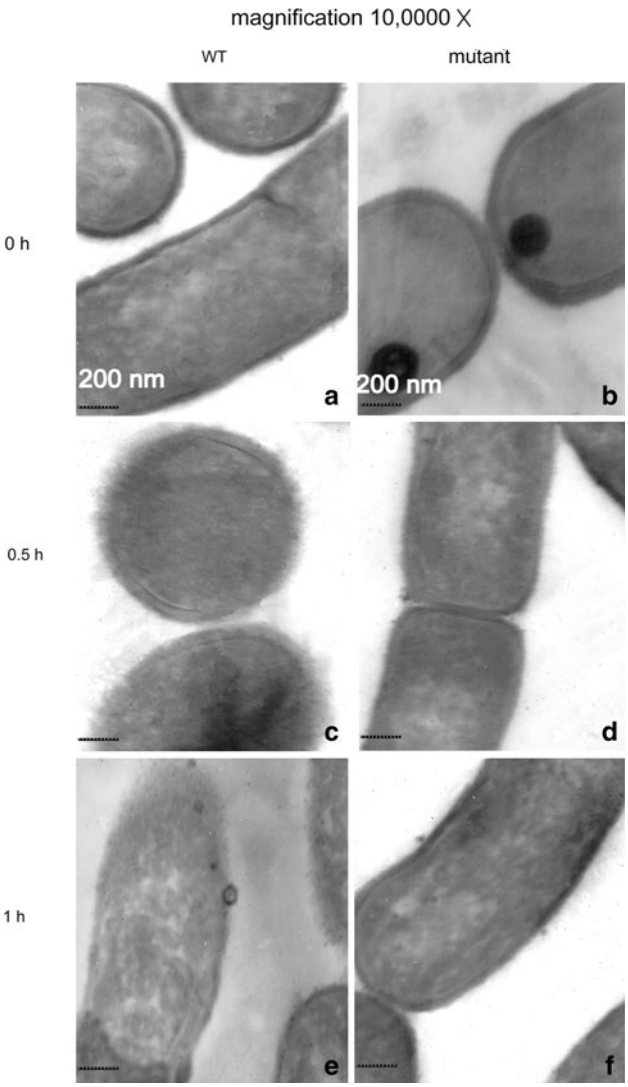

Fig. 7 Transmission electron microscopy examination of the morphological changes of *L. casei* under acid stress. **a**, **c** and **e**, WT; **b**, **d** and **f**, mutant. Both cells were suspended in MRS broth adjusted to pH 3.5 by lactic acid for 0 h (**a** and **b**), 0.5 h (**c** and **d**) and 1 h (**e** and **f**), respectively. *Scale bars* of 200 nm for ×10,000 views were shown on the *down-left corner* of each photograph

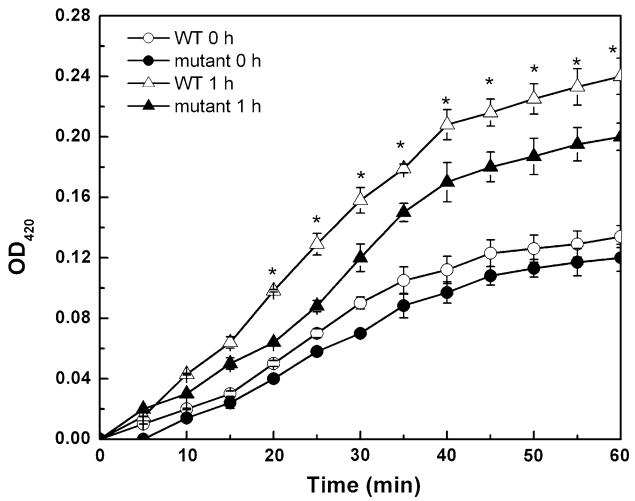

Fig. 8 Changes in inner membrane permeability of *L. casei* before (*circle*) and after (*triangle*) acid stress. Both cell types were grown under steady-state chemostat and then shocked with lactic acid at pH 3.5 for 0 h and 1 h. *Error bars*: SD ($n = 3$). Statistically significant differences ($p < 0.05$) were determined by Student's t test and are indicated with *asterisks*

the resistance of the membrane to sheer forces [11]. In our study, membrane characteristics were investigated using different fluorescence assay methods that provide information on these two diffusion modes. The rate of lateral diffusion in the membrane was determined by monitoring the intermolecular excimerization of pyrene. In this case, the mutant retained a dramatically higher K_a than the WT, indicating that the former had a lower membrane micro-viscosity than the WT. In a corresponding assay, the rotational diffusion of the chains was evaluated by fluorescence anisotropy, with DPH as a probe. As expected, the result was quite similar to those observed with pyrene, signifying a higher membrane micro-viscosity in the WT than in the mutant. Alakomi et al. [1] and Chu-Ky et al. [9] reported that lactic acid stress induced serious membrane rigidification with a highly disorganized status. The rigid state of the membrane could reduce the fluidizing effect of lactic acid so that the deleterious effect of lactic acid may be minimized by preventing its entry. Chu-Ky et al. [9] also suggested that the disorganized state of the cell membrane was caused by protein denaturation which resulted in high cell mortality and was due, at least in part, to differences in acid tolerance between the two strains.

The regulation of membrane fatty acid profiles is also one approach available to the cell to combat environmental stresses. *L. casei*, similar to *Streptococcus mutans*, *S. gordonii*, and *S. salivarius* [13, 14], increased the levels of long-chained, mono-UFA under acid stress, suggesting that modulation of the fatty acid composition is necessary for survival at low pH. The acid-resistant mutant exhibited a higher U/S ratio than the WT (Fig. 5a), indicating an induced mechanism for survival through an increased degree of unsaturation of its membrane fatty acids.

These results lead to an interesting discussion on the relationship between membrane fluidity and fatty acid composition. Change in the SFA:UFA ratio is the most common cellular mechanism used to modulate membrane fluidity [23]. However, our results seem to be inconsistent with this mechanism as although both the WT and the mutant increased the degree of unsaturation under acid stress, no increase in membrane fluidity was observed (Figs. 3, 4). There is currently no clear explanation of the relationship between membrane fluidity and fatty acid profiles during acid stress. It is very possible that

mechanisms in addition to alterations in membrane fatty acids may be implicated in contributing to membrane fluidity at low pH. Cellular fatty acid composition is a result of a sum of complex phenomena maintaining optimal viability of the cell under various conditions. Therefore, it is difficult to understand the adjustment mechanisms linking fatty acid composition to various stress factors. Fozo and Quivey recently reported that mono-UFA are necessary for the maintenance of ΔpH across the membrane, and it is likely that this function is conserved among a number of specific microorganisms [12]. In addition to fatty acid distribution, fatty acid chain length is another important alteration that is used to increase survival in acidic environments. Both the mutant and the WT showed increased fatty acid chain length, with the mutant containing slightly longer fatty acid chains than the WT (Fig. 5b). A previous study showed that an increased chain length of fatty acids in oral bacteria *S. gordonii* DL1 and *S. salivarius* 57.1 protected the cells from acid stress [14]. It has also been reported that longer chains more easily span the width of the bilayer, thereby promoting acyl chain packing and making the membrane environment more gel-like; in contrast, shorter chains are unable to span the bilayer and cannot form hydrophobic interactions with other lipids and proteins. As a result, the fluidity and instability of the bilayer increase due to the motion of the free acyl chain ends [7]. Therefore, the increased fatty acid length contributes to the decrease in cell membrane fluidity, which is in agreement with our results in terms of the pyrene excimerization rate (K_a) and rotational diffusion of DPH.

It is inspiring to have determined that the ratio of CPA in the mutant was higher than that in the WT. The role of CPA in tolerance to acid stress has been investigated in *L. lactis* MG1363 and *E. coli* [6, 8], and increased CFA content has been observed during heat shock [5] or osmotic stress [16] in *L. lactis*. A previous study also found that cyclization of fatty acids is generally regarded as a tool to reduce membrane fluidity and prevent the penetration of undesirable molecules [20].

Cell integrity is critical in maintaining cell viability and metabolic function, particularly under stress. The mutant exhibited better cell integrity than the WT, indicating that the mutant membrane could be more impermeable to lactic acid than that of the WT, partially explaining the higher tolerance of the mutant to lactic acid. This observation also indicates that the increased permeability of the WT induced by lactic acid results in a stronger proton influx. This change leads to dissipation of both the proton motive force and the capacity for pH homeostasis [4, 24].

The *L. casei* cell membrane maintains the proper physiological functions of the cell, thereby contributing to survival at low pH. In our study, we compared the physiological alterations of *L. casei* at the membrane level during acid stress. Our results provide detailed information on the response and tolerance mechanisms of *L. casei* used to survive in a high acid environment that may facilitate the development of new strategies aimed at enhancing the industrial utility of this species under acid stress.

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