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Physiological and proteomic analysis of *Lactobacillus casei* in response to acid adaptation

Chongde Wu · Guiqiang He · Juan Zhang

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Abstract The aim of this study was to investigate the acid tolerance response (ATR) in *Lactobacillus casei* by a combined physiological and proteomic analysis. To optimize the ATR induction, cells were acid adapted for 1 h at different pHs, and then acid challenged at pH 3.5. The result showed that acid adaptation improved acid tolerance, and the highest survival was observed in cells adapted at pH 4.5 for 1 h. Analysis of the physiological data showed that the acid-adapted cells exhibited higher intracellular pH (pH_i), intracellular NH_4^+ content, and lower inner permeability compared with the cells without adaptation. Proteomic analysis was performed upon acid adaptation to different pHs (pH 6.5 vs. pH 4.5) using two-dimensional electrophoresis. A total of 24 proteins that exhibited at least 1.5-fold differential expression were identified. Four proteins (Pgk, LacD, Hpr, and Galm) involved in carbohydrate catabolism and five classic stress response proteins (GroEL, GrpE, Dnak, Hspl, and LCAZH_2811) were up-regulated after acid adaptation at pH 4.5 for 1 h. Validation of the proteomic data was performed by quantitative RT-PCR, and transcriptional regulation of all selected genes showed a positive correlation with the proteomic patterns of the identified proteins. Results presented in this study may be useful for further elucidating the acid tolerance mechanisms and may help in formulating new strategies to improve the industrial performance of this species during acid stress.

Keywords *Lactobacillus casei* · Acid stress · Proteomic analysis

C. Wu (✉) · G. He
Key Laboratory of Leather Chemistry and Engineering, Ministry of Education, College of Light Industry, Textile & Food Engineering, Sichuan University, Chengdu 610065, China
e-mail: cdwu@scu.edu.cn

C. Wu · J. Zhang
Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

Introduction

Lactobacillus casei is a faculatative heterofermentative lactic acid bacterium, and it is widely distributed in various habitats, including fermented products, the intestinal tract, the oral cavity, and even soil and lake [21, 27]. It has been traditionally recognized as a probiotic due to its health-promoting and nutritional properties [31]. However, *L. casei*, similar to other lactic acid bacteria, encounters various stress conditions including acid, oxygen, salt and temperature during industrial processing and in the gastrointestinal tract. Among various environmental stresses, acid stress is one of the important survival challenges, and acid tolerance is accepted as one of the desirable properties for selecting potentially probiotic strains [20].

In response to acid stress, lactic acid bacteria employ various mechanisms to fight against acid damage, including the maintenance of intracellular pH (pH_i) homeostasis and cell membrane functionality, and induction of stress-response proteins [12, 19, 22, 26, 28]. Based on these results, many strategies have been proposed to engineer acid stress resistance, and increased robustness of lactic acid bacteria was obtained. For example, in our previous research, we found that acid stress led to a significant upregulation of DNA repair protein RecO in *L. casei*, and the acid-resistant mutant exhibited higher expression level compared with the corresponding control strain [23]. Inspired by this observation, we set out to engineer

the expression of RecO in *L. lactis* and investigated the protective role of RecO during environmental stresses. As expected, the recombinant strain exhibited increased tolerance to acid, salt and oxygen stresses [25]. Broadbent et al. [4] reported that acid stress led to the accumulation of histidine in *L. casei*. Validation of the data was performed by experiments which suggested that *L. casei* survival at pH 2.5 was improved at least 100-fold by addition of 30 mM histidine to the acid challenge medium. In addition, acid tolerance response (ATR) seems to increase the viability of cells during lethal pH by pre-exposure of the cells to sublethal pH. There are many studies of ATR in microorganisms, such as *Lactococcus lactis*, *Bifidobacterium longum*, *Bifidobacterium animalis* [7, 13]. However, mechanisms underlying acid tolerance are complex and remain largely unclear. In the present study, a comprehensive analysis in *L. casei* was conducted on the basis of the physiological and proteomic data to further elucidate acid tolerance mechanisms employed by *L. casei*.

Materials and methods

Strains and growth conditions

The strain used in this study was *Lactobacillus casei* lbz-2 (CCTCC No. M2010292). Cultures were grown statically in MRS broth (Oxoid) at 37 °C. To investigate the physiological and proteomic responses of *L. casei* to acid stress, the cultures were incubated with an inoculum size of 2 % into a BIOFLO 110 chemostat (New Brunswick Scientific) with a working volume of 600 ml [23]. Cells collected from a steady-state chemostat were subjected to further physiological and proteomic analyses.

Acid stress experiments

To investigate the acid tolerance response, cells grown at steady-state chemostat (OD_{600} = 3.0) were centrifuged at 10,000×*g* (4 °C) for 5 min, washed twice with saline and then resuspended in fresh MRS broth adjusted to pH 6.5 (control), 6.0, 5.5, 5.0, 4.5, 4.0 with 25 % lactic acid. Cells pre-adapted at different pHs for 1 h were harvested and washed as noted and suspended in MRS adjusted to pH 3.5 (acid stress). After acid stress for 1 h, the cell suspensions were serially diluted and spotted in triplicate onto MRS agar plates to determine the viability.

Measurement of intracellular pH (pH_i)

Cells adapted or un-adapted at pH 4.5 were subjected to acid challenge at pH 3.5 for 1 h, and then intracellular pH (pH_i) in acid-adapted and acid-challenged cells was measured by the fluorescence method developed by Breeuwer et al. [3] using 5 (and 6-)-carboxyfluorescein succimidyl ester as the fluorescent probe. Calibration curves on the relationship between extracellular pH and intracellular pH were established to exclude artifacts caused by environmental conditions. Loading of cells with 5 (and 6-)-carboxyfluorescein succimidyl ester, determination of pH_i, and calibration of pH_i all followed the procedure described previously [3].

Determination of intracellular ammonia

Cells grown at steady-state chemostat (OD_{600} = 3.0) were harvested and subjected to different acid treatments. Then, the acid-adapted and acid-challenged cells were washed twice with 200 mM phosphate-buffer saline (PBS, pH 7.5), and then resuspended in the same buffer. The solution was sonicated on ice for 10 min and followed by centrifugation at 12,000×*g* for 10 min. The amount of ammonia in the supernatant was analyzed with the ammonia assay kit (Sigma, USA) according to the manufacturer's instructions.

Measurement of inner membrane permeability

Permeability of the inner membrane was assessed by measuring the access of *o*-nitrophenyl-*β*-D-galactoside (ONPG) to the cytoplasm essentially described previously [16]. The acid-challenged cells with or without acid adaptation were rinsed once by centrifugation (3,000×*g*, 15 min), and resuspended in 10 mM sodium phosphate buffer (pH 7.4) to an OD_{600} of 1.0. ONPG was added to a final concentration of 100 μg/ml into quartz cuvettes containing 2 ml of cell suspension, and substrate cleavage product by β-galactosidase was monitored by light absorption measurement at 420 nm in a spectrophotometer (UV-2450, Kyoto, Japan).

2D gel electrophoresis, gel image analysis and protein identification

Cells grown at steady-state chemostat were centrifuged at 10,000×*g* for 5 min, washed twice with saline (0.9 % NaCl) and then resuspended in modified MRS broth (adjusted to pH 4.5 with 25 % lactic acid). After challenged for 1 h, the cells were washed for three times with distilled water to remove the residual acidified medium. The whole-cell extracts were prepared using the BioRad kit and the concentration of each protein sample was determined using the BioRad Protein Assay Kit (BioRad) with BSA as a standard. Each sample was applied to immobilized pH gradient (IPG) strips (18 cm, pH 4–7, BioRad) with a final concentration of 100 μg protein in 350 μl rehydration buffer. Isoelectric focusing (IEF) of the protein samples was performed according to the method described by

Table 1 Primers used for validation of 2DE data by RT-PCR

Protein	Gene	Primer	Oligonucleotide sequence (5′–3′)
Tagatose-1,6-bisphosphate aldolase	*lacD*	Forward	AGGTGATCAAGATCACGAAGGA
		Reverse	CAGACAGGAAAATGAATGGCAG
Galactose mutarotase-related enzyme	*galm*	Forward	TGTTGAACCATTAAACAGCCC
		Reverse	GTTAGCGAGAATCACGAGACC
60 kDa chaperonin (groEL protein)	*groEL*	Forward	CCTGGCATTGAGAAGCAAAATA
		Reverse	TTGGCAGCCGATAACTACGACT
Chaperone protein dnaK	*dnaK*	Forward	TGACGAATTGCACAAGATTAGC
		Reverse	GCATCCCTTCAACAACAGAGAG
Small heat shock protein	*hspl*	Forward	TGACGAATTGCACAAGATTAGC
		Reverse	GCATCCCTTCAACAACAGAGAG
Esterase C	*estC*	Forward	CACAATTTTGTCTTGTACGGTG
		Reverse	GTGGCGAATCCAATAATCTCTC
	16S rRNA	Forward	GAGGCAGCAGTAGGGAATCTTC
		Reverse	CGACCATTCTTCTCCAACAACAG

Wu et al. [28]. After IEF, the IPG strips were equilibrated sequentially in a buffer (Tris–HCl contained 6 M urea, 30 % v/v glycerol, and 2 % SDS) that contained 1 % DTT or 2.5 % iodoacetamide for 15 min each. The equilibrated IPG strips were placed on 12 % SDS polyacrylamide gels. Electrophoresis performed at 12 °C was carried out at 1 W/gel for 1 h and followed by 10 W/gel until the dye front reached the gel bottom using an Ettan DALT-12 unit (GE Healthcare). The 2D gels were fixed and stained with a modified silver stain [15].

The stained gels were scanned using Imagescanner (GE Healthcare). Comparative analysis of the protein spots was performed using Image Master 6.0 2D platinum software (GE Healthcare). Proteins that displayed at least 1.5-fold variations were taken into account for further analysis. For spot-picking, preparative gel containing 1,000 μg of protein extract was run and stained with 0.1 % Coomassie blue R-250. The protein spots with significant change were excised using gel plugs, transferred to Eppendorf tubes, then digested with 20 μl of 10 ng/μl proteomics sequencing grade trypsin at 37 °C for 16 h and rehydrated in 500 μl of 50 mM NH_4HCO_3 (pH 8.0). Supernatants of 0.5 μl were spotted directly onto the MALDI plate for proteins identified by mass spectrometry as described previously [14].

Gene expression analysis by quantitative RT-PCR

For quantitative RT-PCR analysis, differently expressed proteins were selected. RNA was isolated using a RNA extraction kit (TaKaRa, Japan). Complementary DNA (cDNA) was synthesized using a One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, Japan). The primers used for RT-PCR assay are listed in Table 1. The 16S rRNA was used as the internal control for quantification. RT-PCR assay was performed using the SYBR Premix EX TaqTM Kit (TaKaRa, Japan) with at least three biological replicates. The PCR was carried out using A LightCycler 480 II Real-time PCR System (Roche, Germany) with the procedures described by Wu et al. [24]. The $2^{-\Delta\Delta C_T}$ value method was used to compare the expression of the genes, and the expression levels of all the tested genes were normalized against the expression level of the internal control gene (16S rRNA) [18, 30].

Statistical analysis

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

Results and discussion

Acid tolerance response of *L. casei*

Acid tolerance response (ATR) is a common mechanism utilized by lactic acid bacteria, and it makes these microbes less susceptible to acid stress by prior exposure of the cells to moderately acidic conditions [4, 7]. To investigate the optimal condition for ATR induction, different acid-adaptation conditions were compared (Fig. 1). Cells pre-exposed to different adaptation pHs were challenged at pH 3.5 for 1 h, and the survival rates were determined. The results showed that the ATR could be triggered by exposure to a wide range of sublethal pH values. The survival rate of the control cells (pre-adaptation at pH 6.5) at pH 3.5 was 4.5 %. While it increased gradually with the decrease of adaptation pH, and pre-exposure at pH 4.5 exhibited the highest survival (52.4 %). The results showed that ATR in *L. casei* could be triggered by

exposure to pH 4.5 for 1 h, and it was chosen as the acid-adaptation treatment. Broadbent et al. [4] investigated different acid-adaptation treatments on the survival of *L. casei* ATCC 334, and the results showed that cells pre-adapted at pH 4.5 for 20 min displayed superior survival during acid challenge at pH 2.0 for 140 min. In addition, a 70-fold higher survival was obtained in *B. longum* challenged at pH 3.5 by pre-incubation of cells at pH 4.5 for 2 h [13]. These results also illustrated the importance of optimizing conditions for ATR induction in a particular strain.

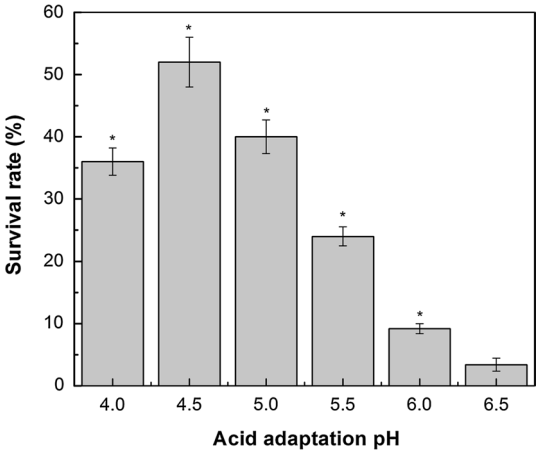

Fig. 1 Effect of acid adaptation on the survival of *L. casei* during acid challenge at pH 3.5 for 1 h. Cells collected from a steady-state chemostat held at pH 6.5 were pre-exposed for 1 h at pH 6.5 (control), 6.0, 5.5, 5.0, 4.5, and 4.0 and then acid challenge at pH 3.5. *Error bars* indicate standard deviations ($n = 3$). Statistically significant differences ($p < 0.05$) were determined by Student's t test and are indicated with an *asterisk*

Changes in intracellular pH and intracellular NH_4^+ concentration

Intracellular pH (pH_i) plays an important role during the growth and metabolism of *L. casei*, and it affects the uptake of nutrients, protein and nucleic acid synthesis [19]. With the decrease of pH_i, enzyme activities may be decreased, and ultimately proteins as well as DNA damages will take place [5]. Thus, the ability to maintain pH_i homeostasis during acid stress is essential for the survival of cells. Figure 2 shows the changes in intracellular pH and intracellular NH_4^+ concentration of *L. casei* during acid stress. After acid stress (pH 3.5) for 1 h, pH_i sharply decreased from 7.24 to 5.45. However, the cells pre-adapted at pH 4.5 for 1 h exhibited higher pH_i (pH_i 6.28) after acid challenge (Fig. 2a). To further validate the result, intracellular NH_4^+ content was determined (Fig. 2b). As expected, acid adaptation induced the increase in intracellular NH_4^+ content from 0.27 to 0.40 nmol/(mg protein). In addition, after acid stress at pH 3.5 for 1 h, a significantly higher ($P \leq 0.05$) NH_4^+ content (0.55 nmol/(mg protein)) was observed in the pre-exposed cells, compared to that in cells without pre-adaptation (0.41 nmol/(mg protein)) (Fig. 2b). These results show that pre-exposure of cells at a mildly acidic condition may protect cells against acid stress by maintaining higher pH_i and NH_4^+ content. Generally, induction of the activity of proton-translocating ATPase is a common mechanism utilized by lactic acid bacteria to regulate the pH_i [8, 24]. Jin et al. [13] analyzed the gene expression profiles in *B. longum* by RNA-sequencing, and many genes involved in the regulation of pH_i were up-regulated. Thus, it was reported that blocking H^+, discharging H^+, and neutralizing H^+ were the mechanisms of ATR utilized by *B. longum* [13].

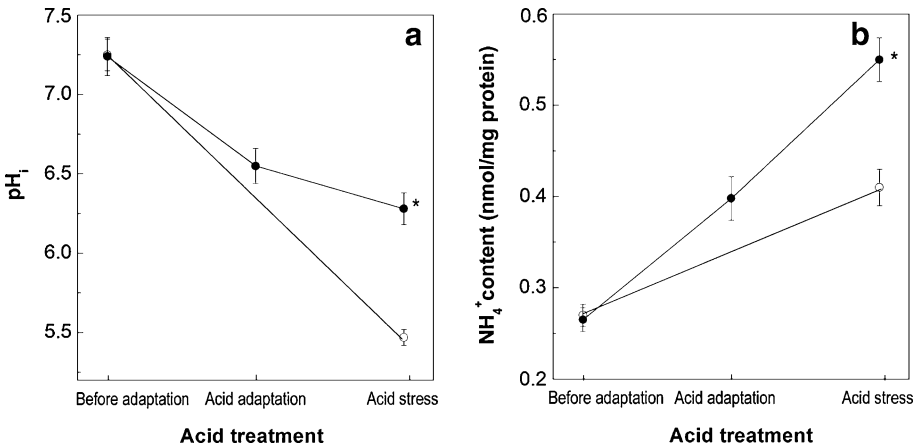

Fig. 2 Changes in intracellular pH (**a**) and intracellular NH_4^+ content (**b**) during acid stress. Cells collected from a steady-state chemostat held at pH 6.5 were challenged at pH 3.5 for 1 h. *Open circles* represent cells shocked at pH 3.5 without pre-adaptation, while *closed circles* represent cells pre-adapted at pH 4.5 for 1 h prior to acid stress at pH 3.5. *Error bars* indicate standard deviations ($n = 3$). Statistically significant differences ($p < 0.05$) were determined by Student's t test and are indicated with an *asterisk*

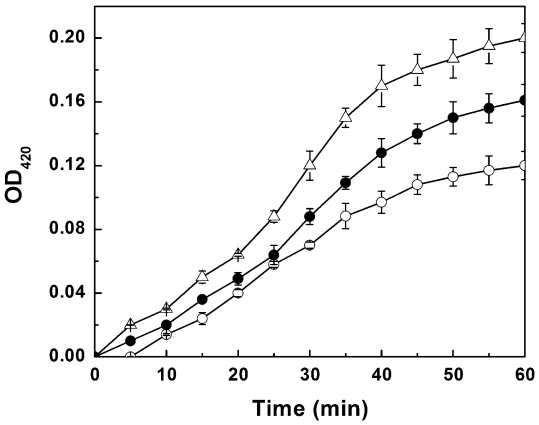

Fig. 3 Change in inner permeability of *L. casei* during acid stress. Cells collected from a steady-state chemostat held at pH 6.5 were harvested and subjected to acid treatment. The acid-challenged cells with or without pre-adaptation were resuspended in 10 mM phosphate buffer (pH 7.4) to an OD_{600} of 1.0. ONPG was added to the cell suspension and the absorbance at 420 nm was monitored for 60 min. *Open circles* present inner membrane permeability of cells at pH 6.5, while *closed circles* and *triangles* present inner permeability of cells at pH 3.5 with and without acid pre-adaptation, respectively. *Error bars* indicate standard deviations ($n = 3$)

Changes in inner permeability during acid stress

Inner membrane permeability was evaluated using the β-galactosidase substrate ONPG as probe. When ONPG passes the inner membrane, it can be cleaved by β-galactosidase, resulting in the appearance of yellow color. Thus, absorbency at 420 nm and its increased rate indicate inner permeability. As shown in Fig. 3, the cells had relatively low level of permeability before acid stress (pH 6.5). However, after acid challenge at pH 3.5 for 1 h, the inner membrane permeability increased, and the cells without pre-adaptation at pH 4.5 exhibited higher level of membrane permeability (Fig. 3). These results suggest that acid stress leads to the increase in membrane permeability, and acid adaptation contributes to protect the cell integrity during acid stress. Generally, cell integrity is critical in maintaining cell viability and metabolic function, particularly under stressed conditions, and the increased permeability during acid stress may lead to a stronger proton influx [2]. The low membrane permeability obtained by acid adaptation could be more impermeable to lactic acid, partially explaining the higher tolerance in the adapted cells during acid stress. Our previous research with *L. casei* wild type and its acid-resistant mutant also suggested that the mutant displayed lower membrane permeability and better cell integrity during acid stress [26].

Comparative proteomic analysis of *L. casei* based on 2DE

To further elucidate the ATR mechanisms employed by *L. casei*, comparative proteomic analysis was performed.

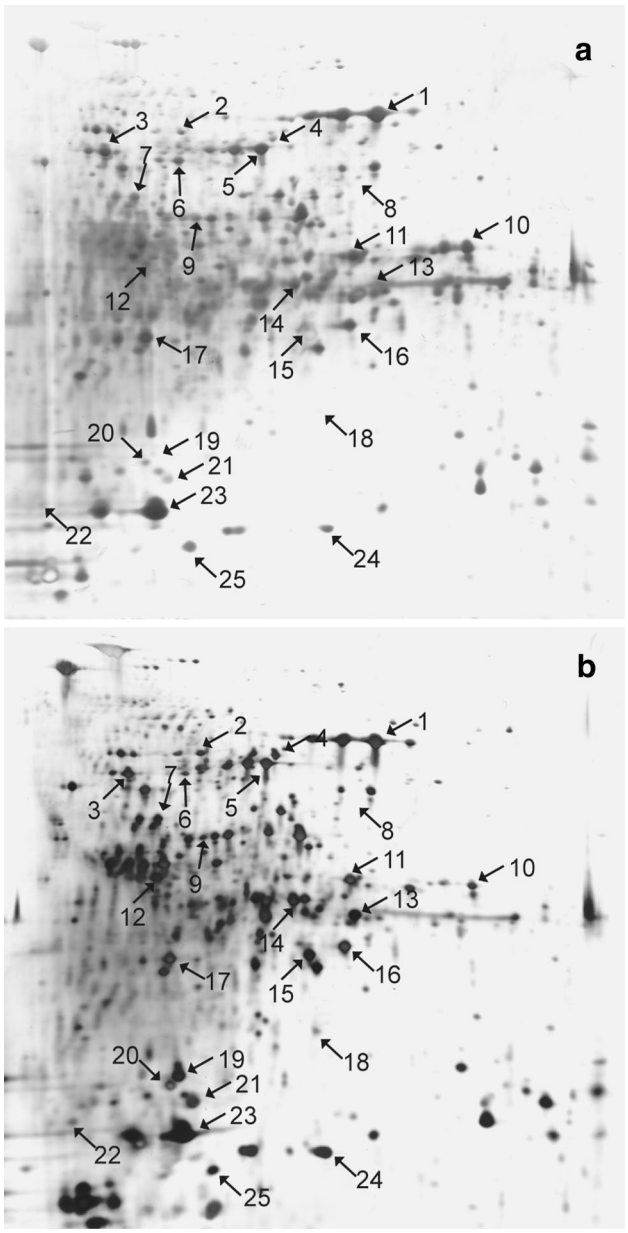

Fig. 4 Two-dimensional electrophoresis gels of the whole cytoplasm proteins of *L. casei* exposed at pH 6.5 (**a**) and pH 4.5 (**b**) for 1 h. The identified proteins are indicated by an *arrow* and *number* and listed in Table 2

Cytosolic proteins were extracted from cells exposed to pH 6.5 and pH 4.5. Following extraction, 2DE was performed and the representative maps are shown in Fig. 4. The gel maps were analyzed and the proteins differentially expressed more than 1.5-fold were labeled with numbers and subjected to identification. A total of 24 proteins were identified and the identified proteins grouped into cellular roles according to COGs are summarized in Table 2. As shown in Table 2, after acid adaptation at pH 4.5 for 1 h, the expressions of 18 proteins up-regulated

Table 2 Differentially expressed proteins identified in *L. casei* under acid stress

Spot no.	NCBI accession no.	Putative function	Gene locus	Gene	Mass	pI	Fold change in fold intensity relative to pH 6.5
Carbohydrate transport and metabolism							
1	gil190712210	Phosphoglycerate kinase	LCABL_11310	pgk	42,211.2	5.64	1.52 ± 0.13
5	gil301067579	Tagatose-1,6-bisphosphate aldolase	LCAZH_0609	lacD	36,400.9	5.25	2.33 ± 0.37
9	gil300439987	Galactose mutarotase-related enzyme	LCAZH_2563	galm	32,378.1	5.04	1.72 ± 0.27
23	gil8307834	Hpr		pstH	9,247.1	4.91	1.90 ± 0.09
Translation, ribosomal structure and biogenesis							
6	gil190712872	Elongation factor Ts (EF-Ts)	LCABL_17980	tsf	31,681.3	5.0	−1.63 ± 0.17
12	gil22266146	Putative elongation factor Tu		tuf	28,210.6	4.54	2.21 ± 0.32
15	gil190712870	Ribosome recycling factor	LCABL_17960	frr	20,580.8	5.43	−2.60 ± 0.19
25	gil190712653	50S ribosomal protein	LCABL_15780	rpmF	11,564.9	5.06	−1.7 ± 0.12
Posttranslational modification, protein turnover, chaperones							
7	gil190713480	60 kDa chaperonin (protein Cpn60) (groEL protein)	LCABL_24200	groEL	57,392.9	4.89	2.09 ± 0.26
13	gil227187398	Molecular chaperone GrpE	HMPREF0530_2227	grpE	24,651.5	5.47	3.38 ± 0.45
17	gil239629755	Peptidyl-prolyl cis–trans isomerase	LBPG_02891		21,335.7	4.91	−1.92 ± 0.24
18	gil190712852	Chaperone protein dnaK(Heat shock protein 70)	LCABL_17780	dnaK	67,522.6	4.77	2.41 ± 0.16
19	gil190711872	Small heat shock protein	LCABL_07530	hsp1	17,804.6	4.98	3.17 ± 0.35
21	gil300440230	Molecular chaperone (small heat shock protein)	LCAZH_2811		16,495.1	5	3.70 ± 0.42
Cell cycle control, cell division, chromosome partitioning							
20	gil190712775	Cell division initiation protein DivIVA	LCABL_15090	divIVA	15,042.4	4.93	2.87 ± 0.26
Transcription							
22	gil259648959	Cold shock protein	LC705_01149	cspC	7,198.4	4.6	1.80 ± 0.21
24	gil300439624	Translational regulateor protein, xre family	LCAZH_2180		13,977.3	5.46	3.86 ± 0.53
Energy production and conversion							
3	gil300438867	Inorganic pyrophosphate/exopolypho sphatase	LCAZH_1400		33,760.9	4.74	1.66 ± 0.12
4	gil190712611	Pyruvate dehydrogenase complex, E1 component, alpha subunit	LCABL_15360	pdhA	40,787.7	5.29	2.95 ± 0.36
8	gil205270992	L-lactate dehydrogenase		ldh3	32,664.7	5.49	−1.90 ± 0.07
Unknown function							
2	gil116494244	Hypothetical protein LSEI_0696	LSEI_0696		38,957.2	5.02	3.06 ± 0.26
10	gil22087374	Esterase C	LCAZH_03550	estC	29,106.8	5.98	−3.10 ± 0.42
11	gil190713229	ABC transporter related	LCABL_21680		27,109.2	5.33	1.62 ± 0.25
16	gil190712608	Peptide deformylase PDF (polypeptide deformylase)	LCABL_15330	def	20,686.6	5.44	1.52 ± 0.14

and 6 proteins down-regulated compared with that at pH 6.5.

Proteins involved in carbohydrate metabolism

Four proteins (spots 1, 5, 9, and 23) involved in carbohydrate transport and metabolism were up-regulated during acid adaptation. The intensity of tagatose-1,6-bisphosphate aldolase (LacD) increased by 2.3-fold during acid adaptation. LacD is a key enzyme in galactose metabolism, which catalyzes the conversion of D-tagatose 1,6-diphosphate into glyceraldehyde 3-phosphate, which subsequently participates in glycolysis. The enhanced expression of LacD may result in the increase in ATP production to support the extrusion of H^+ during acid stress [23, 27]. Hpr is a heat-stable phosphocarrier protein involved in sugar phosphotransferase system (PTS). The increased expression of Hpr may lead to higher activity in glucose PTS and ATP generation through glycolysis, thus enhancing the ability of the cells to maintain pH_i. In addition, galactose

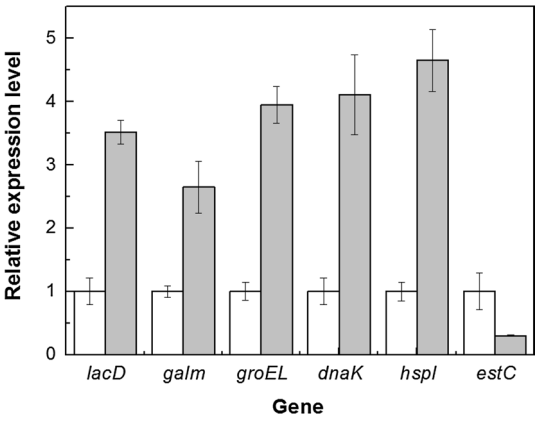

Fig. 5 Comparison of gene expression at the mRNA level in *L. casei* exposed at pH 6.5 (*white bars*) and pH 4.5 (*gray bars*) for 1 h. *Error bars* indicate standard deviations ($n = 3$)

mutarotase-related enzyme (spot 23), which might be involved in galactose metabolism [28], was up-regulated as well. Upregulation of these proteins implies that glycolysis in *L. casei* is enhanced after acid adaptation, and similar results have been reported in previous research [28]. Glycolysis increases the production of ATP, and subsequently supports the H^+ extrusion through H^+-ATPase under acidic conditions. Thus, higher viability was observed in cells after pre-adaptation in sublethal condition.

In addition, three proteins Pdh E1, Ldh3, and EstC were also identified and exhibited differential expression during acid adaptation. Pdh E1 is one of the three subunits of the pyruvate dehydrogenase multienzyme complex, and is responsible for catalyzing pyruvate decarboxylation followed by transfer of the hydroxyethyl group to thiamine diphosphate. The down-regulated expression of lactic acid dehydrogenase (Ldh3) may lead to decreased amount of lactic acid that reduced the injury induced by lactic acid. EstC is a protein of unknown function, and it belongs to the general class of carboxylic ester hydrolases (EC 3.1.1). The breakdown of milk fat by lipases and esterases is one of the main biochemical events that occur during cheese ripening, and it contributes to flavor development [10, 28].

Expression factors and general stress shock proteins

A total of eleven proteins involved in DNA amplification, transcription, translation and protein synthesis were identified, and seven proteins were up-regulated after acid adaptation (Table 2). Of them, five classical stress-response proteins (GroEL, GrpE, DnaK, Hspl, and LCAZH_2811) were identified, and similar results were also obtained in previous research [17]. Generally, the production of stress-response proteins is one of the essential strategies for cells to acquire tolerance or adapt to acidic environments. GroEL is able to capture and refold non-native substrate proteins up to 50–60 kDa, and to protect them from aggregation with other non-native proteins [29]. A 3.38-fold increase in expression of GrpE was observed after acid treatment at pH 3.5 for 1 h. Hörmann et al. [11] demonstrated that GrpE in combination with GroEL exerted great impact on interacting with the glycolytic enzymes at low pH and increased the stability of proteins in the presence of acid challenge. Increased expression of GroEL was also observed in *L. paracasei*, and the overproduction of GroESL in *L. lactis* and *L. paracasei* exhibited enhanced tolerance to multiple-stress [9]. Dnak plays a key role in the maturation of synthesized proteins, and protein degradation and repair. Previous studies also demonstrated that some heat shock proteins were induced by acid stress [17, 27]. Besides, Dnak is believed to serve as a "cellular thermometer" that transduces signals to other cellular factors in response to heat stress [6]. Abdullah-Al-Mahin et al. [1] heterologously expressed *E. coli dnaK* in *L. lactis*, and the mutant exhibited increased tolerance to acid, salt and ethanol stresses. These results demonstrate that overproduction of some stress-response proteins are common strategies for cells to fight against acid stress.

q-PCR

To investigate whether the proteins showing altered levels on 2DE are in good accordance with the changes at the transcriptional level, six genes whose encoded proteins were found differentially regulated on 2DE were selected and the mRNA transcript levels were measured using the q-PCR (Fig. 5). Interestingly, transcriptional regulation of all selected genes showed a positive correlation with the proteomic patterns of the identified proteins.

In this study, a combined physiological and proteomic method was employed to investigate the response of *L. casei* upon acid stress. Acid stress induces a global regulation, and a number of changes take place to reduce the acidic injury. The results demonstrated the stress response of *L. casei* during acid stress, and may facilitate the understanding the protective mechanisms utilized by *L. casei*.

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