Ann Microbiol (2017) 67:669–677
DOI 10.1007/s13213-017-1295-x

CrossMark

ORIGINAL ARTICLE

A physiological comparative study of acid tolerance of *Lactobacillus plantarum* ZDY 2013 and *L. plantarum* ATCC 8014 at membrane and cytoplasm levels

Yilin Guo[1] · Ximei Tian[1] · Renhui Huang[1] · Xueying Tao[1] · Nagendra P. Shah[2] · Hua Wei[1,3] · Cuixiang Wan[3]

Received: 8 April 2017 / Accepted: 4 August 2017 / Published online: 23 August 2017
© Springer-Verlag GmbH Germany and the University of Milan 2017

Abstract This study aimed to disclose the acid tolerance mechanism of *Lactobacillus plantarum* by comparing *L. plantarum* ZDY 2013 with the type strain *L. plantarum* ATCC 8014 in terms of cell membrane, energy metabolism, and amino acid metabolism. *L. plantarum* ZDY 2013 had a superior growth performance under acidic condition with 100-fold higher survival rate than that of *L. plantarum* ATCC 8014 at pH 2.5. To determine the acid tolerance physiological mechanism, cell integrity was investigated through scanning electron microscopy. The study revealed that *L. plantarum* ZDY 2013 maintained cell morphology and integrity, which is much better than *L. plantarum* ATCC 8014 under acid stress. Analysis of energy metabolism showed that, at pH 5.0, *L. plantarum* ZDY 2013 enhanced the activity of Na^+/K^+-ATPase and decreased the ratio of NAD^+/NADH in comparison with *L. plantarum* ATCC 8014. Similarly, amino acid metabolism of intracellular arginine, glutamate, and alanine was improved in *L. plantarum* ZDY 2013. Correspondingly, the activity of arginine deiminase and glutamate decarboxylase of *L. plantarum* ZDY 2013 increased by 1.2-fold and 1.3-fold compared with *L. plantarum* ATCC 8014 in acid stress. In summary, it is demonstrated that the special physiological behaviors (integrity of cell membrane, enhanced energy metabolism, increased amino acid and enzyme level) of *L. plantarum* ZDY 2013 can protect the cells from acid stress.

Keywords *Lactobacillus plantarum* · Acid tolerance · Cell membrane · Energy metabolism · Amino acids

✉ Cuixiang Wan
cuixiangwan@ncu.edu.cn

[1] State Key Laboratory of Food Science and Technology, Nanchang University, 235 Nanjing Donglu, Nanchang, Jiangxi 330047, China

[2] Food and Nutritional Science, School of Biological Science, The University of Hong Kong, Pokfulam Road, Hong Kong, China

[3] Jiangxi-OAI Joint Research Institute, Nanchang University, 235 Nanjing Donglu, Nanchang 330047, People's Republic of China

Introduction

Lactic acid bacteria (LAB) have been used to produce fermented food over the past decades and have been developed as probiotics, which are generally recognized as safe (GRAS) (De Vries et al. 2006), for their health-promoting functions in the human gastrointestinal tract (Duary et al. 2010). Probiotics must maintain viability in the gut at a high concentration (at least 10^6 cfu/mL) to be beneficial to the human host (Ferrando et al. 2015). Therefore, the stronger resistance of LAB strains against various extreme environments is a prerequisite for the development of probiotic supplements.

LAB are exposed to various stress conditions, such as acid, temperature, osmotic stress, or freeze drying in fermented food as well as in the gastrointestinal tract (Guchte et al. 2002). The tolerance of LAB to these stressors is critical for screening potential candidates for LAB application. As probiotic candidates, bacteria have to survive under extreme acidic conditions or digestion by various enzymes in the entire gastrointestinal tract (Wall et al. 2007). Given that acid stress is a pivotal issue for microbial survival, acid tolerance is generally considered as one of the criteria for selection of potential probiotics (De Angelis and Gobbetti 2004; Parvez et al. 2006).

Few publications have focused on the acid tolerance mechanism of LAB such as *Lactococcus lactis* (Rallu et al. 2000), *Lactobacillus bulgaricus* (Hernandez-Hernandez et al. 2012), *Lactobacillus casei* (Broadbent et al. 2010), *Lactobacillus*

plantarum (Hamon et al. 2014), and *Lactobacillus reuteri* (Teixeira et al. 2014) or on the aspect of proteomic analysis level. More routinely studied are the acid tolerance, H^+-ATPase proton pump, glutamate decarboxylase (GAD) system, and arginine deiminase (ADI) system of bacteria involved in pH homeostasis in Gram-positive bacteria (Cotter and Hill 2003). For example, the activity of H^+-ATPase increases under acidic conditions to support the acid tolerance of *Bacillus* spp. (Shobharani and Halami 2014); the GAD system contributes to acid tolerance of *Listeria monocytogenes* in gastric fluid (Cotter et al. 2001); and ADI protects *Bacillus cereus* ATCC14579 (Senouci-Rezkallah et al. 2011), *Escherichia coli* (Lin et al. 1995), and *L. monocytogenes* (Lin et al. 1995; Ryan et al. 2009) against acid stress through the production of ammonia. On the other hand, alterations in cell membrane and metabolic pathways (e.g., energy metabolic pathway and amino acid metabolism) also control acid tolerance (Cotter and Hill 2003). For instance, an increased proportion of long-chained, monounsaturated fatty acids were produced in *Streptococcus mutans* UA159 in response to acid stress (Fozo and Quivey 2004); NADH oxidase, an enzyme responsible for oxidative stress, and which could possibly contribute to acid resistance, its activity of *L. lactis* (De Felipe et al. 1998), and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Marty-Teysset et al. 2000) increased under acidic conditions; addition of glutamine increased the survival rate of *L. reuteri* 100–23 at pH 2.5 (Teixeira et al. 2014). To our best of our knowledge, few literature reports have disclosed information on the physiological changes of LAB due to acid stress, especially in *L. plantarum*.

Among a variety of LAB species, *L. plantarum* is one of the most widely used starters or probiotics in many products (Brinques and Ayub 2011). In our previous study, a strain of *L. plantarum* ZDY 2013 was proven to be an ideal probiotic candidate because of its tolerance to the extreme environment of the gastrointestinal tract. Moreover, *L. plantarum* ZDY 2013 underwent a global acid tolerance reaction in terms of mRNA levels (Huang et al. 2015), indicating that some unknown physiological changes takes place under extreme acidic conditions. To explore the acid stress mechanisms of *L. plantarum* ZDY 2013 through a physiological response, we used the type strain of *L. plantarum* ATCC 8014 as a standard. In the present study, we investigated the acid tolerance ability, cell integrity, and changes in energy and amino acid metabolism based on the different acid stress responses of *L. plantarum* ZDY 2013 and *L. plantarum* ATCC 8014.

Materials and methods

Bacteria strains, media, and growth conditions

L. plantarum ZDY 2013, isolated from a traditional fermented acid beans in our previous study (Huang et al. 2015), and a type strain, *L. plantarum* ATCC 8014, were used in this study. Both strains were incubated in MRS broth (Beijing Solarbio Science & Technology, Beijing, China) at 37 °C for 24 h under anaerobic condition. Growth conditions in MRS were applied in all subsequent experiments. For acid tolerance analyses, the pH of MRS broth was adusted to 6.2, 4.5 and 3.5.

Acid tolerance analyses and acid treatment

Growth of both *L. plantarum* strains was assessed under different acid conditions (pH 6.2, 4.5 and 3.5) in MRS broth, and the optical density (OD) at 600 nm was monitored at different time points using a microplate reader (VersaMaxTM Tunable microplate reader; Molecular Devices, Sunnyvale, CA). Additionally, the survival of *L. plantarum* ZDY 2013 was evaluated at pH 2.5 for 1.0 h in 0.01 M PBS with *L plantarum* ATCC 8014 for comparison.

Cells of *L. plantarum* ZDY 2013 and *L. plantarum* ATCC 8014 at mid-exponential growth phase were centrifuged at 10,000 *g* for 1 min, and incubated in PBS (pH 3.5, 4.5 and 5.0) at 37 °C for 1.0 h. Then, the acid-treated and untreated cells were used for further study.

Scanning electron microscopy

The morphological structure of acid treated and untreated cells were examined using scanning electron microscopy (SEM). The samples were prepared as described by Bron et al. (2004), with some modifications. Briefly, cells were harvested by centrifugation at 5000 *g* for 10 min and fixed in 2.5% (*v*/v) glutaraldehyde for 4 h at 4 °C. Subsequently, the cells were washed three times with deionized water, then dehydrated with ethanol by using 30%, 50%, 70%, 80% and 90% ethanol for one time, successively, and finally with absolute ethyl ethanol for three times, and then freeze-dried. The samples were fixed on an aluminum foil and sprayed with gold.

Measurement of membrane permeability

Membrane permeability was measured using the β-galactosidase substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) as a probe, which can be degraded by β-galactosidase to produce o-nitrophenol (yellow color) (Zhu et al. 2014). Cells were rinsed once by centrifugation (5000 *g*, 5 min) and resuspended in 0.01 M PBS (pH 7.4) to an OD_{600} of 1.0, then ONPG was added to a final concentration of 1.5 mM. After incubation at 37 °C for 30 min, the cell suspension was monitored at 420 nm using a spectrophotometer (Precision and Scientific, Shanghai, China).

Measurement of Na^+/K^+-ATPase activity

The Na^+/K^+-ATPase assay was carried out using the Na^+/K^+-ATPase assay kit (Suzhou Comin Biotechnology. Suzhou, China) following the manufacturer's protocol. The activity of the Na^+/K^+-ATPase was expressed in Na^+/K^+-ATPase catalyzing ATP and the formation of 1 μmol inorganic phosphorus per hour per milligram of total protein. Protein concentration was determined by the method of Coomassie Brilliant Blue staining, using bovine serum albumin as a standard protein.

Determination of intracellular NAD^+/NADH ratio

Cellular metabolism was stopped by putting cell suspension into liquid nitrogen for 3 min then the content of intracellular NAD^+/NADH was determined using the NAD^+/NADH assay kit (Suzhou Comin Biotechnology) following the manufacturer's protocol.

Determination of intracellular amino acids

For the extraction of intracellular amino acids, 20 mL stationary phase cells was treated under different acid conditions (pH 6.2, 4.5 and 3.5) in 0.01 M PBS broth at 37 °C for 1.0 h, harvested by centrifugation at 12,000 *g* for 10 min, washed twice and resuspended in 1 mL of 0.2 M PBS, then boiled for 15 min. Cell debris was discarded after the centrifugation (12,000 *g*, 10 min, 4 °C). The supernatants were treated at room temperature after adding 1 mL 10% TCA for 10 min. Then the mixture was centrifuged at 12,000 *g* for 10 min at 4 °C, and the supernatants were analyzed with amino-acid analyzer (Sykam, Munich, Germany).

Measurement of ADI activity

ADI activity was measured as described by De Angelis et al. (2002) with some modifications. Cells resuspended in 0.05 M Tris-HCl buffer (pH 7.5) were sonicated for 10 min with an ultrasonic cell disruptor (Xinzhi, Ningbo, China) and centrifuged at 10,000 *g* for 10 min at 4 °C; the supernatant, containing cell wall and cytoplasm, was collected and considered as enzyme extract.

The reaction mixture (2.5 mL) consisted of enzyme extract, 0.05 M arginine and 0.05 M acetate buffer (pH 5.5). Controls without enzyme extract were included. After incubation at 37 °C for 1 h, the reaction was stopped by adding 500 μL 2 M HCl, and the precipitate was removed by centrifugation (10,000 *g* for 10 min at 4 °C). The citrulline content of the supernatant was determined as described by Archibald (1944) 1 mL supernatant was added to 1.5 mL acid mixture of H_3PO_4-H_2SO_4 (3:1, *v*/v) and 250 μL diacetyl monoxime (1.5%, w/v), mixed, and boiled in the dark for 30 min. After cooling for 10 min, the absorbance was measured at 460 nm. The standard curve for citrulline was determined by applying the same procedure to five standard solutions of citrulline (0, 10, 20, 30 and 40 μg/mL). One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 μmol citrulline per hour per milligram of total protein. Protein concentration in the enzyme preparation was determined as described previously.

Measurement of GAD activity

GAD activity was measured as previously described for *L. lactis* (Johnson et al. 1997; Xu et al. 2003) with minor modifications. Cells were re-suspended in 2 mL cold McIlvaine buffer (pH 4.7) combined with 0.1 mM pyridoxal phosphate and 1 mM mercaptoethanol, sonicated for 10 min and centrifuged at 10,000 *g* for 10 min at 4 °C; the supernatant was taken as GAD extract.

The reaction mixture consisted of enzyme extract, 10 mM L-glutamate (pH 4.8) and 0.1 mM pyridoxal phosphate. Controls without enzyme extract were included. After incubation at 30 °C for 10 h, the reaction was stopped by addition of 200 μL 0.2 M sodium borate buffer, 1 mL 6% phenol solution and 400 μL sodium hypochloride coupled with ice water bath. Color development was carried out in boiling water for 10 min, then immediately put it in an ice water bath for 20 min. The optical density was read at 630 nm. The standard curve for γ-amino butyric acid (GABA) was determined by applying the same procedure to five standard solutions of GABA (0, 2, 4, 6 and 8 mM GABA). One unit of the activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol GABA per hour per milligram of total protein. Protein concentration in the enzyme preparation was determined as described previously.

Statistical analysis

All results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using two-way ANOVA procedure of SPSS 13.0 software (SPSS, Chicago, IL). Data was considered statistically significant when $P < 0.05$.

Results

Growth curve and acid tolerance ability of *L. plantarum* ZDY 2013 and ATCC 8014

To analyze the mechanism of acid tolerance of different *L. plantarum*, the growth curve of *L. plantarum* ZDY 2013 and ATCC 8014 in standard or acid conditions, and their cell survival in extreme acidic environment was investigated. At the initial pH of 6.2 (Fig. 1a), no significant difference

between two strains was observed ($P > 0.05$). However, the biomass of *L. plantarum* ZDY 2013 was 32% higher than that of *L. plantarum* ATCC 8014 from 10 h when the initial pH was 4.5 (Fig. 1a). The growth of *L. plantarum* ATCC 8014 was severely inhibited at pH 3.5 (Fig. 1a), whereas *L. plantarum* ZDY 2013 grew well and exhibited a more than two-fold biomass increase. This finding clearly showed that *L. plantarum* ZDY 2013 had superior growth performance in the acidic condition. As shown in Fig. 1b, the survival rate of both cells decreased in PBS at pH 2.5. When exposed to acid stress for 1 h, the viable cells reached 1.9×10^6 cfu/mL in *L. plantarum* ZDY 2013, which was more than 2 logs higher than that of *L. plantarum* ATCC 8014.

Differentiation of cell integrity of *L. plantarum* strains under acid stress

Cell morphology of *L. plantarum* ZDY 2013 and ATCC 8014 was investigated by SEM (Fig. 2). Cells grown under standard conditions exhibited a rod-shaped, smooth-surface morphology in both *L. plantarum* ZDY 2013 and ATCC 8014 (Fig. 2a,c). When exposed to pH 3.5 for 1 h, the cells of *L. plantarum* ZDY 2013 showed a slight tendency to clump together, and their surfaces appeared to be less smooth, with some cavities (Fig. 2b). By contrast, the cells of *L. plantarum* ATCC 8014 showed significant changes under acidic environment; many cells appeared cracked (Fig. 2d), whereas *L. plantarum* ZDY 2013 retained the relative integrity of cell morphology at pH 3.5.

To further compare the cell integrity of both strains, cell membrane permeability in series acid stress was evaluated by using ONPG as a substrate. As shown in Fig. 3, no difference in membrane permeability of either strain was found above pH 4.5 ($P > 0.05$). Nevertheless, *L. plantarum* ZDY 2013 presented significantly less membrane permeability at pH 3.5 when compared with *L. plantarum* ATCC 8014 ($P < 0.001$), again indicating that *L. plantarum* ZDY 2013 maintained cell morphology and integrity much better than *L. plantarum* ATCC 8014 under acid stress.

Na^+/K^+-ATPase

Na^+/K^+-ATPase is an integral membrane enzyme that plays a key role in maintaining electrochemical gradients. To investigate whether the Na^+/K^+-ATPase activity was related to the acid tolerance in *L. plantarum*, the activity of two strains was evaluated under different acid stress (Fig. 4). No obvious difference ($P > 0.05$) was found in the activity of Na^+/K^+-ATPase between *L. plantarum* ZDY 2013 and ATCC 8014 at either pH 6.2 or 4.5. However, the activity of *L. plantarum* ZDY 2013 and ATCC 8014 reached 8.2 ± 0.4 and 6.1 ± 0.4 U/mg, increasing by 1.88-fold and 1.62-fold at pH 5.0 when compared with pH 6.2. While exposed to pH 4.5, the activity was reduced to 6.4 ± 0.4 and 5.2 ± 0.4 U/mg, respectively.

Intracellular NAD^+/NADH ratio

Most energy in glucose metabolism is released by the respiratory chain, and the physiological change that occurs during

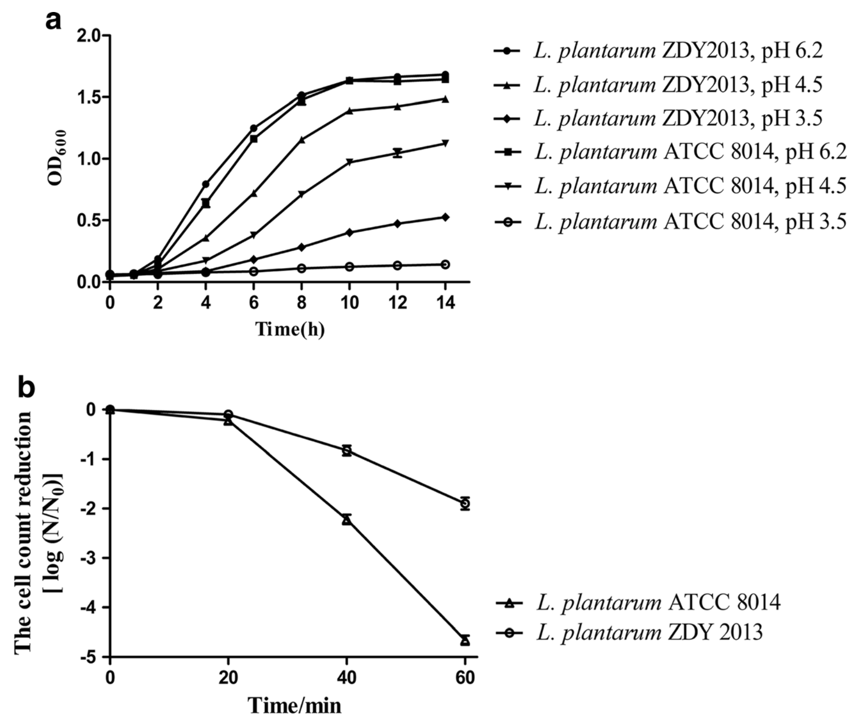

Fig. 1a,b Tolerance analyses of *Lactobacillus plantarum* ZDY 2013 and *L. plantarum* ATCC 8014 cells under acid stress. **a** Growth of the two strains was calculated by measuring OD_{600} in MRS at pH 6.2, 4.5, or 3.5. **b** The cell count reduction was calculated by log (N/N_0); N represented the survival counts, whereas N_0 represented the initial cell counts under acid stress of pH 2.5. All tests were performed in triplicate

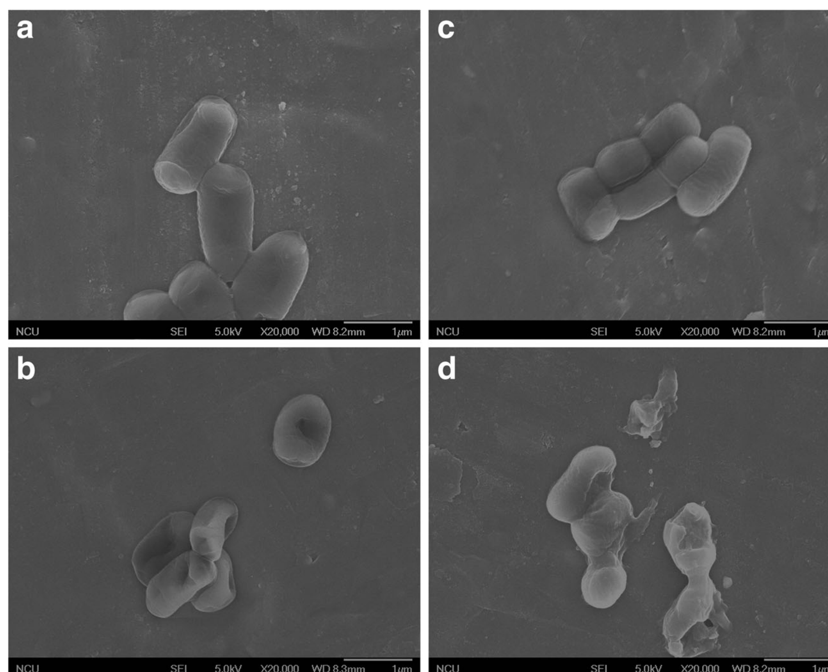

Fig. 2a–d Morphological changes of *Lactobacillus plantarum* under acid stress as viewed by scanning electron microscopy (SEM). *L. plantarum* ZDY 2013 treated with MRS at **a** pH 6.2 or **b** pH 3.5 after 3 h; *L. plantarum* ATCC8014 treated with MRS at **c** pH 6.2 or **d** pH 3.5 after 3 h

respiration might be a consequence of the shift in the NAD^+/NADH ratio (Lechardeur et al. 2011). In this study, the ratio of NAD^+/NADH in *L. plantarum* ZDY 2013 and ATCC 8014 under acid stress was investigated (Fig. 5). At pH 6.2, pH 4.5 and pH 3.5, no significant difference was found in the ratio of NAD^+/NADH of the two strains ($P > 0.05$). At pH 5.0, NAD^+/NADH ratio in *L. plantarum* ZDY 2013 and *L. plantarum* ATCC 8014 increased to 6.8 ± 0.4 and 10.5 ± 1.2 ($P < 0.01$), respectively.

Amino acid changes

To investigate the effect of amino acid metabolism on acid tolerance mechanisms of *L. plantarum*, the chang in intracellular amino acid content of the two strains was monitored. Under standard conditions, no significant difference was found in arginine, glutamate, and alanine content ($P > 0.05$), whereas at pH 3.5, arginine content increased to 7.8 ± 0.8 and 3.9 ± 0.2 mg/mg (1.95-fold and 1.30-fold over pH 6.2) in *L. plantarum* ZDY 2013 and ATCC 8014, respectively (Fig. 6). *L. plantarum* ZDY 2013 maintained almost the same content of glutamate as in standard conditions at pH 4.5, whereas it decreased by 2.9-fold in *L. plantarum* ATCC 8014 (Fig. 6). A higher concentration of alanine accumulated in *L. plantarum* ZDY 2013 than in *L. plantarum* ATCC 8014 (Fig. 6). Before acid stress, the concentration of alanine was at 57.4 ± 6.7 and 42.1 ± 4.4 mg/mg in *L. plantarum* ZDY 2013 and ATCC 8014, respectively, and the level increased by 2.1-fold in the former but decreased by 0.7-fold in the latter. Proline and aspartate were detected in *L. plantarum* ZDY 2013 (2.6 ± 0.3 and 24.1 ± 2.8 mg/mg, respectively) but not in *L. plantarum* ATCC 8014 during acid stress. These results suggested that the accumulation of arginine, glutamate, and alanine might contribute to the acid tolerance response in *L. plantarum* ZDY 2013.

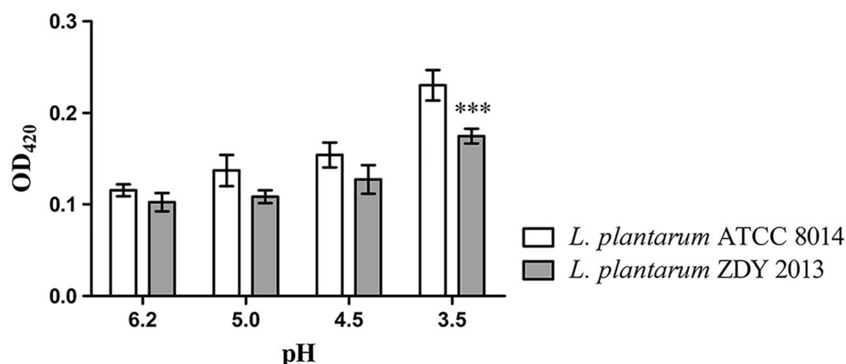

Fig. 3 Changes in membrane permeability of *L. plantarum* at different pH conditions. *White bars L. plantarum* ATCC 8014, *gray bars L. plantarum* ZDY 2013. All tests were performed in triplicate. Statistically significant difference was at $P < 0.05$

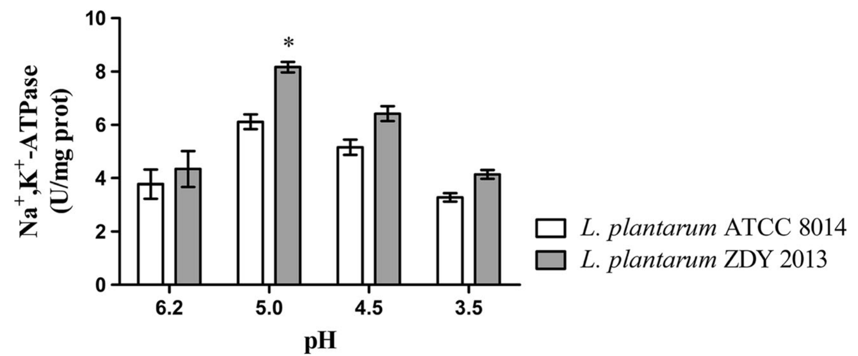

Fig. 4 Effects of acid stress on the activity of Na^{+}/K^{+}-ATPase activity in *L. plantarum*. *White bars L. plantarum* ATCC 8014; *gray bars L. plantarum* ZDY 2013. All tests were performed in triplicate. Statistically significant difference was at $P < 0.05$

ADI activity

The effect of pH on ADI activity was further determined in both strains. As shown in Fig. 7, at pH 5.0, ADI activity increased to 30.0 ± 1.8 and 25.09 ± 1.8 U/mg in *L. plantarum* ZDY 2013 and ATCC 8014, respectively. The activity decreased at pH below 5.0 in both strains, but no significant difference was found between them ($P > 0.05$) at pH 6.2. This result demonstrated that ADI was involved in the acid tolerance mechanism response in *L. plantarum* ZDY 2013.

GAD activity

Acid stress might affect the GAD activity of cells of *L. plantarum* ZDY 2013 and ATCC 8014. As shown in Fig. 8, at pH 4.5, GAD activity of *L. plantarum* ZDY 2013 and ATCC 8014 increased by 3.3-fold and 2.7-fold, respectively, compared with that at pH 6.2. Although GAD activity increased in the two strains at pH 5.0, no significant difference was found between them ($P > 0.05$) at pH 6.2.

Discussion

L. plantarum has a great potential to survive in acid stress environments either in vitro or in vivo; therefore, the acid tolerance properties of *L. plantarum* have attracted much attention because of its broad application in fermented food and probiotic supplements. In our previous study, *L. plantarum* ZDY 2013 was found to initiate a complex metabolic network involving cell membrane components, cellular metabolism, and energy production under acid stress, as reported at the molecular level by Huang et al. (2015). However, the related physiological mechanisms of the acid tolerance response are yet to be clarified. In this study, we performed a comparative and systematic study of two *L. plantarum* strains, namely, *L. plantarum* ZDY 2013 and *L. plantarum* ATCC 8014, and disclosed their acid tolerance response with regard to the cell membrane, energy metabolism, and amino acid metabolism.

Generally, the fluidity and integrity of the bacterial cytoplasmic membrane affects the viability of cells and their metabolic functions, particularly under stress conditions (Mykytczuk et al. 2007), since the cell envelope plays an important role in cellular growth, host defense, and in maintaining the stability of the intracellular environment (Wu et al. 2012). In our study, *L. plantarum* ZDY 2013 retained the relative integrity of cell morphology and the structure of intracellular membrane, which protects cells against damage in acid stress. On the other hand, a type strain, i.e., *L. plantarum* ATCC 8014, did not have the normal cell integrity of ZDY 2013 in acid stress; for instance, the survival rate of *L. plantarum* ZDY 2013 was approximately 100-fold higher than that of *L. plantarum* ATCC 8014 in pH 2.5 after 1 h.

Similar to many other microorganisms under acid stress, *L. plantarum* might start pH homeostasis activities including H^{+}-ATPase proton pump, alterations in cell membrane, and

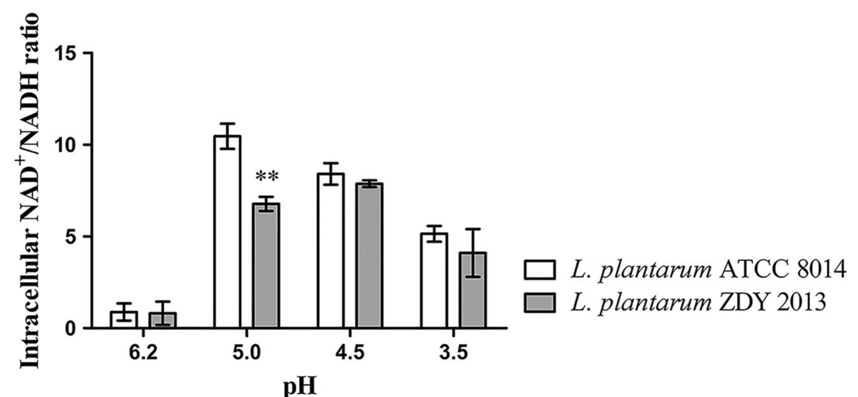

Fig. 5 Change in NAD^{+}/NADH ratio in *L. plantarum* under acid stress. *White bars L. plantarum* ATCC 8014; *gray bars L. plantarum* ZDY 2013. All tests were performed in triplicate. Statistically significant difference was at $P < 0.05$

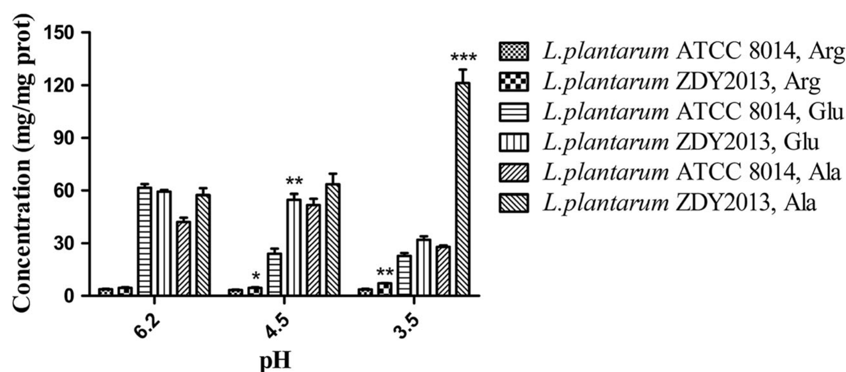

Fig. 6 Changes in intracellular arginine, glutamate, and alanine concentration *L. plantarum* ZDY 2013 and *L. plantarum* ATCC 8014. Concentration of intracellular arginine, glutamate, and alanine was determined. The unit of mg/mg indicates the amount of amino acids (mg) per milligram of protein. All tests were performed in triplicate. Statistically significant difference was at $P < 0.05$

metabolic pathways (Cotter and Hill 2003) to enhance its survival efficiency. Na^+/K^+-ATPase is a ubiquitous integral membrane enzyme (Towle 1984), which maintains the ion gradients of Na^+ and K^+ by consuming energy through ATP hydrolysis (Jutfelt 2006). In our study, we confirmed that *L. plantarum* ZDY 2013 strengthened proton cell pumping as supported by a 1.88-fold increase in Na^+/K^+-ATPase activities from an initial level that was significantly higher than that of *L. plantarum* ATCC 8014 in the same pH of 5.0 ($P < 0.05$). A similar result was reported also in *L. reuteri* (Lee et al. 2008). The activity of Na^+/K^+-ATPase decreased at pH 4.5. Wang et al. (2002) revealed that the active transport of Na^+ and K^+ was inhibited at pH 4.6–5.0 in their experiment. In our previous work, the gene transcription levels in ATP synthase (*atpA* and *atpC*) and glycerol-3-phosphate dehydrogenase (*gspA*) related to cellular energy metabolism were enhanced during acid stress (Huang et al. 2015).

Normally, NAD(H) plays an important role in cell death (Ying 2008), and can affect numerous enzymatic activities involved in the glycolysis pathway and tricarboxylic acid cycle (Fernie et al. 2004). At pH 5.0, the increased ratio of NAD^+/NADH in *L. plantarum* ZDY 2013 and *L. plantarum* ATCC 8014 might relieve the inhibition of glyceraldehyde 3-phosphate dehydrogenase activity because a higher intracellular NAD^+/NADH ratio could affect the enzymes using NAD or pyruvate as substrate (Lechardeur et al. 2011; Wendisch et al. 2006). An increased intracellular NAD^+/NADH ratio and decreased levels of the content intracellular NAD^+ and NADH could contribute to the increased of ATP/ADP ratio and enhance the energy metabolism (Lin et al. 2009).

Amino acid metabolism plays an important role in maintaining the homeostasis of intracellular pH, generating metabolic energy, and enhancing the cell resistance to environmental stress of LAB (Wu et al. 2013). In our work, we demonstrated that the arginine and alanine content of cells increased significantly for *L. plantarum* ZDY 2013 ($P < 0.001$), but was maintained or reduced for *L. plantarum* ATCC 8014 at pH 3.5. *L. plantarum* ZDY 2013 shifted the metabolic pathway by increasing the flux to arginine under acid conditions, and, based on arginine deiminase system, higher intracellular arginine concentration under acid conditions may be involved in acid tolerance in *L. plantarum* ZDY 2013. Glutamate content was maintained or decreased in both *L. plantarum* ZDY 2013 or *L. plantarum* ATCC 8014 at pH 4.5, respectively. Several previous research studies have demonstrated that

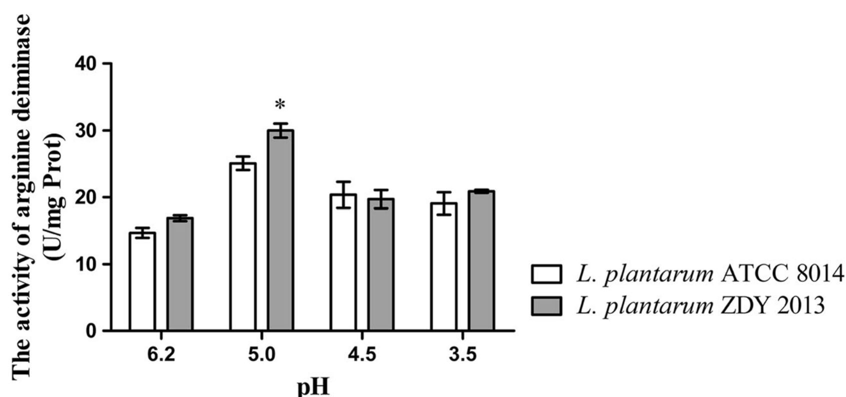

Fig. 7 Arginine deiminase (ADI) activity of *L. plantarum* ZDY 2013 and *L. plantarum* ATCC 8014 under acid stress. All tests were performed in triplicate. Statistically significant difference was at $P < 0.05$

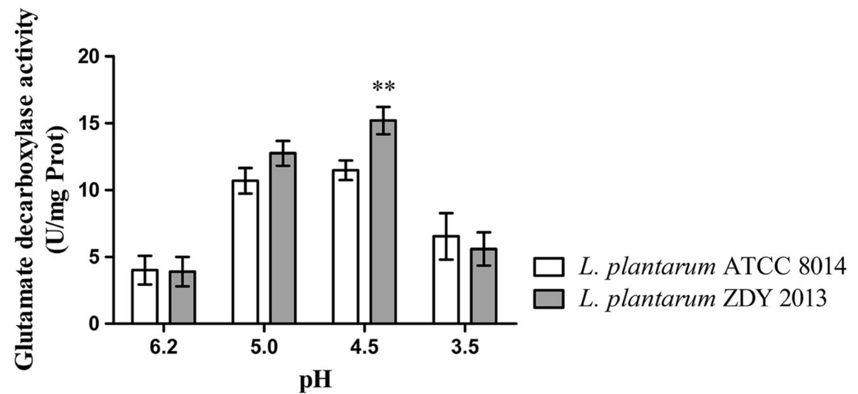

Fig. 8 Glutamate decarboxylase (GAD) activity of *L. plantarum* ZDY 2013 and *L. plantarum* ATCC 8014 under acid stress. *White bars L. plantarum* ATCC 8014, *gray bars L. plantarum* ZDY 2013. All tests were performed in triplicate. Statistically significant difference was at $P < 0.05$

many amino acids may protect cells against acid stress (Senouci-Rezkallah et al. 2011). Senouci-Rezkallah et al. (2011) investigated the effects of adding glutamate and arginine on acid tolerance of *B. cereus* ATCC 14579, and the results showed that cell survival at pH 4.0 increased by 1-log or 2-log populations, respectively.

Deiminase and decarboxylases were mainly involved in the acid stress response of LAB (Fernández and Zúñiga 2006). Our data revealed that, at pH 6.2 to 5.0, ADI activity increased and then decreased gradually until pH 3.5, but it was still relatively higher than the initial level. Arginine was converted to NH_3 in the ADI pathway, which contributed to the alkalization of cell cytoplasm, protecting it from acid stress damage (Lin et al. 1995; Ryan et al. 2009). Moreover, ADI was involved in the extrusion of cytoplasmic protons of *B. cereus* ATCC 14579 and regulation of intracellular pH of *Lactobacillus fermentum* IMDO 130101, helping cells to survive longer under acid stress (De Angelis et al. 2002; Senouci-Rezkallah et al. 2011). As for GAD, we proved that it was increased significantly ($P < 0.05$) until pH 4.5 and went down at the initial level. Fernández and Zúñiga (2006) reported that, at pH 4.7, optimal activity of GAD degraded Glu into GABA. Sanders et al. (1998) concluded that GAD activity of *L. lactis* was a particularly effective system for protecting cells from acid stress.

Conclusions

By comparing the morphology and survival ratio of *L. plantarum* ZDY 2013 and ATCC 8014 strains, the physiological behavior of their Na^+/K^+-ATPase and $NAD^+/NADH$, amino acid content, and ADI and GAD activity, we might ascertain that the acid stress response of *L. plantarum* ZDY 2013 may be complicated. Together with our previous findings, further work should be done to determine the systematic regulative effect of amino acids on the acid tolerance of *L. plantarum* ZDY 2013.

Acknowledgments This project was sponsored by the National Natural Science Foundation of China (NSF31170091, 81760102, 31360377 and 31260363), the Ganpo Talent Engineering 555 Project, the Academic and Technical Leaders Training Program for Major Subjects of Jiangxi Province (P. R. China), the Research Project of Jiangxi Provincial Education Department (GJJ13098; P. R. China), and the Postdoctoral Science Foundation Funded Project of China (M570567).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Disclosures The manuscript does not contain clinical studies or patient data.

References

Archibald RM (1944) Determination of citrulline and allantoin and demonstration of citrulline in blood plasma. J Biol Chem 156:121–142

Brinques GB, Ayub MAZ (2011) Effect of microencapsulation on survival of *Lactobacillus plantarum* in simulated gastrointestinal conditions, refrigeration, and yogurt. J Food Eng 103:123–128

Broadbent JR, Larsen RL, Deibel V, Steele JL (2010) Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress. J Bacteriol 192:2445–2458

Bron PA, Marco M, Hoffer SM, Van Mullekom E, De Vos WM, Kleerebezem M (2004) Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. J Bacteriol 186:7829–7835

Cotter PD, Gahan CG, Hill C (2001) A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol Microbiol 40:465–475

Cotter PD, Hill C (2003) Surviving the acid test: Responses of gram-positive bacteria to low pH. Microbiol Mol Biol Rev 67:429–453

De Angelis M, Gobbetti M (2004) Environmental stress responses in *Lactobacillus*: a review. Proteomics 4:106–122

De Angelis M, Mariotti L, Rossi J, Servili M, Fox PF, Rollán G, Gobbetti M (2002) Arginine catabolism by sourdough lactic acid bacteria: Purification and characterization of the arginine deiminase pathway enzymes from *Lactobacillus sanfranciscensis* CB1. Appl Environ Microbiol 68:6193–6201

De Felipe FL, Kleerebezem M, de Vos WM, Hugenholtz J (1998) Cofactor engineering: a novel approach to metabolic engineering

in *Lactococcus lactis* by controlled expression of NADH oxidase. J Bacteriol 180:3804–3808

De Vries MC, Vaughan EE, Kleerebezem M, de Vos WM (2006) *Lactobacillus plantarum*—survival, functional and potential probiotic properties in the human intestinal tract. Int Dairy J 16: 1018–1028

Duary RK, Batish VK, Grover S (2010) Expression of the *atpD* gene in probiotic *Lactobacillus plantarum* strains under in vitro acidic conditions using RT-qPCR. Res Microbiol 161:399–405

Fernández M, Zúñiga M (2006) Amino acid catabolic pathways of lactic acid bacteria. Crit Rev Microbiol 32:155–183

Fernie AR, Carrari F, Sweetlove LJ (2004) Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. Curr Opin Plant Biol 7:254–261

Ferrando V, Quiberoni A, Reinhemer J, Suárez V (2015) Resistance of functional *Lactobacillus plantarum* strains against food stress conditions. Food Microbiol 48:63–71

Fozo EM, Quivey RG (2004) Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. Appl Environ Microbiol 70:929–936

Hamon E, Horvatovich P, Marchioni E, Aoudé-Werner D, Ennahar S (2014) Investigation of potential markers of acid resistance in *Lactobacillus plantarum* by comparative proteomics. J Appl Microbiol 116:134–144

Hernandez-Hernandez O, Muthaiyan A, Moreno FJ, Montilla A, Sanz ML, Ricke S (2012) Effect of prebiotic carbohydrates on the growth and tolerance of *Lactobacillus*. Food Microbiol 30:355–361

Huang R, Tao X, Wan C et al (2015) *In vitro* probiotic characteristics of *Lactobacillus plantarum* ZDY 2013 and its modulatory effect on gut microbiota of mice. J Dairy Sci 98:5850–5861

Johnson BS, Singh NK, Cherry JH, Locy RD (1997) Purification and characterization of glutamate decarboxylase from cowpea. Phytochemistry 46:39–44

Jutfelt F (2006) The intestinal epithelium of salmonids: transepithelial transport, barrier function and bacterial interactions. PhD Thesis, Department of Zoology, Göteborg University

Lechardeur D, Cesselin B, Fernandez A et al (2011) Using heme as an energy boost for lactic acid bacteria. Curr Opin Biotechnol 22:143–149

Lee K, Lee HG, Pi K, Choi YJ (2008) The effect of low pH on protein expression by the probiotic bacterium *Lactobacillus reuteri*. Proteomics 8:1624–1630

Lin FM, Qiao B, Yuan YJ (2009) Comparative proteomic analysis of tolerance and adaptation of ethanologenic *Saccharomyces cerevisiae* to furfural, a lignocellulosic inhibitory compound. Appl Environ Microbiol 75:3765–3776

Lin J, Lee IS, Frey J, Slonczewski JL, Foster JW (1995) Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. J Bacteriol 177:4097–4104

Marty-Teysset C, de la Torre F, Garel J (2000) Increased production of hydrogen peroxide by *Lactobacillus delbrueckii* subsp. *bulgaricus* upon aeration: involvement of an NADH oxidase in oxidative stress. Appl Environ Microbiol 66:262–267

Mykytczuk NC, Trevors JT, Leduc LG, Ferroni GD (2007) Fluorescence polarization in studies of bacterial cytoplasmic membrane fluidity under environmental stress. Prog Biophys Mol Biol 95:60–82

Parvez S, Malik KA, Ah KS, Kim HY (2006) Probiotics and their fermented food products are beneficial for health. J Appl Microbiol 100:1171–1185

Rallu F, Gruss A, Ehrlich SD, Maguin E (2000) Acid- and multistress-resistant mutants of *Lactococcus lactis*: Identification of intracellular stress signals. Mol Microbiol 35:517–528

Ryan S, Begley M, Gahan CG, Hill C (2009) Molecular characterization of the arginine deiminase system in *Listeria monocytogenes*: regulation and role in acid tolerance. Environ Microbiol 11:432

Sanders JW, Leenhouts K, Burghoorn J, Brands JR, Venema G, Kok J (1998) A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation. Mol Microbiol 27:299–310

Senouci-Rezkallah K, Schmitt P, Jobin MP (2011) Amino acids improve acid tolerance and internal pH maintenance in *Bacillus cereus* ATCC14579 strain. Food Microbiol 28:364–372

Shobharani P, Halami PM (2014) Cellular fatty acid profile and H^+-ATPase activity to assess acid tolerance of *Bacillus* sp. for potential probiotic functional attributes. Appl Microbiol Biotechnol 98:9045–9058

Teixeira JS, Seeras A, Sanchez-Maldonado AF, Zhang C, Su MS, Gänzle MG (2014) Glutamine, glutamate, and arginine-based acid resistance in *Lactobacillus reuteri*. Food Microbiol 42:172

Towle D (1984) Regulatory functions of Na^++K^+-ATPase in marine and estuarine animals. In: Pequeux A (ed) Osmoregulation in estuarine and marine animals. Springer, Berlin, pp 158–170

van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E (2002) Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek. 82:187–216

Wall T, Bth K, Britton RA, Jonsson H, Versalovic J, Roos S (2007) The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative Cell Wall-altering esterase. Appl Environ Microbiol 73:3924–3935

Wang WN, Wang AL, Chen L, Liu Y, Sun RY (2002) Effects of pH on survival, phosphorus concentration, adenylate energy charge and Na^+-K^+ ATPase activities of *Penaeus chinensis* Osbeck juveniles. Aquat Toxicol 60:75

Wendisch VF, Bott M, Eikmanns BJ (2006) Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids and amino acids. Curr Opin Microbiol 9:268–274

Wu C, Zhang J, Du G, Chen J (2013) Aspartate protects *Lactobacillus casei* against acid stress. Appl Microbiol Biotechnol 97:4083–4093

Wu C, Zhang J, Wang M, Du G, Chen J (2012) *Lactobacillus casei* combats acid stress by maintaining cell membrane functionality. J Ind Microbiol Biotechnol 39:1031–1039

Xu J, Jiang B, Xu S (2003) Rapid determination of glutamate decarboxylase activity from lactic acid bacteria by spectrometric method and its applications. Wei Sheng Wu Xue Tong Bao 31:66–71

Ying W (2008) NAD^+/NADH and $NADP^+$/NADPH in cellular functions and cell death: regulation and biological consequences. Antioxid Redox Signal 10:179–206

Zhu X, Dong N, Wang Z, Ma Z, Zhang L, Ma Q, Shan A (2014) Design of imperfectly amphipathic α-helical antimicrobial peptides with enhanced cell selectivity. Acta Biomater 10:244