Stress adaptation and cross-protection of *Lactobacillus plantarum* KLDS 1.0628

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**ABSTRACT**

Various stresses are commonly encountered by probiotics in their ecological niches. Effects of adaptation treatments on the heat tolerance of *Lactobacillus plantarum* KLDS 1.0628 were evaluated. Cells pre-adapted to heat, cold, oxidative, acid, bile salt, and osmotic stresses were evaluated for tolerance and cross-protection against subsequent challenge. Except for cold, pre-adaptation to sublethal levels of stress significantly improved the cell viability after heat challenge compared to the control. Heat-adapted cells showed the highest heat stress tolerance, with their tolerance factor against heat challenge reaching 31.38-fold after pre-adaptation at 45°C for 1 h. Moreover, adaptation treatments improved tolerance to the same stress and evoked cross-protection against heterologous challenges, inducing a significantly lower ratio of saturated to unsaturated fatty acid content in the cell membrane of *L. plantarum* KLDS 1.0628. The results indicated that adaptation treatments are potential strategies for enhancing the survival rate of probiotics and exerting their functional properties.

**Adaptación al estrés y protección cruzada de *Lactobacillus plantarum* KLDS 1.0628**

**RESUMEN**

Los probióticos suelen estar expuestos a diversos tipos de estrés en sus nichos ecológicos. Este estudio se propuso evaluar los efectos que conllevan distintos tratamientos de adaptación en la tolerancia al calor de *Lactobacillus plantarum* KLDS 1.0628. Para ello se valoraron células preadaptadas al calor, el frío, la oxidación, el ácido, la sal biliar y el estrés osmósmico, con el fin de determinar la tolerancia y la protección cruzada contra el desafío posterior. Se constató que, excepto en el caso del frío, en comparación con el control la preadaptación a niveles subletrales de estrés mejoró significativamente la viabilidad de la célula después del desafío con calor. Tras la preadaptación a 45°C durante 1 h, las células adaptadas al calor mostraron la mayor tolerancia al estrés térmico, registrando un factor de tolerancia contra el desafío de calor que alcanzó 31.38 veces. Además, los tratamientos de adaptación mejoraron la tolerancia al propio estrés y evocaron una protección cruzada contra desafíos heterólogos, induciendo una ratio significativamente menor de contenido de ácidos grasos saturados a insaturados en la membrana celular de *L. plantarum* KLDS 1.0628. En conclusión, los resultados indican que los tratamientos de adaptación son estrategias potenciales para aumentar la tasa de supervivencia de los probióticos y potenciar el ejercicio de sus propiedades funcionales.

**1. Introduction**

*Lactobacillus plantarum* is a Gram-positive and facultative anaerobic lactic acid bacteria (LAB) species that is a commensal microorganism in the human gastrointestinal tract and widely exists in the food environment (Wang et al., 2018). *L. plantarum* not only can tolerate the simulated digestive tract environment but also has good probiotic characteristics (Cao et al., 2019).

Many bacteriocins are synthesized by food-grade *L. plantarum*, which offers the possibility of deliberately manipulating the microbial ecosystem (Cotter et al., 2005). Extensive studies have shown that *L. plantarum* contributes to inhibiting pathogenic bacterial infection, regulating the host immune system, and ameliorating lipid metabolism (Zhao et al., 2018). Hence, *L. plantarum* is widely used as a starter culture and is usually marketed commercially as a probiotic (Papadopoulou et al., 2018; Salar-Bezhadi et al., 2013).

In recent years, there have been numerous studies on the functional properties of probiotic LAB given the growing market of probiotics (Aoudia et al., 2016; Ma, Yu et al., 2020). Nevertheless, probiotics are inevitably subjected to multiple harsh conditions during production, processing, storage, and passage through the gastrointestinal tract (Marcial-Coba et al., 2018). In particular, there are some high-temperature technological processes in the industrial application of *Lactobacillus*, including spray drying, pasteurization, etc. Heat stress is a common difficulty for survival and requires retention of a sufficient amount of viable probiotic LAB amidst these environmental challenges (Chen et al., 2017). Furthermore, heat stress will seriously affect the growth, metabolism and functional properties of probiotics.

To confer health benefits, stress tolerance is a prerequisite for probiotic LAB to survive and maintain high viability throughout processing, storage and consumption (Ma, Xu et al., 2020). Therefore, many recent studies have focused on the development of different approaches to improve the survival of probiotics, such as screening resistant strains, adding extra nutrients, microencapsulation and inducing the cellular stress response. Among these approaches,
adaptive tolerance responses have been observed in many Lactobacillus, which help to ameliorate the adverse effects and improve the viability of the cells in subsequent lethal challenge (Ricciardi et al., 2012). Lactobacillus has multiple defence mechanisms to cope with various stress conditions, including preserving cell energy and changing cell membrane compositions (Papadimitriou et al., 2016). The role of pre-adaptation with sublethal thermal shock in heat-challenged cells has been reported (Kang et al., 2015). Furthermore, previous research also indicated that a variety of adaptations could induce homologous tolerance as well as cross-protection against other types of stress challenges (Papadimitriou et al., 2016).

The adaptive response and cross-protection of Lactobacillus have attracted wide attention in recent years. However, to the best of our knowledge, few studies have examined the stress response and tolerance of L. plantarum to multiple environmental stresses. The aim of the present work was to evaluate the effect of adaptation treatments on the viability of L. plantarum KLDS 1.0628 after heat challenge. The cross-protection of heat-, cold-, H2O2-, acid-, bile salt- and NaCl-adapted cells against stress challenges was evaluated. Additionally, changes in the cell membrane fatty acid composition were examined.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Lactobacillus plantarum KLDS 1.0628 was previously isolated from traditional fermented pickles in Heilongjiang Province, China, and kept at the Key Laboratory of Dairy Science (KLDS), Ministry of Education, Northeast Agricultural University. The strain was identified according to morphological, biochemical and physiological characteristics and then identified by 16S rDNA gene sequencing, followed by BLAST homology searching using the NCBI database (Du et al., 2018). L. plantarum KLDS 1.0628 was stored at –80°C, inoculated (1%, v/v) into De Man, Rogosa, and Sharpe (MRS) broth and then cultured at 37°C for 18–22 h. The activated cells were used in further experiments after three consecutive transfers in sterile MRS broth.

2.2. Growth characteristics of Lactobacillus plantarum KLDS 1.0628 under different stress conditions

One hundred millilitres of fresh MRS broth was inoculated with 1% (v/v) of an overnight culture of L. plantarum KLDS 1.0628 and further incubated at 37°C for 4 h until the optical density at 600 nm reached 0.4–0.5. Cultures were heated to 37 (control), 40, 45, 50, 55, and 60°C for 1 h and then restored to 37°C. For cold stress, the cultures were incubated at 4, 10, 15, 20, and 25°C for 1 h. Furthermore, bacterial cells were harvested by centrifugation at 5000 × g for 10 min at 4°C, washed twice with sterilized phosphate-buffered saline at pH 7.1, and then resuspended in fresh MRS broth supplemented with 0 (control), 0.2, 0.5, 1, 2, and 5 mmol/L H2O2 for 1 h at 37°C. For acid stress, the washed cells were subcultured in fresh MRS broth adjusted to pH values of 2.5, 3, 4, 5, and 6 with 2 M HCl and incubated for 1 h at 37°C. MRS broth supplemented with 0 (control), 1%, 2%, 4%, 6%, and 8% NaCl was prepared to evaluate the osmotic tolerance response of L. plantarum KLDS 1.0628. For bile salt stress, the washed cells were exposed to varying concentrations of oregano bile (0.1%, 0.2%, 0.3%, 0.4%, and 0.5%). Samples (10 mL) were collected every 2 h for 12 h, and changes in optical density were routinely monitored at 600 nm.

2.3. Stress adaptation and challenge assays

After incubation overnight in MRS broth at 37°C, L. plantarum KLDS 1.0628 was inoculated in 100 mL of fresh MRS broth and incubated at 37°C for 4 h until it reached an optical density of 0.4–0.5 at 600 nm. Bacterial cells were harvested by centrifugation (5000 × g, 10 min, 4°C), washed twice with PBS, and then resuspended in the same volume of MRS broth for stress adaptation and stress challenge experiments. The tolerance of L. plantarum KLDS 1.0628 to sublethal levels of heat (45°C, 1 h), cold (15°C, 1 h), oxidative (1 mmol/L, 1 h), acid (pH 4.0, 1 h), bile salt (0.2%, 1 h), and osmotic stresses (2%, 1 h) was evaluated. Cell viability was enumerated after serial dilution of each sample in sterile 0.85% (w/v) sodium chloride solution and spread on MRS agar plates. The cell plates were incubated at 37°C anaerobically for 24–48 h, and the number of colony forming units per millilitre (CFU/mL) was determined. For heat challenge, 1 mL of each of these non-adapted and pre-adapted cells was then transferred to the same volume of MRS broth and subjected to the lethal level of heat challenge (60°C for 1 h). Cells grown in MRS broth at 37°C without any stress treatment were used as controls. Cell viability was also determined after heat challenge.

2.4. Cross-protection assays

Based on the results of preliminary growth tests of L. plantarum KLDS 1.0628 under different conditions, stress adaptations were carried out under the following experimental conditions: 45°C for 1 h, 15°C for 1 h, 1 mmol/L H2O2 for 1 h at 37°C, pH 4.0 (adjusted by 2 M HCl) for 1 h at 37°C, 0.2% bile salt for 1 h at 37°C, 2% NaCl for 1 h at 37°C. Non-adapted cells grown in fresh MRS broth at 37°C for 1 h were used as controls. Then, 1 mL of each of these non-adapted and pre-adapted cells was transferred to the same volume of MRS broth and subjected to the following stress challenge conditions: 60°C for 1 h, –20°C for 14 d, 5 mmol/L H2O2 for 1 h at 37°C, pH 2.5 (adjusted by 2 M HCl) for 1 h at 37°C, 0.5% bile salt for 1 d at 37°C, and 8% NaCl for 1 d at 37°C. The cell viability of L. plantarum KLDS 1.0628 affected by various stress challenges was estimated. The survival rate (%) was calculated by applying Equation (1):

\[
\text{Survival rate} = \frac{\text{The number of viable bacteria after incubation}}{\text{The number of viable bacteria before incubation}} \times 100
\]

(1)

In addition, the tolerance factor against the stress challenge was evaluated as defined in equation (2):

\[
\text{Tolerance factor} = \frac{\text{The survival rate of pre-adapted cells}}{\text{The survival rate of non-adapted cells}}
\]

(2)

2.5. Membrane fatty acid analysis

Cell membrane fatty acids of L. plantarum KLDS 1.0628 were separated and identified as described earlier (Ma, Wang et al., 2020). Samples were analysed using a 6890 N gas chromatograph (Agilent, USA) equipped with an HP-5 elastic quartz capillary column (30 m × 0.25 mm × 0.25 µm, Agilent,
USA). Chromatography was carried out with an injection volume of 1 μL. High-purity helium was used as the carrier gas at a flow rate of 1 mL/min. The column front pressure and interface temperature were controlled at 73.0 kPa and 250°C, respectively. The program temperature rose from 130°C to 230°C. The relative contents of cell membrane fatty acids were expressed as molar percentages (mol %).

2.6. Statistical analysis
All experiments were performed independently in triplicate, and the data were collected. One-way analysis of variance (ANOVA) was performed using SPSS software 17.0, and means were separated by using Tukey’s range test with statistical significance when \( P < .05 \).

3. Results and discussion
3.1. Heat tolerance response of heat-adapted L. plantarum KLDS 1.0628 cells
It was observed that the growth rate of L. plantarum KLDS 1.0628 decreased slightly when the heat treatment temperature was 40 and 45°C (Figure 1a(i)). When the temperature was higher than 50°C, the cell growth rate was significantly inhibited. The viable count of differentially heat-adapted and non-adapted cells of L. plantarum KLDS 1.0628 after heat challenge at 60°C for 1 h is shown in Figure 1(b). The heat adaptations at 40°C and 45°C for 1 h exhibited no significant effect on the viable counts of L. plantarum KLDS 1.0628 compared with that of untreated cells (\( P > .05 \)). The survival rate of L. plantarum KLDS 1.0628 decreased by more than 95% after 1 h of exposure at 55°C (6.73 \( \log_{10} \) CFU/mL) compared with that of non-adapted cells (9.21 \( \log_{10} \) CFU/mL). After heat challenge at 60°C for 1 h, the survival rate of L. plantarum KLDS 1.0628 without heat adaptation was 0.08%. It should be noted that L. plantarum cells subjected to heat adaptation at 45°C for 1 h exhibited higher heat tolerance than other heat adaptation treatments (\( P < .05 \)). In our study, the heat tolerance of L. plantarum 1.0628 could be enhanced by a sublethal level of heat stress, which was similar to the results reported by Kang et al. (2015). Moreover, the results suggested that a heat tolerance response could be induced when exposed to a wide range of sublethal temperatures. This effect is mainly due to heat stress resulting in significant protein denaturation, cell wall, and nucleic acid molecule damage (Salar-Bezhadi et al., 2013). Additionally, LAB can produce a series of heat-induced proteins called heat shock proteins (HSPs) when exposed to high temperatures, which contribute to stabilizing the cytoskeleton and repairing damaged proteins (Sottile & Nadin, 2018).

3.2. Heat tolerance response of cold-adapted L. plantarum KLDS 1.0628 cells
The growth of L. plantarum KLDS 1.0628 during various cold stress treatments was investigated (Figure 2a(i)). When exposed to cold stress, the growth of L. plantarum KLDS 1.0628 was inhibited to different degrees. When the incubation temperature decreased from 37°C to 4°C, the growth rate significantly decreased (\( P < .05 \)), and the lowest growth rate of L. plantarum KLDS 1.0628 was observed when the temperature was 4°C. Based on the results presented in Figure 2(b), the cold adaptation at 4°C for 1 h showed significantly lower viability of L. plantarum KLDS 1.0628 than that of the control (\( P < .05 \)), but no significant difference was observed when cells were subjected to cold stress at 4°C and 15°C for 1 h (\( P > .05 \)). Additionally, after heat challenge at 60°C, the viability of cold-adapted cells did not significantly differ from that of control untreated cells (\( P > .05 \)). Previous studies have also shown that cold adaptation can enhance the stress tolerance of L. plantarum K25, and isobaric tags for relative and absolute quantification proteomic analysis confirmed that this phenomenon is mainly due to changes in stress tolerance gene expression and proteins related to DNA repair, transcription and translation (Liu et al., 2020). Moreover, the two-component system and quorum sensing are also involved in the cell cold-adaptation process.

3.3. Heat tolerance response of oxidative-adapted L. plantarum KLDS 1.0628 cells
The impact of different \( \text{H}_2\text{O}_2 \) concentrations on the growth of L. plantarum KLDS 1.0628 is presented in Figure 3(a). The growth rate of L. plantarum KLDS 1.0628 decreased slowly with increasing \( \text{H}_2\text{O}_2 \) concentration when the concentration of \( \text{H}_2\text{O}_2 \) ranged from 0.2 mmol/L to 1 mmol/L. A detrimental effect on cell growth was also observed when the cultures were treated with 2 or 5 mmol/L \( \text{H}_2\text{O}_2 \). Compared to that of untreated cells, the survival rate of L. plantarum KLDS 1.0628 decreased by 90.10% after 2 mmol/L oxygen adaptation for 1 h (Figure 3(b)). Biological macromolecules such as proteins,
nucleic acids, and lipids may be damaged by ROS, resulting in cell senescence and death (Papadimitriou et al., 2016). Generally, ROS are scavenged to protect cells from oxidative stress through antioxidant defence systems (Zhang et al., 2018). Among all heat-challenged cells, \( L.\) \( \text{plantarum} \) KLDS 1.0628 cells that were pre-treated with 1 mmol/L showed the highest heat tolerance to 60°C for 1 h. However, all of the oxidative-adapted cells suffered a more than 3.05 \( \log_{10} \) CFU/mL decrease in viable counts after heat challenge at 60°C. These results revealed that pre-adaptation to oxygen stress at lower concentrations could enhance the heat tolerance of \( L.\) \( \text{plantarum} \) KLDS 1.0628. This idea was confirmed by Zhang et al. (2020), who found that an adaptive response of \( \text{Lactobacillus} \) to one stress could lead to cross-protection against another stress by changing the physiological characteristics and basic metabolic pathways.

### 3.4. Heat tolerance response of acid-adapted \( L.\) \( \text{plantarum} \) KLDS 1.0628 cells

The growth of \( L.\) \( \text{plantarum} \) KLDS 1.0628 subjected to acid stresses was recorded as shown in Figure 4. There were no remarkable differences in the growth of \( L.\) \( \text{plantarum} \) KLDS 1.0628 when the pH was between 6.0 and the control (6.5) (Figure 4(a)). When the pH was decreased from 6.0 to 5.0 or 4.0, the cell growth of \( L.\) \( \text{plantarum} \) KLDS 1.0628 became slow. The growth rate of \( L.\) \( \text{plantarum} \) cells declined markedly when the initial pH value was adjusted to 3.0, and \( L.\) \( \text{plantarum} \) KLDS 1.0628 incubated at pH 2.5 showed growth arrest. In addition, the pre-adapted cells at different pH values (3.0, 4.0, and 5.0) for 1 h exhibited lower viability than that of untreated cells (Figure 4(b)). After heat challenge at 60°C for 1 h, \( L.\) \( \text{plantarum} \) KLDS 1.0628 pre-adapted to acid (pH 4.0) achieved the highest viable count of 5.69 \( \log_{10} \) CFU/mL. The number of heat-challenged cells at 60°C after pre-adaptation at pH 3.0, 4.0, and 5.0 was significantly higher than that of the control (\( P < .05 \)), suggesting that an adaptive acid tolerance response (ATR) was observed in \( L.\) \( \text{plantarum} \) KLDS 1.0628. The mechanism of LAB against acid stress can be summarized as follows: increasing the activity of proton pump \( H+\text{-ATPase} \) activity, production of alkali (arginine deaminase pathway), the expression of stress protein, and the change of membrane fatty acid composition (Huang et al., 2016), which may have a correlation with the enhancement of heat tolerance of the strain.

### 3.5. Heat tolerance response of bile salt-adapted \( L.\) \( \text{plantarum} \) KLDS 1.0628 cells

The growth of \( L.\) \( \text{plantarum} \) KLDS 1.0628 was not noticeably affected by the addition of 0.1% bile salt within the incubation time (Figure 5(a)). Along with an increased concentration of bile salt, a significant reduction in the growth rate of \( L.\) \( \text{plantarum} \)
KLDS 1.0628 was observed ($P > .05$). Furthermore, L. plantarum KLDS 1.0628 can also survive and grow when the concentration of bile salt was up to 0.5%. Moreover, 0.1% bile salt adaptation for 1 h exhibited no significant difference in the viability of L. plantarum KLDS 1.0628 compared to that of the control ($P < .05$). However, a significant decrease in cell count was detected when pre-adapted for 1 h in the presence of 0.2% or 0.3% bile salt for 1 h (Figure 5(b)). In addition, the 0.1% bile salt stress treatment did little to enhance the heat tolerance of L. plantarum KLDS 1.0628. Remarkably, L. plantarum KLDS 1.0628 pre-adapted to 0.2% bile salt had significantly higher viability than that of other 0.3% bile salt adaptation and control untreated cells after heat challenge at 60°C for 1 h. This phenomenon may be due to the differentially expressed proteins, including those involved in stress response, DNA repair, and peptidoglycan biosynthesis, providing tolerance towards environmental challenges (Kaur et al., 2017).

### 3.6. Heat tolerance response of osmotic-adapted L. plantarum KLDS 1.0628 cells

There was hardly any significant impact on the growth of L. plantarum KLDS 1.0628 when the addition of NaCl was 1% (Figure 6(a)). However, it is obvious that the proliferation of L. plantarum KLDS 1.0628 was inhibited in the presence of 2%, 4%, or 6% NaCl, and L. plantarum KLDS 1.0628 could not grow when exposed to NaCl at a higher concentration of 8% ($P < .05$). There were no significant differences in the viable counts of L. plantarum KLDS 1.0628 between the treatment with low osmotic concentrations (1% and 2%) for 1 h and non-adapted cells (Figure 6(b)). Cells pre-adapted to 4% NaCl osmotic stress for 1 h resulted in a strong prolongation of cell viability, followed by the pre-adaptation of 4% NaCl. Previous research indicated that the downregulation of carbamoyl phosphate synthase in carbohydrate metabolism of L. plantarum induced a bacterial regulation mechanism to save energy (Wang et al., 2016). Nevertheless, the osmotic adaptation of 1% NaCl showed no marked influence on the bacterial number compared to the control.

### 3.7. Effects of cross-protection on the stress tolerance L. plantarum KLDS 1.0628

The survival rates (%) and tolerance factors of non-adapted and pre-adapted cells of L. plantarum KLDS 1.0628 under...
different stress conditions were summarized and are shown in Table 1 and Figure 7, respectively. Except for cold adaptation, the other five pre-adaptation treatments used in this study can all activate the defence mechanism of L. plantarum KLDS 1.0628 against thermal lethal conditions compared with non-adapted cells (Table 1). The highest survival rate was observed in heat-adapted cells (2.51%). Prior bile salt and acid adaptation also significantly improved the survival rate of L. plantarum KLDS 1.0628 cells from 0.08% to 1.63% and 0.99%, respectively (P < .05). The tolerance factors of heat-, bile salt- and acid-adapted cells against heat challenge increased 31.38-, 20.41-, and 12.34-fold, respectively (Figure 7(a)). Pre-exposure to other types of stresses also induced cross-adaptation against heat challenge, which indicates that the adaptive mechanism of lactic acid bacteria under different types of stress has a certain degree of overlap (Zhang et al., 2018). However, homologous pre-adaptation appears to be more effective. Studies have indicated that cell characteristics are altered to cope with heat stress, such as cell morphology, membrane fatty acid composition, surface charge, and adhesion ability (Haddaji et al., 2017).

It was observed that both heat and peroxide adaptation had no effect on cold tolerance (Table 1). Adaptation to cold, acid, bile salt, and osmotic stress resulted in significant cross-protection against lethal levels of cold stress, and the cold tolerance increased 6.19-, 4.73-, and 4.09-fold, respectively (Figure 7(b)). It has been confirmed that cold shock proteins (CSPs) are able to participate in transcription, translation, and protein folding and can bind to single-stranded nucleic acids and break down secondary structures formed at low temperatures, thereby increasing the cold tolerance of cells (Papadimitriou et al., 2016). CSPs can also be induced when exposed to stresses other than cold stress. Our results are in agreement with the report by Streit et al. (2008), who found that pre-adaptation to acid improved the cold tolerance of L. delbrueckii and L. acidophilus subsp. bulgaricus.

After 1 h of exposure to 5 mmol/L H$_2$O$_2$, the survival of acid-adapted cells was the highest (0.51%), followed by osmotic-, heat-, and oxidative-adapted cells, which may be due to the transcription of genes related to oxidative stress tolerance being regulated in response to sublethal stress. Furthermore, it has been revealed that antioxidant-related genes contribute to coping with oxidative stress (Zhao et al., 2018). In addition, the oxidative stress tolerance of L. plantarum KLDS 1.0628 was increased 16.85- and 5.61-fold, respectively, after pre-adaptation to acid and sodium chloride (Figure 7(c)).

The acid-adapted cells displayed a higher survival of 0.10% and acid tolerance (4.79-fold) compared to that of other pre-treatments after exposure to acid challenge. In addition, H$_2$O$_2$, bile salt and NaCl pre-treatment also enhanced the acid tolerance of L. plantarum KLDS 1.0628. However, prior heat and cold

Table 1. The survival rates (%) of non-adapted and pre-adapted cells of L. plantarum after stress conditions.

| Stress adaptation | Stress challenge | Heat (60°C, 1 h) | Cold (~20°C, 14 d) | H$_2$O$_2$ (5 mmol/L, 1 h) | Acids (pH 2.5, 1 h) | Bile salt (0.5%, 1 d) | NaCl (8%, 1 d) |
|-------------------|-----------------|-----------------|-----------------|-------------------------|----------------|-----------------|---------------|
| Blank             |                 | 0.08 ± 0.02 a   | 0.11 ± 0.02 a   | 0.03 ± 0.00 a           | 0.02 ± 0.00 a | 0.09 ± 0.01 a   | 0.06 ± 0.01 a  |
| Heat (45°C, 1 h)  |                 | 2.51 ± 0.00 f   | 0.08 ± 0.02 a   | 0.14 ± 0.01 c           | 0.02 ± 0.00 a | 0.63 ± 0.05 c   | 0.67 ± 0.01 c  |
| Cold (15°C, 1 h)  |                 | 0.11 ± 0.02 a   | 0.68 ± 0.04 c   | 0.05 ± 0.01 b           | 0.02 ± 0.00 a | 0.31 ± 0.02 b   | 0.52 ± 0.04 b  |
| H$_2$O$_2$ (1 mmol/L, 1 h) | | 0.58 ± 0.01 c   | 0.11 ± 0.01 a   | 0.13 ± 0.01 c           | 0.04 ± 0.01 b | 0.29 ± 0.03 b   | 0.56 ± 0.03 b  |
| Acid (pH 4.0, 1 h) |                 | 0.99 ± 0.04 d   | 1.18 ± 0.07 d   | 0.51 ± 0.02 e           | 0.10 ± 0.01 d | 1.79 ± 0.06 e   | 1.56 ± 0.03 d  |
| Bile salt (0.2%, 1 h) |               | 1.64 ± 0.07 e   | 0.52 ± 0.04 b   | 0.05 ± 0.00 b           | 0.06 ± 0.00 c | 2.35 ± 0.09 f   | 1.75 ± 0.05 e  |
| NaCl (2%, 1 h)    |                 | 0.40 ± 0.02 b   | 0.48 ± 0.03 b   | 0.17 ± 0.01 d           | 0.03 ± 0.01 b | 1.13 ± 0.09 d   | 2.19 ± 0.03 f  |

Different lowercase letters (a–f) in the same column denote significant differences (P < .05) between different stress adaptations.

Las distintas letras minúsculas (a–f) en la misma columna denotan diferencias significativas (P < .05) entre las diferentes adaptaciones de estrés.
adaptation did little to enhance the acid tolerance, which is similar to the results reported by Chen et al. (2017) and is different from Kulkarni et al. (2018). The results illustrated that acid adaptation can be used as a potential strategy to improve cell survival in response to multiple lethal stresses. Prior bile salt adaptation of L. plantarum KLDS 1.0628 also contributed to inducing a 2.94-fold increase in acid tolerance (Figure 7(d)).

Bile stress tolerance is important for probiotic LAB to survive in the gastrointestinal tract and confer beneficial impacts on human health. After a 1 d exposure to 0.5% bile salts, the survival of all pre-adapted L. plantarum KLDS 1.0628 was significantly higher than that of the non-adapted control (P > .05) (Table 1). The results observed were comparable with previous studies in which the adaptive response induced by bile salt stress contributed to ameliorating the negative impacts of various lethal challenges (Noriega et al., 2004). As shown in Figure 7(e), the highest bile salt tolerance factor induced by stress adaptation treatments was observed in the following decreasing order: bile salt > acid > osmotic > cold = peroxide. The deconjugation of bile salts by bile salt hydrolases (BSHs) could be an effective molecular mechanism against bile salt stress (Bustos et al., 2018). The crucial role of membrane phospholipid cardiolipin and F1F0-ATPase in bile acid adaptation by LAB is well established (Bi et al., 2016). These results could also be explained by the changes in gene expression in different biological processes, including carbohydrate, amino acid, peptide metabolism, transcription factors, and transmembrane transport (Ma et al., 2018).

LAB often encounter the challenge of high salt concentrations when applied to the processing of foods such as cheese fermentation. Osmotic stress results in intracellular water...
outflow and cytoplasmic separation, causing cells to stop growing or even die (Gandhi & Shah, 2015). Studies have shown that the expression of proteins involved in carbohydrate metabolism, amino acid metabolism, nucleotide metabolism, and ATP-binding cassette (ABC) transporter was changed when exposed to salt stress (Li et al., 2019). To maintain cellular osmotic equilibrium, compatible solutes (osmolytes) such as betaine, carnitine, choline, and proline were accumulated in LAB using self-synthesis or absorption from the external environment (Papadimitriou et al., 2016). It was also shown that all the various pre-adaptations had significantly higher (P < .05) survival rates than those of non-adapted cells (0.06%) after exposure to sodium chloride (8%) for 1 d. Furthermore, the osmotic stress tolerance (36.44-fold) and highest survival rate (2.19%) were obtained following pre-adaptation with sodium chloride, followed by bile salt-, acid-, and heat-adapted cells (Figure 7f).

### 3.8. Cell membrane fatty acid analysis

Gas chromatography was carried out to explore the effect of different stress adaptations on the cell membrane fatty acid composition, including saturated fatty acids (SFAs) and unsaturated fatty acids (USFAs). In this study, myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and cyclopropane fatty acid (cyc C19:0) were the main fatty acids in the cell membrane of *L. plantarum* KLDS 1.0628, accounting for more than 97% of the total cell membrane fatty acids (Figure 8). The results showed that USFAs accounted for 58.63% (control), 61.26% (heat group), 58.38% (cold group), 58.96% (peroxide group), 60.45% (acid group) and 60.11% (bile salt group) and 60.64% (NaCl) group of the total cell membrane fatty acids. Moreover, the ratio of SFA to USFA varied with different stress adaptation treatments. A similar trend was observed for the SFA/USFA ratio of *Lactobacillus casei* under stress conditions (Wu et al., 2012). In general, the ratio of SFA/USFA was lower when *L. plantarum* KLDS 1.0628 was subjected to all types of pre-adaptation treatments. Previous studies have shown that bacterial membrane fluidity is reduced by changing the composition of fatty acids to protect proton flow and maintain pH homeostasis (Huang et al., 2016). Furthermore, the content of cyc C19:0 increased remarkably in all stress adaptation groups compared to that for the control cells, which may be because *L. plantarum* KLDS 1.0628 had the ability to regulate the elasticity, fluidity and flexibility of its cell membrane to resist various environmental stresses (Wang et al., 2018). Before evaluation of their functional properties in a complex food matrix or host with a harsh environment, it is crucial to investigate the stress responses of *L. plantarum* in less complex laboratory media with limited interfering factors. Some reports have shown the physiological response of *L. plantarum* to stress (Aoudia et al., 2016; Hlaing et al., 2018). Moreover, exposure to environmental stresses may alter the physiological characteristics of *L. plantarum*. These results further demonstrated the possible existence of stress response mechanisms regulated by the distribution of SFAs and USFAs in cell membranes.

### 4. Conclusions

The present study suggested that pre-adaptation to sublethal levels of heat, oxidative, acid, bile salt, and osmotic stresses enhances the heat tolerance of *L. plantarum* KLDS 1.0628. Furthermore, different types of adaptation could induce cross-protection of *L. plantarum* KLDS 1.0628 against homologous and heterologous challenges. Exposure to these stresses significantly decreased the SFA/USFA ratio in the cell membrane of *L. plantarum* KLDS 1.0628. The above results indicated that pre-adaptation strategies have the potential to enhance the stress tolerance of *L. plantarum* KLDS 1.0628 against multiple stresses. Further research is needed to clarify the molecular mechanism of the adaptation response and cross-protection of *L. plantarum* KLDS 1.0628 to various stresses.

**Disclosure statement**

The authors declare no conflict of interests.

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**References**

Aoudia, N., Rieu, A., Briandet, R., Deschamps, J., Chluba, J., Jego, G., Garrido, C., & Guzzo, J. (2016). Biofilms of *Lactobacillus plantarum* and *Lactobacillus fermentum*: Effect on stress responses, antagonistic effects on pathogen growth and immunomodulatory properties. *Food Microbiology*, 53(Pt A), 51–59. https://doi.org/10.1016/j.fm.2015.04.009

Bi, J., Liu, S., Du, G., & Chen, J. (2016). Bile salt tolerance of *Lactococcus lactis* is enhanced by expression of bile salt hydrolase thereby producing less bile acid in the cells. *Biotechnology Letters*, 38(4), 659–665. https://doi.org/10.1007/s10529-015-2018-7

Bustos, A. Y., de Valdez, G., Fadda, S., & Taranto, M. P. (2018). New insights into bacterial bile resistance mechanisms: The role of bile salt hydrolase and its impact on human health. *Food Research International*, 112, 250–262. https://doi.org/10.1016/j.foodres.2018.06.035

Cao, P., Wu, L., Wu, Z., Pan, D., Zeng, X., Guo, Y., & Lian, L. (2019). Effects of oligosaccharides on the fermentation properties of *Lactobacillus*
Ma, K., Wang, G., Zhai, Z., Zhou, P., & Hao, Y. (2018). Global transcriptional analysis and function identification of malolactic enzyme pathway of Lactobacillus paracasei L9 in response to bile stress. Frontiers in Microbiology, 9, 1978. https://doi.org/10.3389/fmicb.2018.01978

Marcial-Coba, M. S., Cieplak, T., Cahu, T. B., Blenow, A., Knochel, S., & Nielsen, D. S. (2018). Viability of microencapsulated Akkermansia muciniphila and Lactobacillus plantarum during freeze-drying, storage and in vitro simulated upper gastrointestinal tract passage. Food & Function, 9(11), 5868–5879. https://doi.org/10.1039/c8fo01331d

Noriega, L., Gueimonde, M., Sanchez, B., Margolles, A., & de Los Reyes-Gavilan, C. G. (2004). Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross-resistance to bile salts in Bifidobacterium. International Journal of Food Microbiology, 94(1), 79–86. https://doi.org/10.1016/j.ijfoodmicro.2004.01.003

Papadimitriou, K., Alejria, A., Bron, P. A., de Angelis, M., Gobbetti, M., Kleerebezem, M., & Kok, J. (2016). Stress physiology of lactic acid bacteria. Microbiology and Molecular Biology Reviews, 80(3), 837–890. https://doi.org/10.1128/MMBR.00076-15

Papadopoulou, O. S., Argyri, A. A., Varzakis, E. E., Tassou, C. C., & Chorianopoulos, N. G. (2018). Greek functional Feta cheese: Enhancing quality and safety using a Lactobacillus plantarum strain with probiotic potential. Food Microbiology, 74, 21–33. https://doi.org/10.1016/j.fm.2018.02.005

Riccioni, A., Parente, F., Guidone, A., Iannelli, R. G., Zotta, T., Abu Sayem, S. M., & Varcamonti, M. (2012). Genotypic diversity of stress response in Lactobacillus plantarum, Lactobacillus paraplantarum and Lactobacillus pentosus. International Journal of Food Microbiology, 157(2), 278–285. https://doi.org/10.1016/j.ijfoodmicro.2012.05.018

Salar-Behzadi, S., Wu, S., Toegel, S., Hofrichter, M., Altenburger, I., Unger, F. M., Wirth, M., & Viernstein, H. (2013). Impact of heat treatment and spray drying on cellular properties and culturability of Bifidobacterium bifidum BB-12. Food Research International, 54(1), 93–101. https://doi.org/10.1016/j.foodres.2013.05.024

Sotille, M. L., & Nadin, S. B. (2018). Heat shock proteins and DNA repair mechanisms: An updated overview. Cell Stress and Chaperones, 23(3), 303–315. https://doi.org/10.1007/s12921-017-0843-4

Streit, F., Delettre, J., Corrieu, G., & Beal, C. (2008). Acid adaptation of Lactobacillus delbrueckii subsp. bulgaricus induces physiological responses at membrane and cytosolic levels that improves cryotolerance. Journal of Applied Microbiology, 105(4), 1071–1080. https://doi.org/10.1111/j.1365-2672.2008.03848.x

Wang, P., Wu, Z., Wu, J., Pan, D., Zeng, X., & Cheng, K. (2016). Effects of salt stress on carbohydrate metabolism of Lactobacillus plantarum ATCC 14917. Current Microbiology, 73(4), 491–497. https://doi.org/10.1007/s00284-016-1087-8

Wang, W., He, J., Pan, D., Wu, Z., Guo, Y., Zeng, X., Lian, L., & Nychas, G.-J. (2018). Metabolomics analysis of Lactobacillus plantarum ATCC 14917 adhesion activity under initial acid and alkaline stress. PLoS ONE, 13(5), e0196231. https://doi.org/10.1371/journal.pone.0196231

Wu, C., Zhang, J., Wang, M., Du, G., & Chen, J. (2012). Lactobacillus casei combats acid stress by maintaining cell membrane functionality. Journal of Industrial Microbiology & Biotechnology, 39(7), 1031–1039. https://doi.org/10.1007/s10295-012-1104-2

Zhang, C., Lu, J., Yang, D., Chen, X., Huang, Y., & Gu, R. (2018). Stress influenced the aerotolerance of Lactobacillus rhamnosus Hsrmy 1301. Biotechnology Letters, 40(4), 729–735. https://doi.org/10.1007/s10529-018-2523-6

Zhang, H., Wang, Q., Liu, H., Kong, B., & Chen, Q. (2020). In vitro growth performance, antioxidative activity and cell surface physiological characteristics of Pedicoccus pentosaceus R1 and Lactobacillus fermentum R6 stressed at different NaCl concentrations. Food & Function, 11 (7), 6376–6386. https://doi.org/10.1039/c9fo02309g

Zhao, J., Tian, F., Yan, S., Zhai, Q., Zhang, H., & Chen, W. (2018). Lactobacillus plantarum CCFM10 alleviating oxidative stress and restoring the gut microbiota in d-galactose-induced aging mice. Food & Function, 9(2), 917–924. https://doi.org/10.1039/c7fo01574g