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Phenylalanine, Cysteine and Threonine Improve Acid Tolerance of Lactococcus Lactis NZ9000 During Acid Stress

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***Abstract* – In this study, the environmental stress response properties of *L. lactis* NZ9000 by exogenous Phenylalanine, Cysteine and Threonine during acid stress conditions was investigated and to evaluate how amino acid metabolic pathway strategies can be used to increase the robustness of LAB against environmental stresses. Cells cultivated to mid-exponential phase were challenged with acid stress for various times, and the growth performance and survival rate of cells were determined. Results obtained showed that *L. lactis* strain induced a growth-phase-dependent acid tolerance response and survival rate. The use of exogenous amino acids leads to improve in growth performance and acid tolerance of *L. lactis* subjected to acid stress. In the presence of Phenylalanine, Cysteine and Threonine, the survival rate of cell was increased 14.12%, 26.60% and 2.87%, respectively higher than the control. Significant differences in the content of membrane-associated fatty acids were also detected. The greater amounts of cytoplasmic amino acids in the membrane fraction of the *L. lactis* suggested the decreased permeability of the cell membrane. As evidenced by intracellular pH homeostasis, amino acid metabolic pathway was activated in response to low pH. In the presence of amino acids, cells contained higher abundance of unsaturated fatty acids and with lower saturated fatty acids. Increased abundance of metabolites suggested that high activation of metabolic flux of amino acid affected the permeability of the cell membrane and possibly accounts for the acid tolerance of *L. Lactis*.**

***Keywords* – *L. lactis*, Phenylalanine, Cysteine, Threonine, Acid Stress.**

I. Introduction

Lactic acid bacteria (LAB) are one of the interesting industrial microorganisms in fermentation engineering. In food products, LAB produce a wide range of metabolites including organic acids, diacetyl, acetoin, antifungal peptides, and bacteriocins that may play a forceful rival to spoilage microorganisms. These products are generally recognized as safe (GRAS) [1-4]. Due to their beneficial effects on health, they are also considered as probiotic [5]. LAB act a significant role in the fermentation of different food products, such as milk, cheese [6,7] or meats [8,9]. During the fermentation process, lactobacilli are commonly faced with several inhibiting factors such as high salt concentration, temperature changes, organic acids and low pH [10]. At low pH, unsaturated lactic acid spread out passively beyond the cell membrane, leading to collapse the electrochemical proton gradient and alternation of membrane permeability, with subsequent disturbance of the substrate transport system [11,12].

The metabolism of sugars is the main process of LAB to obtain energy; therefore lactic acid bacteria are limited to environments sugars availability. LAB have limited biosynthetic capability, thus environments rich in amino acids, vitamins, purines and pyrimidines can satisfy all their nutritional requirements for growth performance and stress tolerate [13,14]. Low pHs is one of most important environmental stresses and affect survival of lactic acid bacteria during fermentation [15,16]. One of the physiological approach to tolerate environmental acid stress is the control of amino acid metabolic pathway by homeostasis of intracellular pH, influx of metabolic energy, redox power, control of pH optima of the proton trans locating (H^+) - ATPase, reduce the energy demand for proton translocation and enzyme complex. Broadbent et al. (2010) showed that accumulation of histidine in cell could protected *Lactobacillus casei* ATCC 334 cell against environmental acid damage [17]. Senouci - Rezkallah et al. (2011) reported that glutamate and arginine effects on acid tolerance of *Bacillus cereus* ATCC14579 [18]. In order to investigate whether other amino acids metabolism is involved in acid tolerance *Lactococcus lactis* NZ9000, in this study the effect of phenylalanine, cysteine and threonine on acid tolerance of *L. lactis* during acid stress was investigated. Results of this study may provide a new strategy to enhance acid tolerance of *L. lactis* during its industrial application.

II. Materials and Methods

1. Growth Conditions and Survival Rate

Bacterial strain *Lactococcus lactis* ssp. *cremoris* NZ9000 was used. The bacterial strain was grown statically at 30 °C without aeration in M17 broth (Oxoid, Basingstoke, UK)

supplemented with glucose (5 g/l), unless stated otherwise. The culture was split into refresh three 100 ml GM17 broth containing 50 mM of amino acids (Phenylalanine, Cysteine, Threonine) separately, adjusted to pH 6.0, 5.0, 4.0 with lactic acid, respectively. The cell suspension was incubated at 30 °C, and samples were withdrawn at specified times to determine survival rate. Cell numbers were determined with spot plating, where 10 µl of serially diluted samples were spotted in triplicate onto M17 agar plates and incubated at 30 °C for 48h. Plates containing 5 – 100 CFU were counted, and cell colony forming units per milliliter was calculated from the average.

2. Measurement of Intracellular pH

Intracellular pH (pH_i) was measured by the fluorescence method developed by Casey et al. [19] using 5 - (and – 6 -) - Carboxyfluorescein Acetoxymethyl Ester as the fluorescent probe. Calibration curves were established to exclude artifacts caused by environmental conditions. Loading of cells with dye 2', 7' - Bis - (2 - Carboxyethyl) - 5 - (and – 6 -) - Carboxyfluorescein Acetoxymethyl Ester (BCECF-AM), determination of pH_i, and calibration of pHi all followed the procedure described previously by McBrian et al. [20].

3. Measurement of the H^+-Atpase Activity

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

4. Fatty Acid Extraction and Analysis

Fatty acids from the cellular materials were extracted according to the method described by [21] with modifications as follows: the cell pellet was resuspended in a glass tube containing 500 µl of 1 mol/l sodium methoxyde in methanol at 4 °C. The preparations were mixed for 1 min and then fatty acid methyl esters (FAME) were then extracted by shaking with 100 µl hexane for 2 min. Samples were pelleted, and the upper organic phase after decanting was taken for analysis. Gas chromatography was carried out on a Hewlett-Packard 6850 gas chromatograph (Agilent Technologies). Two microlitres of sample were injected (splitless, 0.75 min) into an HP-FFAP column (Agilent Technologies) with an HP 6850 automatic injector.

III. Results and Discussion

1. Cell Viability and Acid Survival Rate of L. Lactis During Acid Stress

Acid survival of *L. lactis* added without and with exogenous amino acids (phenylalanine, cysteine or threonine) and subjected to pH 4.0 is shown in Fig. 1. A significant increase in survival rate of cells was observed when the cells were grown in the presence of amino acid at pH 4.0. Furthermore, when cells are exposed to acid stress for longer times, the survival differences showed a significant increase. After 6 hours of acid stress, survival of the control strain without amino acids addition decreased to 0.48% of the initial value while the corresponding values upon addition of phenylalanine, cysteine or threonine were 14.12%, 26.60% and 2.87%, respectively. Thus, exogenous phenylalanine, cysteine and threonine enhanced the acid tolerance of *L. lactis* during acid stress.

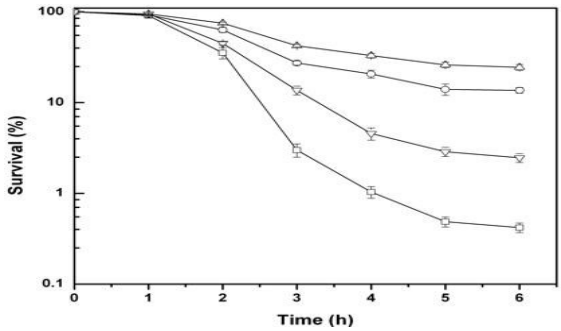

Figure 1: Effect of exogenous Phenylalanine, Cysteine and Threonine addition on the acid tolerance of *L. Lactis* NZ9000. Cells cultivated to mid-exponential phase were challenged with acid stress at pH 4.0 for various times, and the survival rate of cells were determined. Square symbols represent no amino acid was supplemented, while circle symbols represent Phenylalanine, up triangle symbols represent Cysteine and down triangle symbols represent Threonine were added into the culture medium separately. Error bars indicate standard deviations (n = 3).

2. Intracellular pH (pH_i) Homeostasis

In order to verify intracellular pH homeostasis during acid stress, the pH_i content in *L. lactis* at different pHs was measured. As shown in Fig. 2, with decreasing extracellular pH, the pH_i decreased sharply. Moreover, addition of phenylalanine, cysteine and threonine led to the increase of pH_i under acidic conditions. In the presence of phenylalanine, cysteine or threonine in different media, the intracellular pH increased 14.85%, 17.64% and 11.54% (pH 4.0) compared with the corresponding control (without amino acids addition), respectively. These results suggested that exogenous phenylalanine, cysteine and threonine enhanced the pH homeostasis performance of *L. lactis* during acid stress. In order to be able to grow at acidic condition, lactic acid bacteria generally maintain a pH gradient across the cellular membrane while producing ATP by the influx of protons through the F_0F_1 ATPase [22]. *L. lactis* seemed to share a distinctive structural and functional characteristic including a reversed membrane potential, highly impermeable cell membranes and a predominance of secondary transporters. Once protons enter the cytoplasm, appropriate responsive mechanisms are required to alleviate the effects of a lowered internal pH [23]. Thus proton transport systems might mixed up in the regulation of intracellular pH in *L. lactis* and interacts with the F_0F_1 which is used as the driving force [24]. These results suggested that exogenous phenylalanine, cysteine and threonine enhanced the pH homeostasis performance of *L. lactis* during acid stress and the phenylalanine, cysteine and threonine seemed to confer the cells higher capability to maintain pH_i proportions.

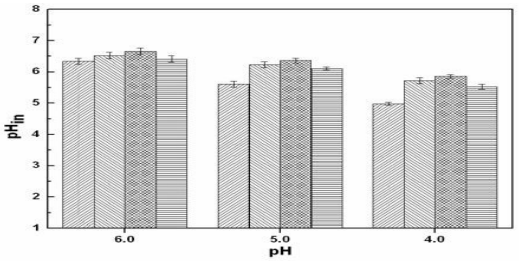

Figure 2: Effect of exogenous Phenylalanine, Cysteine and Threonine addition on pH_i in *L. Lactis* NZ9000. Cells cultivated at various pHs were harvested at mid-exponential phase, and the pH_i concentration were measured. Symbols represent no amino acid was supplemented, while symbols represent Phenylalanine, symbols represent Cysteine and symbols represent Threonine were added into the culture medium separately. Error bars indicate standard deviations (n = 3). Asterisk indicates significant differences with the corresponding control (without amino acid addition) at $p < 0.05$ by the Student's t test.

3. Changes in H^+ - ATPase Activities

The effect of amino acids on H^+-ATPase activity and intracellular ATP pool during acid stress was examined (Fig. 3). Addition of phenylalanine, cysteine and threonine enhanced the H^+-ATPase activity and intracellular ATP concentration at various pHs. At pH 6.0, 5.0 and 4.0, cells exhibited significantly higher H^+-ATPase activity and intracellular H^+-ATPase concentration than the control cells in which amino acids were not added. In cells added with phenylalanine, cysteine or threonine the intracellular H^+-ATPase content increased by 65.10%, 79.99% and 44.04% of the initial value at pH 4.0 respectively. H^+-ATPase is acid tolerance response (ATR) proteins induced by intracellular acidic pH. It expels protons at the expense of ATP hydrolysis and this lead to pH_i homeostasis [25-28]. The growth of *L. lactis* NZ9000 is more affected by acid stress rather than high lactic acid concentrations. This indicates the inability of the cells to accumulate enough H^+ to restore pH_i [29-31]. Results of the present study revealed that ATP formation was not correlated with pH_i and instead a significant correlation was found between cellular level of H^+-ATPase and pH_i. Higher levels of H^+-ATPase is associated with higher acid tolerance of cells induced with sublethal levels of acid. Increased H^+-ATPase activity can counteract any intracellular acidification that accompanies acid influx (Fig. 3) and finite limit to the extent that H^+-ATPase action can enhance the charge component of ΔpH [32]. Membrane ATPases are major engines of acid tolerance for *L. Lactis*. Piper et al. (2001) demonstrated that a strong activation of plasma membrane H^+-ATPase occur in weak-acid-stressed cells and acidification of the cytosol due to the intracellular dissociation of organic acid can be counteracted by increasing H^+-ATPase-catalysed proton extrusion from the cell [33] (Fig. 3). These results further suggest the possible mechanisms involved acid tolerance in *L. lactis* by H^+-ATPase proton pump activities against acid stress.

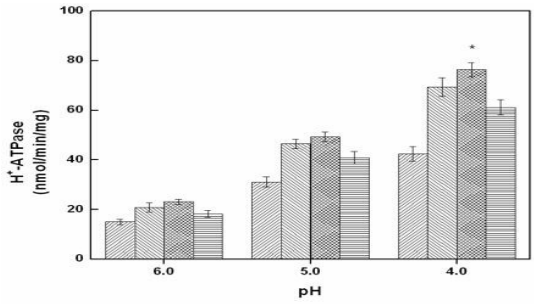

Figure 3: Effect of exogenous Phenylalanine, Cysteine and Threonine addition on H^+-ATPase activity in *L. Lactis* NZ9000. Cells cultivated at various pHs were harvested at mid-exponential phase, and the H^+-ATPase activity were measured. Symbols represent no amino acid was supplemented, while symbols represent Phenylalanine, symbols represent Cysteine and symbols represent Threonine were added into the culture medium separately. Error bars indicate standard deviations (n = 3). Asterisk indicates significant differences with the corresponding control (without amino acid addition) at $p < 0.05$ by the Student's t test.

4. Fatty Acid Metabolism Changes

As Shown in Fig. 4, the composition of membrane fatty acid profiles in *L. lactis* was influenced by the addition of amino acids during acid stress. The content of saturated (C14:0, C16:0, and C18:0) and unsaturated (C14:1t11, C16:1c9, C16:1c11, C17:cyc9, C16:2c9, 12, C18:1c11, C18:1t11, C18:2c9, 12, C19cyc11 C16:1, C18:1t, C18:1c, C16:2, C18:2, and C19-cyc) fatty acids changed with the in the presence of phenylalanine, cysteine and threonine. Cells in the presence of amino acids displayed increased unsaturated fatty acids while the relative content of saturated fatty acids (C14:0, C16:0, and C18:0) decreased. Addition of phenylalanine, cysteine or threonine led to an increase in long chain unsaturated fatty acid in contrast to short chain saturated fatty acids (U/S ratio). Several studies have demonstrated that fatty acid composition of bacterial membranes can be varied depending on environmental conditions [34-38]. In LAB the proportion of unsaturated fatty acids (UFA) of membrane phospholipids are changed in response to environmental acid stresses [39]. Low pH increased accumulation of C18:1 and C19cyc and loss of the saturated fatty acids. The increase in unsaturated fatty acids and the decrease in saturated fatty acids at low pH caused an increase in membrane fluidity. Loffhagen et al. (2007) demonstrated that UFA decreases the rigidity of lipids in bacterial cells [40]. The contribution of unsaturated fatty acids modification in acid resistance and high content of C19:0, Δ under low pH condition has been reported in *L. bulgaricus* [41]. In this study it was found that unsaturated fatty acids are essential in maintaining optimal membrane fluidity and function in *L. lactis* NZ9000 faced high acidic conditions. Our results also provided indirect evidence suggesting that long chain monounsaturated fatty acids are important for *L. lactis* NZ9000 growth under high acid stress. Previous studies also confirmed that LAB generally change the fatty acid composition of their membranes in

response to acidification of the medium [42,43,17]. It seems UFA play an important role in maintaining physiological conditions in cells exposed to extreme environmental acid stress and in these case roles of long chain mono-unsaturated fatty acid is highest.

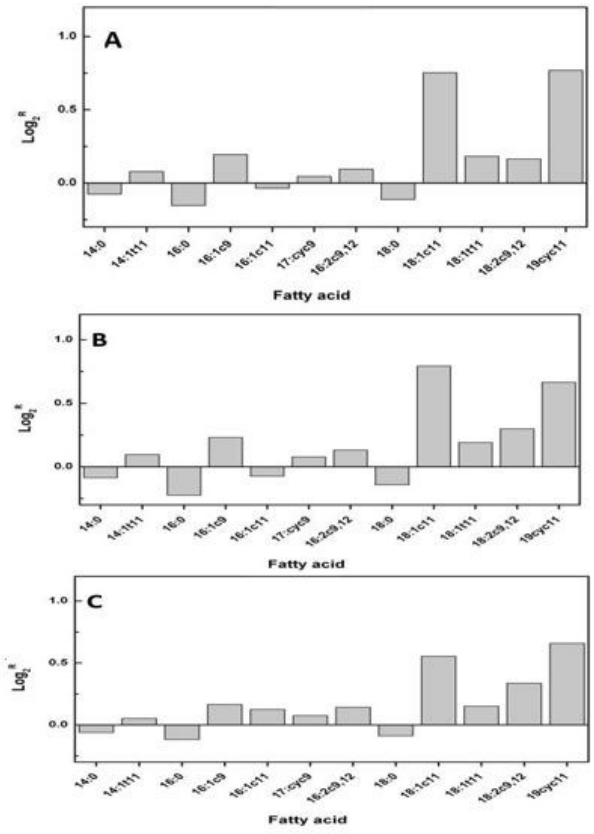

Figure 4: Effect of exogenous Phenylalanine, Cysteine and Threonine addition on membrane fatty acid metabolism during acid stress. Cells grown in mid-exponential growth phase at pH 4.0 were harvested, and the membrane fatty acids concentrations were analyzed. R refers to ratio of relative abundance of fatty acids in *L. Lactis* NZ9000 cultured in the absence and presence of amino acid. ((A) Represents cells cultured in presence of Phenylalanine, (B) represents cells cultured in presence of Cysteine and (C) represents cells cultured in presence of Threonine. Data are the means value of seven biological replicates.)

IV. Conclusion

Results of the present study indicated that in the presence of amino acids, *L. lactis* obviously maintained high level of metabolic efficiency when subjected to acid stress. Also, amino acid metabolic components changed when the cells are exposed to low pH. Furthermore, it was observed that *L. lactis* maintained higher values in maximum metabolic balance and amino acid changing when pH level decreased. As a result, interruption of the metabolic balance weakened the acid stress performance. These results suggest that amino acid metabolic pathway regulates ATR mechanisms in *L. lactis* that ensure high viability and stress tolerance during subsequent acid fermentation. In addition, it will help in developing alternative strategies for pathogen elimination and improving acid tolerance during acid fermentation.

V. Future Prospects

In a prospective study, we seek to establish a basis for future industrial applications, through integrating the *new industrial methods* of industrial strains into the commercial, to enhance environmental tolerance and survive within high concentration acid fermentation.

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Statement of Competing Interests

No conflict of interest associated with this work.

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