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Aspartate protects *Lactobacillus casei* against acid stress

Chongde Wu · Juan Zhang · Guocheng Du · Jian Chen

Abstract The aim of this study was to investigate the effect of aspartate on the acid tolerance of *L. casei*. Acid stress induced the accumulation of intracellular aspartate in *L. casei*, and the acid-resistant mutant exhibited 32.5 % higher amount of aspartate than that of the parental strain at pH 4.3. Exogenous aspartate improved the growth performance and acid tolerance of *Lactobacillus casei* during acid stress. When cultivated in the presence of 50 mM aspartate, the biomass of cells increased 65.8 % compared with the control (without aspartate addition). In addition, cells grown at pH 4.3 with aspartate addition were challenged at pH 3.3 for 3 h, and the survival rate increased 42.26-fold. Analysis of the physiological data showed that the aspartate-supplemented cells exhibited higher intracellular pH (pH$_i$), intracellular NH$_4^+$ content, H$^+$-ATPase activity, and intracellular ATP pool. In addition, higher contents of intermediates involved in glycolysis and tricarboxylic acid cycle were observed in cells in the presence of aspartate. The increased contents of many amino acids including aspartate, arginine, leucine, isoleucine, and valine in aspartate-added cells may contribute to the regulation of pH$_i$. Transcriptional analysis showed that the expression of *argG* and *argH*

increased during acid stress, and the addition of aspartate induced 1.46- and 3.06-fold higher expressions of *argG* and *argH*, respectively, compared with the control. Results presented in this manuscript suggested that aspartate may protect *L. casei* against acid stress, and it may be used as a potential protectant during the production of probiotics.

Keywords Acid stress · *Lactobacillus casei* · Aspartate accumulation · Stress response · Amino acid metabolism

Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group of bacteria for producing fermented foods and are generally recognized as safe. They are normal inhabitants of the oral cavity and the digestive tract in humans (Kleerebezem and Vaughan 2009; Randazzo et al. 2004). LAB are used for the production of a variety of food and feed raw materials such as vegetables, grains, meat, and milk, where they contribute to flavor and texture of the fermented products (Hufner et al. 2007; Smit et al. 2005; Olson 1990). In addition, specific LAB strains are considered as probiotics owing to their health-promoting effects in consumers as living bacteria (Kailasapathy and Chin 2000; Guarner and Schaafsma 1998). However, LAB encountered various environmental stresses during industrial fermentation and processing such as acid, temperature, osmotic stress, and freeze-drying. Besides, to provide health benefits, probiotics must overcome various physical and chemical barriers in the gastro-intestinal tract, including gastric pH, bile, and osmolarity, and the microorganisms need to be viable, active, and abundant, with a concentration of at least 10^7 cfu/ml in the probiotic products (Vinderola et al. 2000; Corcoran et al. 2008). Therefore, maintaining the robustness towards the

Chongde Wu and Juan Zhang contributed equally to this paper.

C. Wu
Key Laboratory for Leather Chemistry and Engineering, Ministry of Education, and College of Light Industry, Textile & Food Engineering, Sichuan University, Chengdu 610065, People's Republic of China
e-mail: wuchongde@163.com

C. Wu · J. Zhang (✉) · G. Du · J. Chen (✉)
Key Laboratory of Industrial Biotechnology, Ministry of Education, and School of Biotechnology, Jiangnan University, Wuxi 214122, People's Republic of China
e-mail: zhangj@jiangnan.edu.cn
e-mail: jchen@jiangnan.edu.cn

stresses is vital for the industrial application of these starter and probiotics cultures. In this case, many effective strategies and new protectants have been developed to enhance the functionality of lactic acid bacteria (Abdullah-Al-Mahin et al. 2010; Li et al. 2010; Zhang et al. 2012a; Kim et al. 2012).

Among various environmental stresses, acid stress is one of the important survival challenges encountered both in industrial process and in the gastrointestinal tract, and acid tolerance is regarded as one of the desirable criteria to select potential probiotics (Parvez et al. 2006). Amino acid metabolism in lactic acid bacteria serves a number of physiological roles such as intracellular pH control, generation of metabolic energy or redox power, and resistance to stresses (Fernández and Zúñiga 2006). Previous researches have demonstrated that many amino acids may protect cells against acid stress. Broadbent et al. (2010) reported that intracellular accumulation of histidine helped *Lactobacillus casei* ATCC 334 resist acid damage, and the survival at pH 2.5 improved at least 100-fold by addition 30 mM of histidine to the acid challenge medium. Senouci-Rezkallah et al. (2011) investigated the effects of glutamate and arginine addition on acid tolerance of *B. cereus* ATCC14579, and the results showed that the survival of cells at pH 4.0 enhanced 1-log or 2-log populations, respectively. Previous research reported that acid stress led to the accumulation of aspartate and arginine and upregulation of proteins involved in aspartate and arginine metabolism in *L. casei* (Wu et al. 2012a; Zhang et al. 2012b). In order to investigate whether aspartate metabolism is involved in acid tolerance *L. casei*, we examined the effect of aspartate addition on the acid tolerance of *L. casei* Zhang, which was isolated from traditional home-made koumiss in Inner Mongolia of China and was considered as a new probiotic bacterium by probiotic tests (Wu et al. 2009). Results provided in this study may provide a new strategy to enhance the acid tolerance and subsequently the industrial utility of lactic acid bacteria.

Materials and methods

Strains and growth conditions

The bacterial strains used in this study were *L. casei* Zhang (WT) (CGMCC no. 1697) and its acid-resistant derivative Lbz-2 (mutant) (CCTCC no. M2010292). The mutant was obtained by adaptive evolution, which was conducted in a BIOFLO 110 Chemostat (New Brunswick Scientific) (Wu et al. 2012a). Both strains were grown statically in de Man, Rogosa, and Sharpe (MRS) broth (Oxoid) at 37 °C.

Lactic acid challenge experiments

Cultures were grown statically in 250-ml flasks containing 50 ml MRS broth at 37 °C. When the cells grow to mid-exponential growth phase, the culture was split into three 100 ml MRS broth containing 50 mM aspartate adjusted to pH 6.5, 5.0, 4.3 with lactic acid, respectively. The growth of the cells in the presence of lactic acid was further monitored.

For acid-shock experiments, cells grown to mid-exponential growth phase in the presence or absence of aspartate were harvested, centrifuged at 10,000×*g* for 5 min, washed, and then resuspended in MRS (adjusted to pH 3.3 with 25 % lactic acid). Aliquots of cell suspension were incubated at 37 °C, and samples were withdrawn at different times to determine viability. Cell numbers were determined with spot plating, where 10 μl of serially diluted samples were spotted in triplicate onto MRS agar plates and incubated at 37 °C for 48 h. Plates containing 5–100 CFU were counted, and colony-forming units per milliliter was calculated from the average.

Intracellular amino acid determination

For the extraction of amino acids, cells grown in mid-exponential growth phase were harvested by centrifugation at 12,000×*g* for 10 min, washed twice, resuspended in 1 ml of 200 mM phosphate-buffer saline (PBS) adjusted to pH 7.0, and then boiled for 15 min. Cell debris was discarded by centrifugation (12,000×*g*, 10 min, 4 °C). The supernatants were treated at room temperature by addition of 1 ml 10 % tricarboxylic acid (TCA) for 10 min. The mixture was then centrifuged (12,000×*g*, 4 °C) again for 10 min, and the supernatants were analyzed with HPLC according to the method of Fountoulakis and Lahm (1998).

Measurement of intracellular pH (pH$_i$)

pH$_i$ was measured by the fluorescence method developed by Breeuwer et al. (1996) using 5- (and 6-)-carboxyfluorescein succimidyl ester as the fluorescent probe. Calibration curves establishing 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 (Breeuwer et al. 1996).

Determination of intracellular ammonia

Cells grown in mid-exponential growth phase were harvested by centrifugation at $12,000 \times g$ for 10 min, washed twice with 200 mM 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 $12,000 \times g$ for 10 min. The amount of ammonia in the supernatant was analyzed with the ammonia assay kit (Sigma, USA) according to manufacturer's instructions.

Determination of intracellular ATP concentration

Intracellular ATP content was determined according to the method described by Wu et al. (2012a). The final ATP concentration was expressed as nanomoles per milligram protein. All values of ATP concentrations were the mean values of at least three independent extraction processes.

Measurement of the H^+-ATPase activity

The H^+-ATPase assay was carried out using the H^+-ATPase assay kit (GENMED) following manufacturer's protocol. The activity of the H^+-ATPase was expressed in nanomoles of the NADH oxidized per minute per milligram of protein.

Fatty acid extraction and analysis

Extraction of bacterial lipids and preparation of fatty acid methyl esters were carried out according to the method of Bligh and Dyer (1959), as modified by Wu et al. (2012b).

Sampling, quenching, and extraction of intracellular metabolites

The metabolism of a culture sample was rapidly inactivated by mixing one volume of culture sample with three volumes of 60 % MeOH and 0.85 % (w/v) ammonium carbonate (pH 5.5) solutions at −40 °C. After quenching, the cells were kept at −40 °C for 30 min, centrifuged (5 min, $3,000 \times g$) with a pre-cooled rotor of −40 °C and washed with the same volume of quenching buffer. The supernatants were diluted with the same volume of cold water, freeze-dried, and stored at −80 °C until further analysis.

As for extraction of metabolites, the cell pellets were directly resuspended in 1 ml of cold absolute and frozen. Next, the sample was thawed on ice and immediately centrifuged ($10,000 \times g$) for 2 min at 4 °C. The supernatant was subsequently transferred to a new tube, and the extracted pellet was re-extracted twice with 0.5 ml of cold methanol and twice with 0.5 ml of cold water. All extracts were combined and diluted with an equal volume of cold water, after which the solution was frozen in liquid nitrogen, freeze-dried, and stored at −80 °C until further analysis. To correct for minor variations occurring during analysis, the internal standard solution (0.14 mg/ml succinic d4 acid, 50 μl) was added to 150 μl aliquots before lyophilization (Ding et al. 2010). Seven biological replicates were performed for each sample.

Sample derivatization and GC/MS analysis

Two-stage chemical derivatization was performed on dried samples according to the method by Ding et al. (Ding et al. 2010). Gas chromatography/mass spectrometry (GC/MS) analysis was subsequently performed as described previously with some modifications (Ding et al. 2010). Briefly, the derivatized sample (1 μl) was analyzed by GC/MS (Agilent 7890A GC /5975C MS) which was equipped with a capillary column (30 m × 0.25 mm i.d.). The injector temperature was 280 °C. The oven temperature was programmed as 80 °C for 2 min and then increased to 300 °C (10 °C/min), holding for 3 min. The ionization energy was set equal to 70 eV. Masses were acquired in the range of m/z 50–600.

Gene expression analysis by quantitative RT-PCR

Cells grown in mid-exponential growth phase were harvested. 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). To investigate the gene expression at a transcription level, real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. The primers used for RT-PCR assay are listed in Table 1. The 16SrRNA was used as the internal control for quantification. RT-PCR assay was performed using the SYBR Premix $EX Taq^{TM}$ Kit (TaKaRa, Japan) with at least three biological replicates. The PCR was carried out by using a LightCycler 480 II real-time PCR System (Roche, Germany) with the following protocol—95 °C for 5 min, followed by at

Table 1 Primers used in this study

Genes		Oligonucleotide sequence (5′–3′)
argG	Forward	GTAAAGGCAATGACCAAGTTC
	Reverse	TGGCATCAATGGAATAAGGAG
argH	Forward	AAACATCGCCTACCAACAATC
	Reverse	AAATACGCCAACAAATAATGACC
16SrRNA	Forward	GAGGCAGCAGTAGGGAATCTTC
	Reverse	CGACCATTCTTCTCCAACAACAG

40 cycles at 95 °C for 5 s, 60 °C for 20 s, and finally a cooling step at 50 °C for 30 s. Each 96-well plate contained serial dilutions of cDNA, which were used to generate the relative standard curves for target genes. The expression levels of all the tested genes were normalized against the expression level of the internal control gene (16S rRNA).

Statistical analysis

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

Results

Acid stress resulted in the accumulation of aspartate in $L.$ $casei$

Figure 1 showed the alterations of intracellular aspartate content in both strains at different pHs (6.5, 5.0, 4.3). The aspartate concentration increased when culture pH decreased. At pH 4.3, the content in the parental strain and the mutant increased 2.5- and 3.0-fold, respectively, compared with the corresponding values at pH 6.5. In addition, the acid-tolerant mutant displayed significantly higher aspartate content during acidic conditions. At pH 6.5, no dramatic difference between the parental strain and the mutant was observed; however, with a lower pH, the differences enlarged. The mutant exhibited 24.5 % and 32.5 % increase in aspartate content compared with the parental
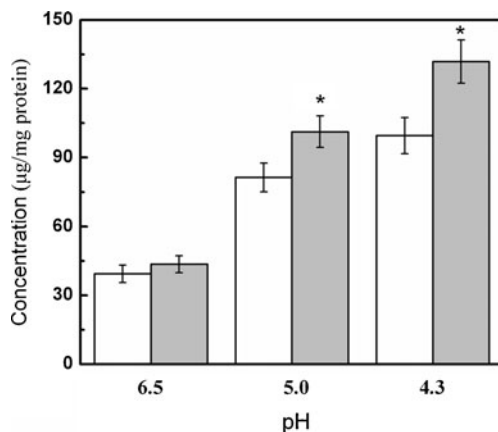

Fig. 1 Changes in intracellular contents of arginine in $L.$ $casei$ and its acid-resistant mutant at different pHs. Both cells were cultivated in MRS at various pHs, and the mid-exponential phase cells were harvested, and the concentrations of the intracellular aspartate were determined. *White bar* represents parental strain, while *gray bar* represents acid-resistant mutant. *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*

strain did when grown at pH 5.0 and 4.3, respectively. These results suggested that acid stress led to increase in aspartate content, and the acid-resistant mutant exhibited higher amount of aspartate.

Exogenous aspartate improved the growth performance and acid tolerance of $L.$ $casei$ during acid stress

Results presented in Fig. 1 showed that acid stress enhanced the accumulation of aspartate, and the mutant contained higher content of aspartate. In order to investigate whether the accumulation of aspartate is involved in acid tolerance of $L.$ $casei$, exogenous aspartate was added into the culture medium, and the growth performance of $L.$ $casei$ was measured at different pHs (6.5, 5.0, 4.3) adjusted by lactic acid. As shown in Fig. 2, no obvious enhancement of cell growth was detected when aspartate was supplemented into MRS broth at pH 6.5. However, under acidic conditions, the cell growth was enhanced by aspartate supplement. In the presence of 50 mM aspartate, the biomass increased 10.8 % (pH 5.0) and 65.8 % (pH 4.3) compared with the corresponding control (without aspartate addition), respectively.

To clarify the effect of exogenous aspartate on the acid tolerance of $L.$ $casei$, acid survival of $L.$ $casei$ during acid stress was investigated. The cells cultivated at various pHs (6.5, 5.0, 4.3) in the presence or absence of aspartate were challenged at pH 3.3 for 3 h, and the survival rates were determined. As expected, a 1.36- and 3.17-fold increase in survival rate was detected when the cells were grown in the presence of aspartate at pH 6.5 and 5.0, respectively (data not shown). However, it was worth noting that a visible increase in survival rate was obtained when the cells were cultured at pH 4.3 with aspartate addition (Fig. 3). Furthermore, when the exposure time in lactic acid is prolonged, the survival differences increased significantly. After 3 h of acid shock, survival of the control strain (without aspartate addition) sharply decreased, and only 0.35 % of the cells survived compared with more than 14.79 % for the cells cultured in the presence of aspartate. These results suggested that exogenous aspartate enhanced the growth performance and acid tolerance of $L.$ $casei$ during acid stress.

Effect of aspartate addition on intracellular pH and intracellular NH_4^+

In order to verify these results, we measured the pH_i and intracellular NH_4^+ content in $L.$ $casei$ at different pHs (Fig. 4). As shown in Fig. 4, as the decrease of extracellular pH, the pH_i decreased sharply. Interestingly, addition of aspartate led to the increase of pH_i, and the aspartate seemed to confer the cells higher capability to maintain pH_i homeostasis. Likely, these results are in agreement with those obtained with ammonia assay, suggesting that acid stress
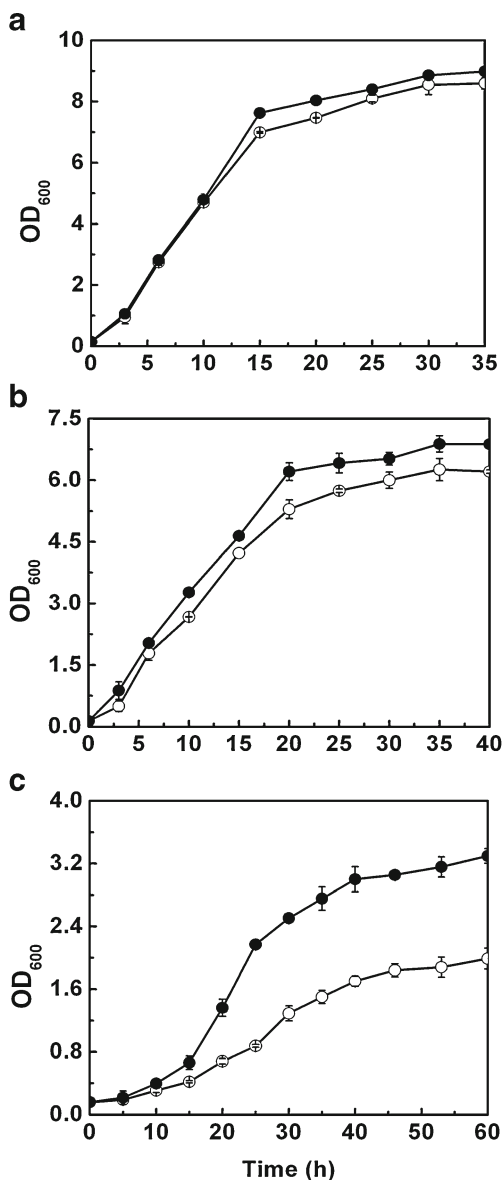
Fig. 2 Effect of aspartate addition on the growth of *L. casei*. The cells were cultivated in MRS at pH 6.5 (**a**), 5.0 (**b**), and 4.3 (**c**) and the growth of the cells were measured by monitoring the OD_{600}. *Open symbols* represent no aspartate was supplemented, while *solid symbols* represent 50 mM aspartate was added into the culture medium. *Error bars* indicate standard deviations (*n*=3)

led to the increase of NH_4^+ content and aspartate enhanced the production of NH_4^+ (Fig. 4). These results demonstrate that aspartate protects the *L. casei* against acid stress by overproduction of NH_4^+ and neutralization of the intracellular protons.

Changes in H$^+$-ATPase and intracellular ATP concentration

H$^+$-ATPase is one of the most important mechanisms that regulate the homeostasis of pH_i. It expels protons at the expense of ATP hydrolysis and consequently the
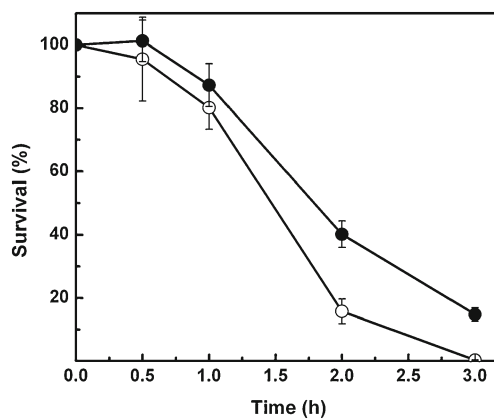

Fig. 3 Effect of aspartate addition on the acid tolerance of *L. casei*. Cells cultivated to mid-exponential phase in MRS broth at pH 4.3 were challenged with acid stress at pH 3.3 for various times, and the survival rate of cells were determined. *Open symbols* represent no aspartate was supplemented, while *solid symbols* represent 50 mM aspartate was added into the culture medium. *Error bars* indicate standard deviations (*n*=3)
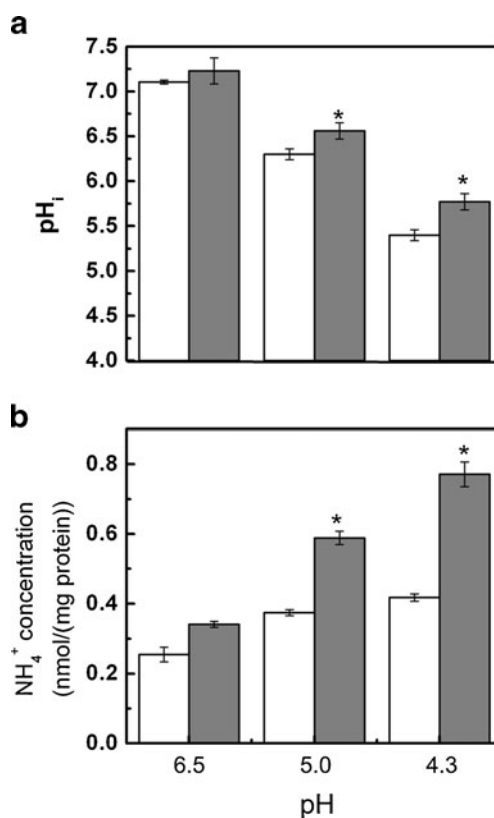

Fig. 4 Effect of aspartate addition on pH_i (**a**) and intracellular NH_4^+ pool (**b**) in *L. casei*. Cells cultivated at various pHs were harvested at mid-exponential phase, and the pH_i and NH_4^+ concentration were measured. *White bar* represents no aspartate addition, while *gray bar* represents aspartate addition. *Error bars* indicate standard deviations (*n*=3). *Asterisk* indicates significant differences with the corresponding control (without aspartate addition) at *p*<0.05 by the Student's *t* test

establishment of pH_i homeostasis (De Angelis and Gobbetti 2004; Cotter and Hill 2003). An experiment was performed to investigate the effect of aspartate addition on H^+-ATPase activity and intracellular ATP pool during acid stress (Fig. 5). The addition of aspartate enhanced the H^+-ATPase activity and intracellular ATP concentration at various pHs, and the aspartate-added cells exhibited significantly higher H^+-ATPase activity and intracellular ATP levels than the control cells when the cells were cultured at pH 6.5, 5.0 and 4.3. These results further suggest the possible mechanisms involved acid tolerance in *L. casei*.

Intracellular metabolites related to central carbon metabolism, amino acids, and fatty acids in *L. casei* during acid stress

Effect of aspartate addition on intracellular metabolites profiles in *L. casei* during acid stress was investigated by metabolomic analysis. A total of 45 metabolites including
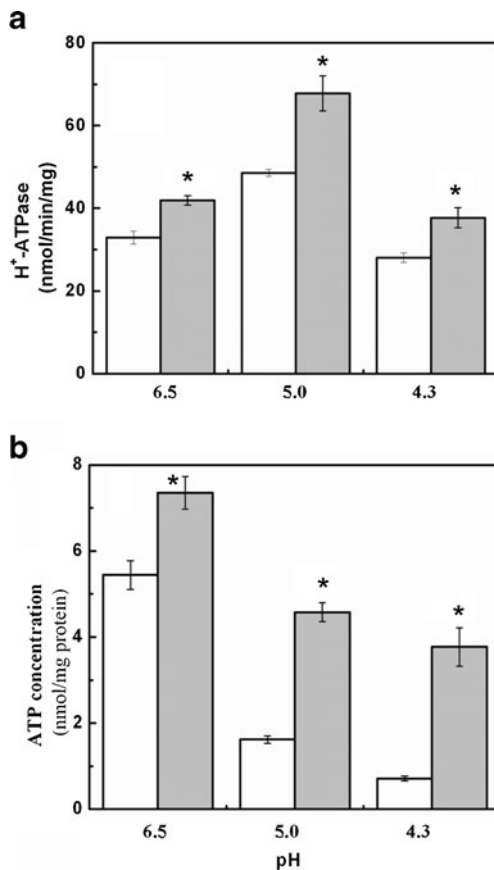

Fig. 5 Effect of aspartate addition on H^+-ATPase activity (**a**) and intracellular ATP pool (**b**) in *L. casei*. Cells cultivated at various pHs were harvested at mid-exponential phase, and the H^+-ATPase activity and intracellular ATP concentration were measured. *White bar* represents no aspartate addition, while *gray bar* represents aspartate addition. *Error bars* indicate standard deviations ($n=3$). *Asterisk* indicates significant differences with the corresponding control (without aspartate addition) at $p<0.05$ by the Student's *t* test

sugars, phosphorylated compounds, amino acids, organic acids, as well as fatty acids were identified and quantified in *L. casei*. The levels of metabolites involved in glycolysis and TCA cycle under acid stress were shown in Fig. 6. Higher levels of glycolytic intermediates (glucose 6-P, fructose 6-P, and glycerate 3-P) and higher levels of tricarboxylic acid cycle intermediates (citrate and malate) were detected in *L. casei* cultured in the presence of aspartate. In addition, lower content of G-3-P and higher content of lactate were also detected in *L. casei* supplemented with aspartate during acid stress. These results suggest that the addition of aspartate during acidic condition protects cells against acid stress and enhances the activities of central carbon metabolism in *L. casei*.

Figure 7 demonstrated the metabolic pathway of amino acid in *L. casei* and the metabolic profiles of the corresponding amino acids during acid stress. As shown in Fig. 7, the addition of aspartate led to significant changes in pools of all detected intracellular amino acids except glycine, serine, and asparagine. As a precursor of many amino acids, the addition of aspartate resulted in increased levels of alanine, arginine, and subsequently higher levels of threonine and ornithine. In addition, higher concentrations of branched-chain amino acids leucine, isoleucine, valine, and intermediate 2,3-dihydroxy-3-methylbutanoate were observed in aspartate-added cells compared with that in control cells. Meanwhile, it was worth noting that the levels of proline and methionine decreased in aspartate-added cells during acid stress.

Effect of aspartate addition on membrane fatty acid profiles in *L. casei* during acid stress was investigated. As shown in Fig. 8, the composition of saturated (C14:0, C16:0, and C18:0) and unsaturated (C16:1, C18:1, C18:2, and C19-cyc) fatty acids changed with the addition of aspartate under acid stress. Cells cultured in the presence of aspartate displayed increased relative abundance of unsaturated fatty acids and decreased relative abundance of saturated fatty acids. Therefore, the addition of aspartate led to increased ratio of unsaturated to saturated fatty acids (U/S ratio) in *L. casei* during acid stress.

Effect of aspartate addition on transcription of argG and argH in *L. casei*

Transcriptional levels of *argG* and *argH* in *L. casei* cultured in MRS broth at various pHs in the presence and absence of aspartate were shown in Fig. 9. The transcriptional levels of both genes increased with the decrease of pH. When incubated at pH 4.3, a 1.9- and 2.4-fold increase was observed in *argG* and *argH* transcription, respectively. Interestingly, higher expression levels of *argG* and *argH* were detected when aspartate was added into the culture medium. In the presence of aspartate, the cells exhibited a 1.46- and 3.06-
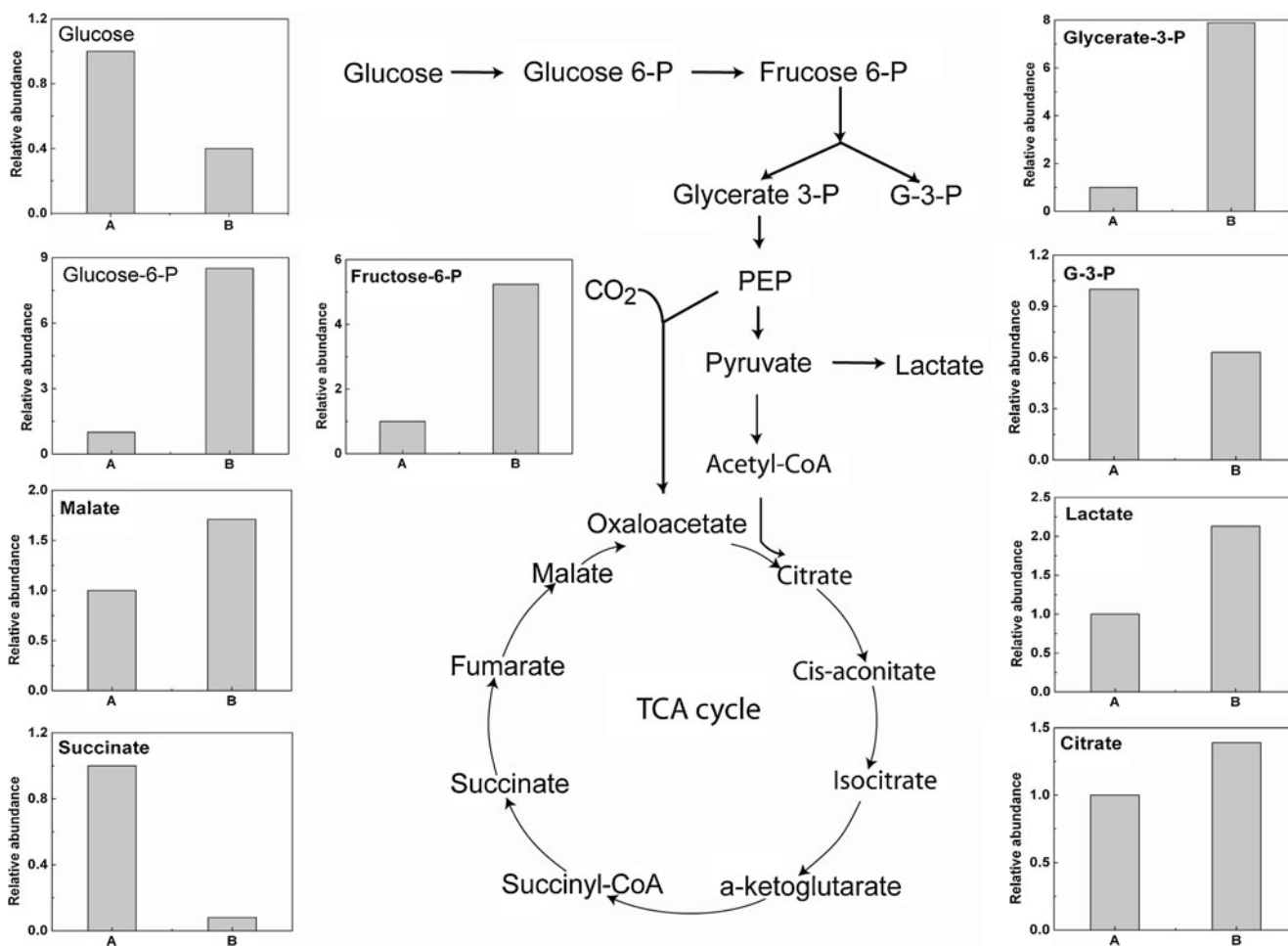
Fig. 6 Comparison of intermediates levels of glycolysis and TCA cycle in *L. casei* during acid stress. Cells grown in mid-exponential growth phase at pH 4.3 were harvested, and the intracellular intermediates levels were analyzed. **A** Represents cells cultured in absence of aspartate, while **B** represents cells cultured in presence of aspartate. Data are the means value of seven biological replicates. *Glucose 6-P*, glucose-6-phosphate; *Fructose 6-P*, fructose-6-phosphate; *Glycerate 3-P*, glyceraldehyde-3-phosphate; *G-3-P*, glycerol-3-phosphate

fold higher expression of *argG* and *argH*, respectively, than those of the control (without aspartate addition) at pH 4.3.

Discussion

In the present study, the effect of aspartate on acid tolerance of *L. casei* was monitored. Exogenous aspartate increased the growth performance and survival rate of *L. casei* during acid stress. In order to elucidate the protective mechanisms of aspartate upon acid stress, more-detailed studies were performed by employing the physiological and metabolomic approaches.

pH_i plays an important role during the growth and metabolism of *L. casei*, and it affects the uptake of nutrients, protein synthesis, glycolysis, and nucleic acid synthesis (O'Sullivan and Condon 1997; Hutkins and Nannen 1993). As the external pH and internal pH decrease, protein activities may decrease, and ultimately protein as well as

DNA damages will take place (Budin-Verneuil et al. 2005). Thus, the ability to maintain pH_i homeostasis during acid stress is essential for the survival of cells. The aspartate-supplemented cells exhibited higher pH_i and intracellular NH_4^+ pool (Fig. 4), suggesting that the addition of aspartate may confer cells higher capability to keep physiological activities and fight against acid stress. Besides, the higher contents of intermediates in central carbon metabolism observed in the aspartate added cells may further validate the results above (Fig. 6).

Several mechanisms regulate the homeostasis of pH_i, and the proton-translocating ATPase is the most important one for lactic acid bacteria (De Angelis and Gobbetti 2004). H$^+$-ATPase expels protons at the expense of consuming ATP. When cultured at mild acid-stressed condition (pH 5.0), cells in the presence or absence of aspartate enhanced the activities of H$^+$-ATPase, while the activity decreased upon the incubation at pH 4.3. This may be due to the optimal pH of ATPase at pH 5.0–5.5 (Bender and Marquis 1987).
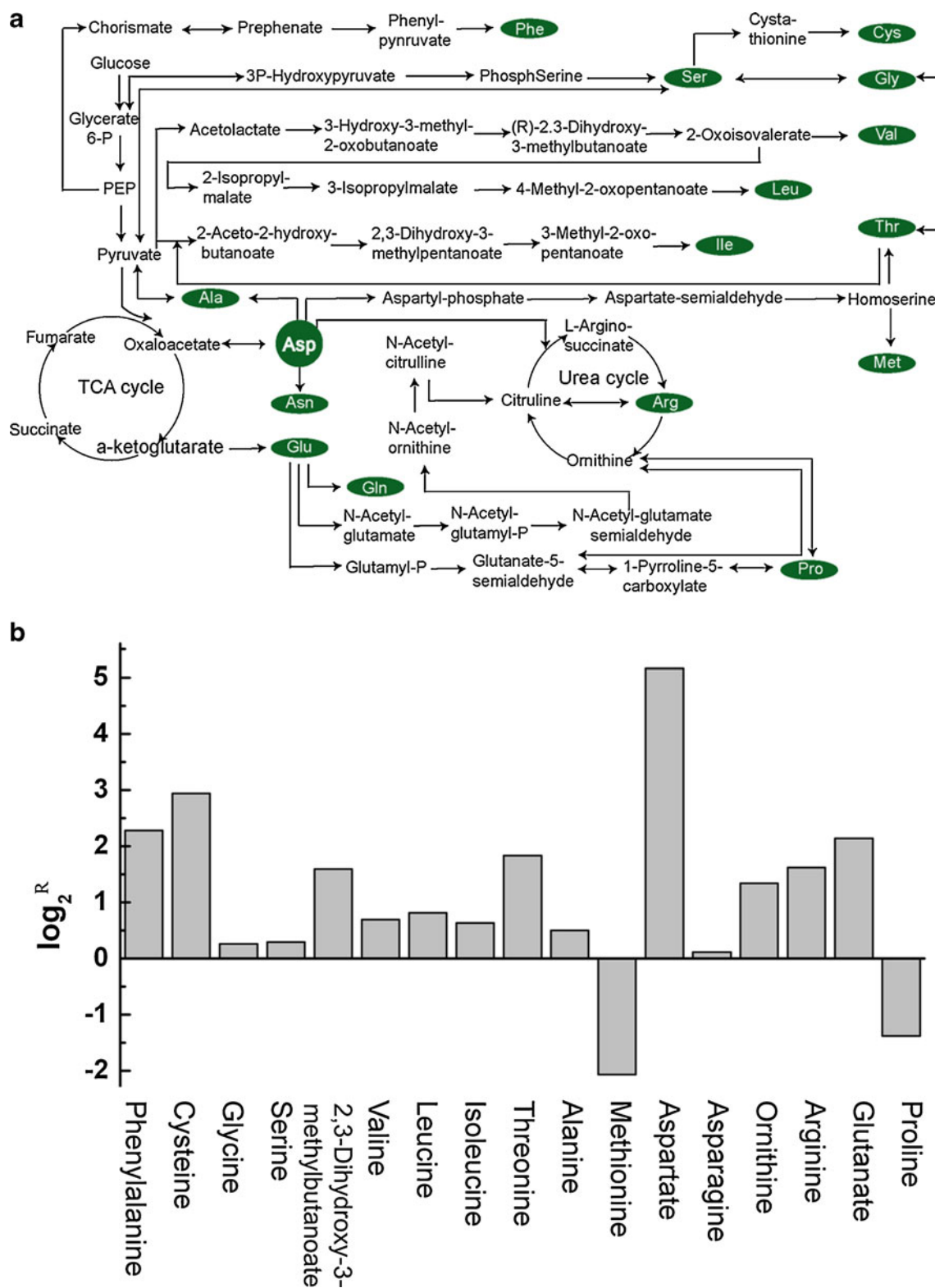
Fig. 7 Effect of aspartate addition on amino acid metabolism during acid stress. **a** Schematic representation of the amino acid pathway in *L. casei*. **b** Metabolic profiles of amino acid during acid stress. Cells grown in mid-exponential growth phase at pH 4.3 were harvested, and the intracellular amino acid contents were analyzed. *R* represents ratio of relative abundance of amino acids in *L. casei* cultured in the absence and presence of aspartate. Data are the means value of seven biological replicates
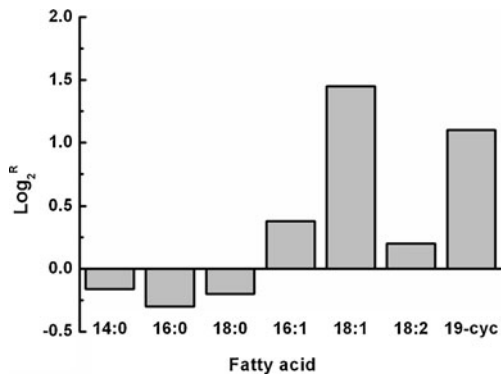
Fig. 8 Effect of aspartate addition on membrane fatty acid metabolism during acid stress. Cells grown in mid-exponential growth phase at pH 4.3 were harvested, and the membrane fatty acids concentrations were analyzed. *R* refers to ratio of relative abundance of fatty acids in *L. casei* cultured in the absence and presence of aspartate. Data are the means value of seven biological replicates
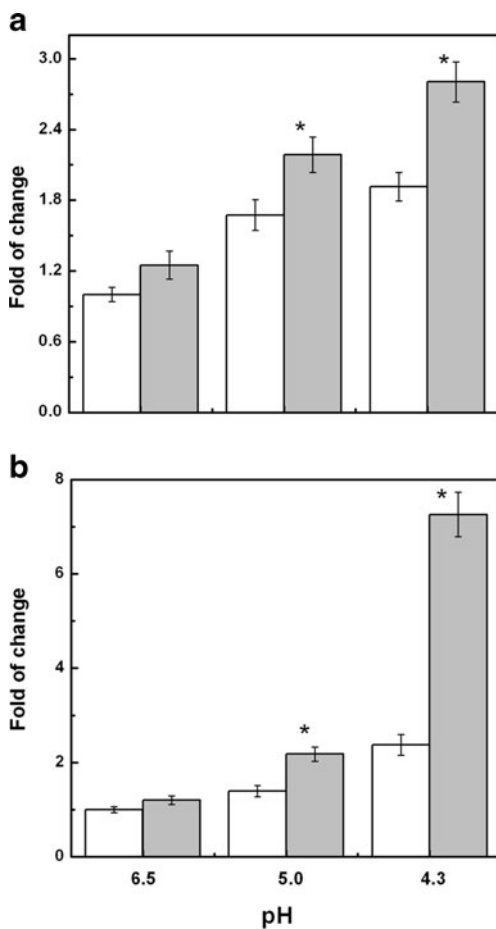

Fig. 9 Transcriptional levels of *argG* (**a**) and *argH* (**b**) in *L. casei* grown at various pHs. Cells cultivated to mid-exponential phase in MRS broth at different pHs were collected for transcriptional assay. *White bars* represent no aspartate was supplemented, while *gray bars* represent 50 mM aspartate was added into the culture medium. *Error bars* indicate standard deviations ($n=3$). *Asterisk* indicates significant differences with the corresponding control (without aspartate addition) at $p<0.05$ by the Student's *t* test

Meanwhile, it is worth noting that, during incubation under acidic conditions, the aspartate-added cells presented higher activity of ATPase (Fig. 5), which contributed to the extrusion of protons and consequently conferred resistance to stress. Likely, a higher intracellular ATP pool in the aspartate-supplemented cells provided more energy for the cells to pump protons out of the cells (Fig. 5). These results suggest that aspartate protects *L. casei* against acid stress by maintaining pH_i homeostasis via physiological regulation.

To further investigate the metabolic regulation of *L. casei* upon aspartate addition during acid stress, a comparatively metabolomic analysis was performed. Generally, the ability of *L. casei* to efficiently transport and metabolize sugar, especially at low pH, is a characteristic that allows these bacteria to grow and persist during acid stress. In this study, the cells supplemented with aspartate displayed higher pools of intermediates involved in glycolysis and TCA cycle, suggesting that the addition of aspartate may increase the metabolic flux of *L. casei* during acid stress. Therefore, the enhanced flux in central carbon metabolism may lead to an increase in ATP production to support the H^+ extrusion under acidic conditions.

Analysis of the intracellular pools of amino acids showed that the aspartate-added cells exhibited higher contents of many amino acids related to aspartate metabolism. An intriguing finding is the regulation of aspartate and arginine metabolism during acid stress. *L. casei* shifted the metabolic pathway by increasing the flux from aspartate to arginine (arginine deiminase system, (ADI)) and decreasing the flux from aspartate to asparagines (Fig. 7). The ADI system has been identified to protect bacteria cells against the damaging effects of acidic condition in a variety of bacteria (Matsui and Cvitkovitch 2010; Mols and Abee 2011; Vrancken et al. 2009). In detail, this pathway allows the cells to neutralize protons by NH_3 production, and the concomitant ATP generation enables extrusion of cytoplasmic protons by H^+-ATPase (Casiano-Colon and Marquis 1988). Excitingly, results presented in Fig. 7 demonstrated that the aspartate-supplemented cells appeared to have higher capability to manipulate the aspartate metabolism, which lead to the formation of ATP and NH_3. In order to further validate the result, a q-PCR experiment was conducted to investigate the expression of key genes involved in aspartate metabolism (Fig. 9). *argG* and *argH* are key genes that participate in aspartate and arginine metabolism (Zhang et al. 2010). Transcriptional data presented in Fig. 9 indicated that acid stress-induced the upregulation of *argG* and *argH* and the addition of aspartate led to enhanced expressions of both genes. The upregulation of these genes may lead to the increase of metabolic flux from aspartate to arginine and resulted in higher pools of ATP and ammonia.

In addition, it is interesting to note that higher contents of BCAA leucine, isoleucine, and valine were observed in

aspartate-added cells. Sánchez et al. and Santiago et al. (Sánchez et al. 2007; Santiago et al. 2012) reported that genes IlvC2, IlvD, and IlvE involved in biosynthesis pathway of BCAA were upregulated by low pH, and deamination of BCAA was postulated as a mechanism of maintaining pH$_i$ of the cells (Len et al. 2004).

Regulation of membrane fatty acids profiles is a way for cells to combat acid stress (Fozo and Quivey 2004; Fozo et al. 2004). Although the adjustment mechanisms linking fatty acid composition to various stress factors were mostly unclear, Wu et al. (2012b) reported that L. casei increased the levels of long-chained, monounsaturated fatty acids, and U/S ratio to fight against acid stress. Our results also showed that the cells supplemented with aspartate exhibited higher survival rate and U/S ratio, indicating that aspartate protected L. casei against acid stress by regulating the membrane fatty acid composition.

In this study, effect of aspartate on the acid tolerance of L. casei was investigated. The addition of aspartate improved the growth performance and increased the survival rate of L. casei during acid stress. In addition, the addition of aspartate led to a global regulation and a number of changes took place during acid stress. A combined physiological and metabolomic approach was performed to elucidate the protective mechanisms of aspartate upon acid stress. Results presented in this manuscript may help us understand acid tolerance mechanisms and help formulate new strategies to enhance the industrial applications of this species.

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