Original Research Article

Pre-cold stress increases acid stress resistance and induces amino acid homeostasis in Lactococcus lactis NZ9000

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Abstract

Purpose: To investigate the effects of pre-cold stress treatments on subsequent acid stress resistance and the viability of Lactococcus lactis during acid fermentation.
Methods: Bacterial strains were grown at 4 °C for 2 h pre-adaptation, and then subjected to various stresses including exposure to 4 °C, 1 mM hydrogen peroxide, 5 % sodium chloride, 7 % ethanol, and lactic acid (pH 5.5) to determine if any of these stress treatments could increase acid stress resistance and induce amino acid homeostasis during acid fermentation.
Results: Among the different abiotic stresses investigated, pre-adaptation of lag-phase cultures to cold shock significantly enhanced cell survival during subsequent acid stress. The stress profile of L. lactis pre-adapted to cold stress revealed induction of amino acid homeostasis and energy balance; however, pre-adaptation responses are induced upon exposure to acid stress alone. Compared to exposure to acid stress only, pre-adaptation to cold stress decreased the redox balance ratio and the formation of hydroxyl radicals, indicating a change in aerobic respiration and oxidative state of the bacteria.
Conclusion: Pre-adaptation to cold stress rescued L. lactis from the deleterious effects of subsequent acid exposure by modifying the amino acid metabolic pathway, leading to an improvement in redox mobility of acid stress response.

Keywords: Pre-cold stress, Acis stress resistance, Lactococcus lactis, Redox balance, Amino acid homeostasis

INTRODUCTION

Lactic acid bacteria (LAB) are of great importance in the biotechnology and food industry. *Lactococcus lactis* has long been used as a starter to improve the stability of silages under aerobic conditions by acidifying the substrate, thereby reducing yeast and mould growth [1]. The development of new functional foods containing bioactive compounds such as probiotics and prebiotics is of great interest to the food industry and regulatory authorities, because in addition to their basic nutritional benefits, they have a significant impact on human health [2]. Therefore, LAB preservation is needed to obtain concentrated viable starters with stress tolerance. Several studies have demonstrated that during fermentation, decreased pH leads to bacterial cell damage and thus decreased viability [3]. However, because *L. lactis* grow in anaerobic environments, fermenting glucose differs in metabolism and production [4] and viability and metabolic activity during

fermentation are affected by stressful conditions [1,5].

Several studies have shown that exposing bacteria to moderate stress activates functional and structural mechanisms that enhance their physiological resistance to subsequent stress [6]. LAB are exposed to various types of stress during the fermentation process such as low temperature [7], high hydrogen peroxide (H_2O_2) [8] and salt (NaCl) [4] levels, and low pH [9]. In virulent *L. lactis* strains, it has been demonstrated that pre-adaptation to sub-lethal acid conditions minimises the lethal effects of subsequent extreme-acid conditions by inducing the activation of amino acid metabolic pathways associated with intracellular pH homeostasis, resulting in enhancement of the acid tolerance response (ATR) system [10]. The association among various stressful conditions such as low temperature and low pH, physiological responses such as cell growth, and biochemical modifications such as the amino acid profile [6] and redox balance can be analysed by metabolic approaches. Improvements in the efficiency of technological processes to develop new functional food processes are increasingly required. Therefore, its is necessary to determine the pre-adaptation properties of salt, cold, acid, oxygen, and ethanol on the *L. lactis* NZ9000 redox system balance, amino acid profile, and acid stress tolerance [3]. Changes in the ability of the ATR to protect *L. lactis* from various stressors have been determined by investigating how changes in amino acid metabolism during fermentation lead to altered bacterial metabolism and physiological function [1]. In this study, to evaluate the protective effects of pre-stress on bacterial growth and viability, cell recovery after exposure to various types of stressors at different pH was assessed through determination of amino acid homeostasis and redox mobility.

EXPERIMENTAL

Bacterial strains and growth conditions

L. lactis ssp. *cremoris* NZ9000 was obtained from Professor Jian Chen (Key Laboratory of Industrial Biotechnology, Jiangnan University, Wuxi, China). The bacterial strain was grown statically at 30°C without aeration in M17 broth (Oxoid, Basingstoke, UK) supplemented with 5 g/L glucose, unless otherwise stated.

Stress treatments

Log-phase cell cultures were centrifuged at 5400×g for 7 min, and the resulting cell pellet was re-suspended in an equal volume of GM17 medium. The cells were subjected to five types of stress: cold stress (4°C), oxidative stress (1 mM H_2O_2), osmotic stress (5 % NaCl), alcohol stress (7 % ethanol), and acid stress (pH 5.5 lactic acid). These conditions are within the range of conditions typically encountered by bacteria during food processing. For this study, these stressors were applied during a 2 h pre-adaptation phase (shock) and then adjusted with lactic acid (pH 4.0) for 6 h (acid stress). Non-stressed strains cultured in GM17 served as positive controls. Serial 10-fold dilutions of pre- and post-stress culture samples were plated to estimate the number of colony-forming units. At the end of the shock and stress treatments, the cultures were divided into two samples and centrifuged at 5400×g for 7 min. The first sample was subjected to further acid stress at pH 5.5, 4.5, and 3.5 and incubated at 30 °C for 6 h. The second sample served as the assay control.

Quantification of the nicotinamide nucleotide ratio

The nicotinamide nucleotide ratio (NAD^+/NADH) was determined using the BioVision NAD^+/NADH Quantification Kit (Research Products, Milpitas, CA, USA) according to the manufacturer's instructions. Bacterial suspensions were centrifuged at 6000×g for 7 min. The pellets were washed twice with cold sterile phosphate-buffered saline (PBS, pH 7.2), centrifuged at 8000×g for 3 min, and lysed using 334 µg microbeads (106 µm in diameter) in a Mini-Beadbeater (Sigma, St. Louis, MO, USA). Samples were centrifuged at 14,000×g for 5 min to obtain clear supernatant. The protein concentration of the supernatant was determined [11] and the NAD^+/NADH measurements were normalised to protein quantity.

Hydroxyl radical measurement

Hydroxyl radical (OH·) levels were measured using hydroxyphenyl fluorescein (HPF) as previously described [12]. Bacterial cultures were centrifuged at 8400×g for 4 min at 4°C, washed twice with an equal volume of sterile PBS, and centrifuged at 8000×g for 3 min. Positive control bacteria were treated with H_2O_2 (25 mM) and negative control bacteria were untreated. For the measurements, all of the samples were first diluted to a concentration of 106 bacteria/mL, after which HPF was added to a final concentration of 10 µM per reaction mixture [13]. The solutions were incubated at 20°C for 75 min in the dark. Fluorescence was measured using a DTX 880 multimode detector (Beckman Coulter, Brea, CA, USA) at an excitation wavelength of 460 nm and an emission wavelength of 520 nm.

Determination of intracellular amino acid content

Bacterial cells were grown to mid-log phase in GM17 culture medium for a 2 h pre-cold stress treatment at 4°C, followed by stress treatment. The cells were collected by centrifugation at 8000×g for 8 min and then washed twice in sterile distilled water. The collected cells were suspended in 0.5 N perchloric acid and disrupted with glass beads. The homogenate was kept on ice for 15 min and centrifuged to obtain the supernatant. The pH of the supernatant was adjusted to 2.0 using lithium hydroxide and then kept on ice for 15 min. The sample was centrifuged to remove the precipitate. The concentration of free amino acids was quantified using a Hitachi L-8900 amino acid analyser (Hitachi, Tokyo).
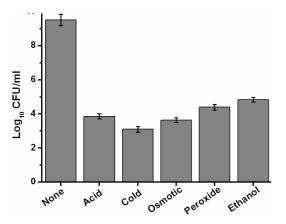
Statistical analysis

Data were subjected to statistical analysis (Student's *t*-test) using SPSS software version 16.0 (SPSS, Chicago, IL, USA). The data are expressed as mean ± standard deviation (SD) and $p < 0.05$ was considered statistically significant.

RESULTS

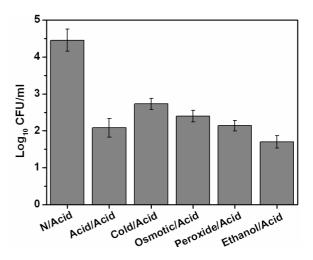### Stress tolerance and survival rate of *L. lactis* subjected to multiple stressors

The response of *L. lactis* to 8 h of acid-, cold-, osmotic-, peroxide- and ethanol-induced stress is presented in Figure 1. Acid, cold, and osmotic stress significantly decreased *L. lactis* survival. As shown in Figure 1, *L. lactis* treated with cold, lactic acid and salt conditions for 8 h had higher survival rates than control bacteria.

Figure 1: Survival rate of *L. lactis* after stress treatments. Survival rate of *L. lactis* after no stress (none) and lactic acid pH 5.5 (acid), 4 °C (cold), 5 % NaCl (osmotic), 1 mM H_2O_2 (peroxide) and 7 % ethanol stress for 8 h, (n = 3)

Stress tolerance and survival rate of *L. lactis* subjected to various pre-stressors and subsequent acid stress

Pre-adaptation to acid, cold, and osmotic shock significantly reduced cell survival during subsequent acid stress exposure (Figure 2). Conversely, pre-adaptation of *L. lactis* to cold (4 °C), osmotic (5 % NaCl), and acid (pH 5.5) shock resulted in a significant increase in survival following acid stress compared to the control. Pre-stress conditions followed by acid stress (pH 4.0) decreased the stress tolerance of *L. lactis* relative to those subjected to acid stress alone. In addition, pre-cold (4 °C), lactic acid (pH 5.5), and salt (5 % NaCl) stress significantly increased the cell survival rate (Figure 2). Significant differences in cell biomass were observed between pre-cold stress shock cultures and subsequent acid stress cultures.

Figure 2: Survival rate after shock and subsequent stress treatment. Survival of *L. lactis* after pre-adaptation to no stress (N), lactic acid pH 5.5 (acid), 4 °C (cold), 5 % NaCl (osmotic), 1 mM H_2O_2 (peroxide) and 7 % ethanol stress for 2 h and then exposure to pH 4.0 acid stress for 6 h (n = 3)
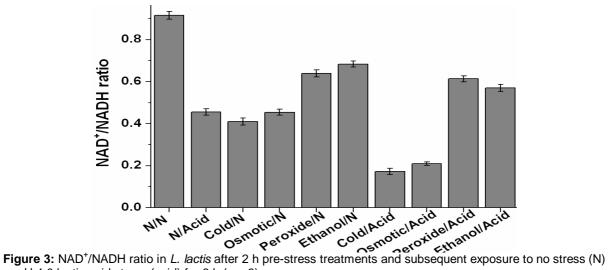
Oxidative performance

Pre-adaptation to cold or osmotic stress alone significantly decreased the ratio of NAD^+/NADH, while exposure to acid stress changed this ratio compared to that of the controls. When *L. lactis* was pre-adapted to cold or osmotic stress and subsequently exposed to acid stress, a significant decrease in the NAD^+/NADH ratio was observed compared to bacterial cultures with no initial stress but subsequent acid stress (Figure 3).

The production of OH· led to a significant decrease in the pre-adaptation capacity of the bacteria to cold and osmotic stress. Exposure to

Figure 3: NAD$^+$/NADH ratio in *L. lactis* after 2 h pre-stress treatments and subsequent exposure to no stress (N) or pH 4.0 lactic acid stress (acid) for 6 h (n = 3)
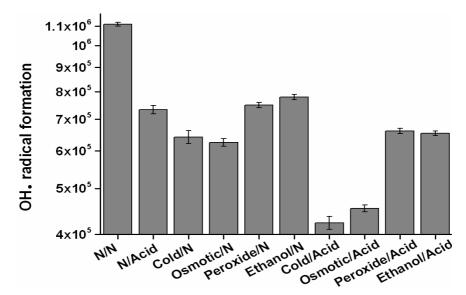

Figure 4: OH· radical ratio in *L. lactis* after 2 h pre-stress treatments and subsequent exposure to no stress (N) or pH 4.0 lactic acid stress (acid) for 6 h (n = 3)

no initial stress and subsequent acid stress significantly changed the amount of OH· produced. When *L. lactis* was pre-adapted to cold or osmotic stress and subsequently exposed to acid stress, OH· production further declined (Figure 4). This indicated that pre-adaptation to cold or osmotic stress induced multiple metabolic cascades that relieved the redox damage caused by acid stress. In addition, these results suggest that oxidative activities in the electron transport chain (ETC) are possible mechanisms for acid adaptation and tolerance in *L. lactis*.

Intracellular amino acid abundance

The amount of intracellular amino acids markedly increased after pre-adaptation to cold stress and subsequent acid stress exposure (Table 1). At pH 3.5, the intracellular amount of cysteine,

phenylalanine, glutamine, aspartate, and threonine was enhanced by 4.12-, 4.01-, 3.34-, 3.11- and 3.01-fold, respectively, relative to the control at pH 3.5, while the amount of proline and methionine was reduced. These results showed that pre-cold stress altered the intracellular amino acid content in a pH-dependent manner. Furthermore, pre-cold stress played an important role in intracellular amino acid homeostasis, an adaptive response to lactic acid stress during fermentation.

DISCUSSION

The effects of pre-cold stress on *L. lactis* NZ9000 resistance to acid stress were evaluated. Pre-adaptation of *L. lactis* to cold stress (storage at 4 °C for 2 h) significantly increased cell viability during subsequent exposure to pH 3.5 acid

Table 1: Amount (nmol/mg protein) of intracellular amino acids in *L. lactis*. Cells were pre-adapted to cold stress (4 °C for 2 h) and then were exposed to pH stress treatments for 6 h

pH condition	pH 5.5		pH 4.5		pH 3.5	
	-Pre-cold	+Pre-cold	-Pre-cold	+Pre-cold	-Pre-cold	+Pre-cold
Asp	882.4	898.8	372.2	451.1	76.6	238.5
Thr	606.1	611.4	191.4	266.1	45.0	135.7
Ser	539.2	569.9	193.8	287.2	106.8	189.2
Asp NH_2	207.8	221.0	68.2	79.2	98.5	120.2
Glu	4317.4	4330.8	2011.1	2576.2	283.9	948.3
Glu NH_2	990.2	1010.1	101.4	111.3	143.4	210.9
Gly	881.4	901.1	471.5	511.4	79.1	156.0
Ala	992.2	998.3	453.9	543.6	82.2	165.4
Val	309.1	311.2	93.8	101.0	52.5	94.1
Met	102.4	99.3	48.2	46.0	11.9	8.3
Ile	149.1	151.1	71.3	78.5	46.2	63.3
Leu	251.7	269.2	113.4	125.2	46.9	99.1
Tyr	89.7	91.2	64.8	75.1	6.8	14.5
Phe	151.6	155.3	81.4	89.2	16.4	66
Orn	241.9	249.2	139.2	167.1	28.9	94.7
Lys	3310.3	3360.4	1911.5	2511.2	302.7	505.6
His	527.7	545.2	219.6	277.3	45.8	142.5
Cys	786.9	815.1	311.7	404.8	139.1	573.2
Arg	3301.2	3313.8	1911.1	2010.5	280.7	819.8
Pro	231.1	224.6	72.4	69.9	19.9	15.3

stress. Therefore, the ATR system may have been maintained in *L. lactis* exposed to pre-cold stress [14]. These results suggest that exposure to cold, lactic acid, and salt conditions diminish the stress tolerance and survival of *L. lactis* under various stress treatments. Significant physiological responses were also induced in response to pre-adaptation, which in turn, protected cells from the adverse effects of further exposure to high-acid conditions.

Pre-adaptation potentially induced the activation of cysteine-, phenylalanine-, glutamate- and arginine-dependent metabolic pathways to maintain intracellular pH homeostasis. Thus, pre-adaptation to cold stress led to the activation of specific metabolic pathways to protect the bacteria from the adverse effects of high-acid conditions and associated secondary effects [15–18]. These results suggest that pre-cold stress may activate intracellular metabolic pathways that enhance stress tolerance and survival of *L. lactis* subjected to subsequent acid stress.

As evidenced by Larsen *et al*, there is a metabolic link between amino acid metabolic pathways and tricarboxylic acid (TCA) cycle [16], via which NADH and succinate are generated. In this study, acid stress exposure caused a decrease in the NAD^+/NADH ratio. However, pre-adaptation to cold stress before acid stress exposure increased this ratio and the formation of OH·, which protected the bacteria from secondary oxidative damage induced by acid exposure [16,19,20]. These results indicate that pre-cold stress directly or indirectly affected the intracellular amino acid content in a pH-dependent manner. Furthermore, pre-cold stress played an important role in intracellular amino acid homeostasis as an adaptive response to lactic acid stress during fermentation.

Metabolic ETC complexes were regulated by different mechanisms in response to pre-adaptation to cold stress and acid stress exposure. Acid stress generally induces the formation of the NADH dehydrogenase complex. Previous studies have shown that metabolic efflux involved in ETC complexes is regulated in *L. lactis* that is pre-adapted to cold stress and subsequently exposed to acid stress. This indicates there are differences in NADH consumption and conversion to NAD^+ in the ETC in response to stressors [21–24], which can be induced by amino acid homeostasis and redox mobility.

CONCLUSION

L. lactis maintains a high level of metabolic efficiency when pre-adapted to cold stress before acid exposure. It also maintains maximum redox balance and amino acid haemostasis when pH is decreased to 3.5. As a result, disruption of redox balance weakens the ability of bacteria to tolerate acid stress. These results also demonstrate that pre-adaptation to cold stress regulates the ATR system in *L. lactis*, resulting in higher viability and acid tolerance in industrial fermentation conditions.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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