Short communication: Protection of lyophilized milk starter *Lactobacillus casei* Zhang by glutathione

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ABSTRACT

Lyophilization is considered an effective way to preserve the activity of milk starters, such as lactic acid bacteria, in which proper protective agents play key roles. In this study, *Lactobacillus casei* Zhang, a probiotic bacterium applied as a milk starter in China, was used to investigate the effects of various cryoprotectants according to cell survival rate and physiological characteristics. The result showed a significant survival improvement to 86.6% when glutathione (GSH) was added as an ideal cryoprotectant. Further study revealed that GSH plays a key role on maintaining higher unsaturation ratio of cell membrane and shorter chain length of saturated fatty acids. In this case, the intact cell structure can be obtained. These findings will contribute not only to deepen the understanding of cells during lyophilization but also to improve the industrial performance of certain milk starters such as *L. casei* Zhang by application of GSH as cryoprotectant.

Key words: lyophilization, cryoprotectant, glutathione, cell structure

Short Communication

The common definition of a probiotic is “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Fuller, 1989). As a well-known probiotic, lactic acid bacteria (LAB) play key roles as traditional dairy starters in the fermented food production, such as cheese and yogurt, and are recognized to be beneficial for health (Calcott, 1986; Carvalho et al., 2003a). The efficiency of LAB as starters for industrial application depends on their viability after long-term storage and delivery (Palmfeldt and Hahn-Hagerdal, 2000). Drying, especially lyophili-

zation, is widely applied, but lyophilization will damage cells, which leads to a decrease of viability throughout the dehydration and storage (Costa et al., 2000). To minimize the cell death, many studies have focused on factors affecting the cryopreservation. Among these factors, cell tolerance greatly depends on the composition of cryoprotective additives before lyophilization. As far as we know, skim milk has been used as an efficient carrier, and a recipe with other cryoprotectants such as monosaccharides, polysaccharides, and polyalcohols can be used to enhance the protection of lyophilized cells (Lamprech and Foster, 1963; Chavarri et al., 1988; Teixeira et al., 1995; Andersen et al., 1999; Carvalho et al., 2003a; Fonseca et al., 2003).

Recently, more attention is turning to the physiological changes of cells taking place during lyophilization. Oxygen is related to viability loss in lyophilized cells by attacking certain cellular components, such as lipids, proteins, DNA, and RNA (Miller and Britigan, 1997). In this case, viability loss is supposed to be a meaningful clue to improve cell activity during lyophilization by enhancing their oxidative resistance. Glutathione (GSH) is a low-molecular-weight thiol compound that plays multiple physiological roles in living organisms to improve their resistances to acid stress (Ricciolo et al., 2000), osmotic stress (Smirnova et al., 2001), and toxic electrophiles (Fu et al., 2006). Besides all of these functions, its outstanding ability to maintain a redox balance (Carmel-Harel and Storz, 2000) inspired us to investigate whether it could improve the survival rate of cells during lyophilization. In our study, *Lactobacillus casei* Zhang, a novel LAB strain isolated from traditional koumiss from Inner Mongolia in China with numerous beneficial functions (Xu et al., 2006; Wang et al., 2008; Ya et al., 2008; Wu et al., 2009), was used as the starter bacteria for lyophilization, and the effect of GSH on releasing the damage caused by lyophilization as well as the protective mechanism were investigated further.

The original cultures of *L. casei* Zhang were stored in 30% (vol/vol) aqueous glycerol at -80°C . Stock cul-

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tures were subcultured twice in de Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) for 12 h at 37°C before use. Subsequently, *L. casei* Zhang was inoculated at the level of 1% (vol/vol) in MRS broth, and again incubated at 37°C for 24 h. To prepare the cell culture for lyophilization, cells were harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C, and washed twice with sterile physiological saline solution (0.85% NaCl, wt/vol). Washed cells were then resuspended in the equivalent volume of the cryoprotective medium. Viable counts of cells, the number of colony forming units, were determined by pour plating each suspension with MRS agar medium and enumerating them after 48 h of incubation at 37°C. The experiments were repeated 3 times as prelyophilized data.

Glucose, sucrose, maltose, trehalose, sorbitol, nonfat milk, glycerin, and mannitol with different concentrations were compared singly or orthogonally to confirm the optimized basic cryoprotective medium. After obtaining the optimized basic cryoprotectants, the following components were added to compare their effects during lyophilization: GSH (0.5%, wt/vol; Sangon, Shanghai, China); GSH (1.0%, wt/vol); vitamin C (1.0%, wt/vol; Sangon); vitamin C (2.0%, wt/vol); GSH (0.5%, wt/vol) + vitamin C (1.0%, wt/vol); GSH (0.5%, wt/vol) + vitamin C (2.0%, wt/vol); GSH (1.0%, wt/vol) + vitamin C (1.0%, wt/vol); GSH (1.0%, wt/vol) + vitamin C (2.0%, wt/vol). The reconstituted cryoprotective medium was autoclaved at 115°C for 10 min and the additives such as GSH and vitamin C were sterilized by filtration.

For lyophilization treatments, each cellular suspension was transferred into a sterilized vial with a concentration of 10^7 to 10^8 cfu/mL, frozen at -80°C for 12 h, and desiccated under vacuum (6.67 Pa) for 30 h in a freeze-drier (Freeze mobile 125 L, Virtis Co. Inc., Gardiner, NY) at room temperature (20°C); the condenser was cooled at -55°C. To evaluate the effect of lyophilization on cell viability of *L. casei* Zhang, the lyophilized samples were withdrawn at room temperature and rehydrated to the original volume with sterile saline solution for 30 min. Afterward, they were serially diluted and drop plated in duplicate on MRS agar medium in 3 replicates. Plates were examined after incubation at 37°C for 48 h. The survival rate was calculated as the ratio between the total number of viable cells before and after lyophilization by the standard plate count method in 3 replicates. Percent survival was calculated as $N/N_0 \times 100$, where N_0 and N represented the number of viable cells before and after lyophilization, respectively.

The water activities of lyophilized samples were measured at room temperature using the Aqualab CX-2 water activity instrument (Decagon Devices Inc.,

Pullman, WA). For fatty acid (FA) extraction and analysis, bacterial lipids were extracted and FAME were prepared (Sasser, 1990). Subsequently, the analysis of FAME was performed (Miller and Berger, 1985). Based on these data, the degree of unsaturation [molar % UFA/molar % SFA (U/S ratio)] and the mean chain lengths were assayed. All experiments were carried out in triplicate.

Preparation for transmission electron microscopy samples was carried out with the classical method at room temperature (Bury et al., 2001). With the stain of uranyl acetate and lead citrate, samples were observed in a JEM 1200 EXII Electron Microscope (Jeol, Tokyo, Japan).

The Student's *t*-test was used to investigate statistical differences. All the experiments were performed in triplicate. Samples with $P \leq 0.05$ were considered statistically different. All statistical analyses were performed with Simplot software package (Systat Software, San Jose, CA).

To investigate the effect of cryoprotective additives on the survival of cells, lyophilization was processed at the same conditions, including cell concentration, growth phase, and rehydration medium. As shown in Figure 1, the viabilities of cells after lyophilization were remarkably different depending on the composition of protective agents. The protective function of nonfat milk showed a survival rate of 20.5% after lyophilization, which was more significant than other normal cryoprotectants such as maltose, trehalose, and glycerin, and even 4.8-fold higher than water.

The data of orthogonal experiment supplied an improved prescription of cryoprotectants (Table 1). Based on the pre-experiment data (data not shown), proper concentrations of cryoprotective additives were selected, and the optimized formula was obtained with a survival rate of 54.5%, which was remarkably higher than single factor.

After the regular formula optimization with commercial feasibility, further study was carried out to enhance the cryoprotectant efficiency based on related mechanism. Cells might encounter oxidative stress during lyophilization. In this case, we investigated the role of GSH to fight against a simultaneous oxidative challenge. Meanwhile, due to its complementary function with vitamin C under oxidative stress (Vatassery et al., 1989; Meister, 1994), the effect of multiple cryoprotectants with vitamin C was analyzed during lyophilization.

Compared with the control obtained, the survival rate of cells in the improved medium containing GSH and vitamin C were much higher. Figure 2A showed that the cell viability increased 15.8% when the concentration of GSH ranged from 0 to 1.0% (wt/vol), and the influence

Figure 1. The effect of basic cryoprotectants on the survival rate of lyophilized cells. Error bars indicate standard deviations (n = 3); NFM = nonfat milk.

of vitamin C with different concentrations was verified to be less beneficial than GSH. Moreover, a higher survival rate was observed when GSH and vitamin C were used together. The most efficient cryoprotectant was a mixture of GSH (1.0%, wt/vol) and vitamin C (1.0%, wt/vol) with the optimized basic medium, yielding a survival rate of 86.8%, which was 20.1 fold higher than in water initially (Figure 2B). Meanwhile, as the water activity has a measurable effect on the storage stability of lyophilized cells (Santivarangkna et al., 2006; Tymczynsyn et al., 2008), it was interesting to find that GSH maintained lower water activity values of samples below 0.1, which benefits their consequent application. To evaluate the performance of lyophilized cells during storage and application, their survival stability was investigated at 4°C as well as 37°C. Cells protected with cryoprotectants containing GSH and vitamin C

had a survival rate of 37.3% at 4°C for 90 d, whereas the survival of cells protected by the basic optimized cryoprotectant (control) and water was 3.06 and 0%, respectively (Figure 3A). Although the mortality of lyophilized cells increased sharply during the storage at 37°C, the protection superiority of GSH and vitamin C was demonstrated by a survival rate of 54.6% after 30 d of treatment, which was 4.1 fold higher than cells protected by basic cryoprotectants. The cell membrane is considered as a key issue to evaluate the cell microenvironment, which also reflects the viability of cells under cold stress (Sajbidor, 1997). Figure 4 demonstrates the alterations of cell membrane FA protected by different cryoprotective media after lyophilization. A total of 9 FA, including tetradecanoic acid (C14:0), pentadecanoic acid (C15:0), hexadecanoic acid (C16:0), heptadecanoic acid (C17:0), octadecanoic acid (C18:0), *cis*-9-octadecenoic acid (C18:1-9), *cis*-11-octadecenoic acid (C18:1-11), 9,12-octadecadienoic acid (C18:2), and cyclopropane (C19:0-9), were determined. In detail, the proportion of membrane SFA of control cells increased from 45.1 to 72.2% (Figure 4A), whereas the proportion of UFA, including C18:1n-7, C18:1n-9, C18:2, and C19:0-9, markedly decreased from 54.9 to 27.8% (Figure 4B). This result further clarifies the lipid oxidation and is a possible reason for cell death during lyophilization. Compared with the control, GSH increased the U/S ratio to 0.79 (Table 2). The 2.02-fold higher U/S ratio indicated that GSH may protect cells by increasing the membrane fluidity under cold stress. Meanwhile, as another factor affecting membrane fluidity, the average chain length of SFA decreased significantly from 17.1 to 16.0 when GSH was applied as the cryoprotectant, which also benefits to the survival of cells under cold stress. Moreover, we found that the proportion of cyclopropane fatty acid C19:0-cyc increased from 0 to 0.7 when GSH was added. As the dihydrosterculic acid (ΔC19:0-9) is reported to be responsible for the elastic-

Table 1. Optimization of basic cryoprotectants¹

Sample (%)	Sucrose	Maltose	Trehalose	Sorbitol	NFM ²	Glycerin	Mannitol	Survival
1	5	5	5	5	10	1	2	51.3 ± 4.1
2	5	5	5	10	20	2	4	47.6 ± 12.9
3	5	10	10	5	10	2	4	52.2 ± 15.3
4	5	10	10	10	20	1	2	44.9 ± 8.3
5	10	5	10	5	20	1	4	46.9 ± 4.2
6	10	5	10	10	10	2	2	49.9 ± 5.6
7	10	10	5	5	20	2	2	54.5 ± 9.2
8	10	10	5	10	10	1	4	49.2 ± 2.6
Control	0	0	0	0	0	0	0	4.3 ± 0.5

¹Data were expressed as mean ± SD, n = 3.
²NFM = nonfat milk.

Figure 2. Survival rate and water activity of lyophilized cells during lyophilization in the presence of different cryoprotectants. (A) The effect of cryoprotectants with single antioxidant and (B) the effect of cryoprotectants with mixed antioxidants. Cells in the optimized basic medium were compared as the control samples. GSH = glutathione; Vc = vitamin C. The bar chart presents the survival rate of each sample, and the line chart refers to the data of water activity. Error bars indicate standard deviations ($n = 3$).

ity and the flexibility of the membrane (Smittle et al., 1974), the increased cyclopropane fatty acid C19:0-cyc may participate in the modulation of membrane fluidity based on its function of double bonds and circular structure (Grogan and Cronan, 1997).

To investigate whether GSH plays a protective role on integrity of cells during lyophilization, cell structure of *L. casei* Zhang was observed by transmission electron micrographs. As shown in Figure 5, cells protected by GSH maintained intact cell structure after lyophila-

Figure 3. Storage stability of cells after lyophilization. (A) Survival rate of lyophilized cells during the later storage at 4°C and (B) survival rate of lyophilized cells during the later storage at 37°C. ▲ = cells protected by optimized cryoprotectants with 1% glutathione (GSH) and 1% vitamin C (Vc); ■ = cells protected by optimized basic medium; ◆ = cells lyophilized with water. Error bars indicate standard deviations ($n = 3$).

Table 2. Composition and the chain length of membrane fatty acids (FA) with lyophilization treatment¹

Item	Composition of membrane FA (mol%)										Average chain length	
	14:0	15:0	16:0	17:0	18:0	18:1w7c	18:1w9c	18:2	Cyc-19	FA-to-SFA ratio	Saturated	Unsaturated
Before ²	0.41 ± 0.07	1.63 ± 0.25	10.62 ± 0.81	ND	3.58 ± 0.52	10.76 ± 0.16	5.39 ± 0.44	1.85 ± 0.60	ND	0.82 ± 0.16	16.23 ± 0.03	18.00 ± 0.02
Control	0.08 ± 0.02	0.08 ± 0.01	6.66 ± 0.04	0.23 ± 0.01	10.07 ± 0.01	9.31 ± 1.04	6.67 ± 0.50	1.99 ± 0.57	ND	0.39 ± 0.02	17.12 ± 0.01	18.00 ± 0.01
GSH ³ (1.0%)	2.56 ± 0.32	0.06 ± 0.01	10.23 ± 0.16	0.23 ± 0.01	3.00 ± 0.24	9.82 ± 0.13	4.74 ± 0.16	2.78 ± 0.06	0.70 ± 0.25	0.79 ± 0.03	15.97 ± 0.07	18.04 ± 0.01
GSH (1.0%)/Vc ³ (1.0%)	2.46 ± 0.06	0.17 ± 0.00	9.91 ± 0.09	0.29 ± 0.01	3.18 ± 0.02	10.04 ± 0.15	4.90 ± 0.00	2.51 ± 0.13	0.59 ± 0.02	0.75 ± 0.01	16.01 ± 0.01	18.03 ± 0.02

¹Data were expressed as mean ± standard deviation, n = 3.
²Cells in the optimized basic medium before and after the lyophilization treatment were used as the before and control sample, respectively.
³GSH = glutathione; Vc = vitamin C. The GSH and Vc samples refer to cells in the optimized basic medium with GSH and Vc of certain concentrations after the lyophilization treatment.

tion, whereas the control cells appeared to be cracked with effused cytoplasm, which confirms the protective role of GSH in this process.

In conclusion, in this study we optimized a basic cryoprotectant formula with commercial operability, and confirmed that GSH can enhance the survival rate of cells in lyophilization significantly. Further study revealed that GSH helps cells to maintain a prolonged shelf life during storage at both 4 and 37°C with low water activity. Interestingly, we found that the lyophilization induces changes in the cellular lipid-containing structure, which varied significantly in both the saturated and unsaturated FA. The results demonstrated

Figure 4. Distribution of membrane SFA (A) and UFA (B) during the lyophilization process. Cells in the optimized basic medium before and after the lyophilization treatment were used as the before and control sample, respectively. The glutathione (GSH) and vitamin C (Vc) samples refer to cells in the optimized basic medium with GSH and Vc of certain concentrations after the lyophilization treatment. Error bars indicate standard deviations (n = 3).

Figure 5. Morphological changes of *Lactobacillus casei* Zhang after lyophilization. Cells were suspended in different cryoprotective medium before lyophilization. After the lyophilization, cells were inspected in different magnifications. (A and C) Cells protected by optimized cryoprotectants with 1.0% glutathione; (B and D) control cells in optimized basic cryoprotectants.

that GSH protects lyophilized cells by reducing the oxidation of membrane FA with increased U/S ratio of cell membrane, and reduce the average length of SFA significantly, which also improved the membrane fluidity during lyophilization. As the exchange between oxidation and reduction status reflects the effect of the induced oxidative stress during lyophilization, the role of GSH to reduce the lipid peroxidation in this case remarkably prevents the membrane disorganization (Nakano and Asada, 1981; Dalton et al., 1986; Dolai et al., 2009) and prompts cells to adapt lyophilization ef-

fectively. Results presented in our study indicated that GSH, which has already been approved for use in food, can be applied as an ideal cryoprotectant especially for the preparation of probiotic direct vat set milk starter with high viability because of its particular physiological functions.

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