Freeze-drying is one of the most commonly used methods of bacteria preservation. During this process, cryoprotectants can greatly reduce cellular damage. Micromolecular cryoprotectants have been widely adopted but have limited selectivity and protective effects. Therefore, explorations of other types of cryoprotectants are needed. This study aimed to explore the possibility of the macromolecular cryoprotectants and combinations of cryoprotectants to maintain bacterial activity. We found that the survival rate of Lactiplantibacillus plantarum AR113 after freeze-drying was 19% higher in the presence of soy polysaccharides than with trehalose, the best-performing micromolecular cryoprotectant. Moreover, a 90.52% survival rate of L. plantarum WCFS1 was achieved using the composite cryoprotectant containing soy polysaccharide and trehalose, which increased by 31.48 and 36.47% compared with adding solely trehalose or soy polysaccharide, respectively. These results demonstrate that macromolecular and micromolecular cryoprotectants have similar effects, and that combinations of macromolecular and micromolecular cryoprotectants have better protective effects. We further observed that the composite cryoprotectant can increase Lactobacilli survival by improving cell membrane integrity and lactate dehydrogenase activity. Our finding provides a new type of cryoprotectant that is safer and more effective, which can be extensively applied in the relevant food industry.

Key words: freeze-drying, macromolecular cryoprotectants, Lactiplantibacillus plantarum

INTRODUCTION

Lactic acid bacteria (LAB) are a class of anaerobic, gram-positive, non-spore-forming food-grade bacteria that are used frequently in the production of healthy foods (de Vos et al., 2004; de Vries et al., 2006). Lactiplantibacillus plantarum is a versatile LAB species that is used safely to produce many dairy, meat, and vegetable fermentation products, but it can also be used to combat pathogens, inhibit the proliferation of harmful bacteria, protect the intestinal tract, and strengthen immunity (Holzapfel et al., 2001; de Vries et al., 2006; Wang et al., 2009; Kerry et al., 2018). To perform these functions, regular consumption of a sufficient number of viable strains is a prerequisite (Mattila-Sandholm et al., 2002). To maintain the high viability during processing, storage, and delivering, the major dosage forms for oral administration are made in powders, tablets, and capsules. Lactiplantibacillus plantarum is grown in liquid medium, and liquid formulations are bulky, inconvenient to transport, and exhibit rapid reductions in viability. Therefore, drying is often used to preserve LAB viability. Of the various drying techniques, freeze-drying is the most common and effective method to maintain the stability of the bacteria during industrial manufacturing (Kandil and El Soda, 2015).

Freeze-drying is divided mainly into 3 processes: freezing, sublimation, and desorption. Ice and bound water molecules are removed through the processes of sublimation and desorption, respectively (Velly et al., 2015; Cui et al., 2018). During freeze-drying process, however, the bacterial cells will be inevitably damaged by freezing and drying. Damage to bacteria caused by freeze-drying can be attributed to 2 major causes: changes in the physical conditions of membrane lipids, resulting in a loss of bacterial membrane integrity, and the inactivation of sensitive proteins, bringing about a loss of key enzyme activities (Ananta et al., 2005; Cui et al., 2018). To avert these deleterious effects dur-
ing freeze-drying, many factors have been considered, including cryoprotectants, the freeze-drying speed and temperature, and the grow medium (Martos et al., 2007; Kandylis, 2010; Kandylis et al., 2014; Dimitrellou et al., 2016).

Cryoprotectants used to maintain high viability (Carvalho et al., 2004; Martos et al., 2007; Kandylis, 2010; Lee et al., 2016). General cryoprotectants include monosaccharides or disaccharides (e.g., trehalose, sucrose), polyols (e.g., sorbitol, mannitol, glycerol), and skim milk (Yang et al., 2006; Cui et al., 2018). Lee et al. (2016) reported that when L. plantarum JH287 was suspended in sucrose, the viability increased to 64.57% relative to the control. However, Zayed and Roos, (2004) observed that the highest viability of Lactiplantibacillus salivarius with sucrose was 13%. The effectiveness of the above-mentioned protective agents varies among strains, and the potential inefficiency of these cryoprotectants remains an urgent problem that requires a solution. In addition, the generally used concentration of these protective agents is 5 to 30% (Lee et al., 2016). As health awareness increases, consumers are eager to avoid sugars such as mannitol and trehalose, so the selectivity of cryoprotectants should be continuously expanded. Therein, in the past, a few researchers tried to use polyethylene glycol and bovine albumin with different properties from micromolecules to increase the survival of LAB (de Valdez et al., 1983); However, these macromolecules were inefficient, presented safety risks, and were more expensive than micromolecular cryoprotectants, and were therefore difficult to apply.

Soy polysaccharides are a natural functionally active ingredient with good antioxidant activity and stability that can be directly extracted from the residual by-product of isolated soybean protein (Maeda and Nakamura, 2009). Besides, due to soy polysaccharides beneficial to humans, its application in food formulations has become a hot topic in the field of food colloids (Tang, 2019). However, few researchers have attempted to use polysaccharides as cryoprotectants. Another approach involves the use complex protective agents. A growing body of research has addressed the attempts to use complex protective agents to improve bacterial survival rates (Lu et al., 2017; Cui et al., 2018; Chen et al., 2019). One composite cryoprotectant containing 25% skim milk powder, 5.5% glycine, 0.8% sodium bicarbonate, 7% xylo-oligosaccharides, and 4.5% arginine was shown to improve the survival rate of Bifidobacterium bifidum to 90.37% (Chen et al., 2019). Chun et al. (2012) reported that a composite cryoprotectant containing 28% skim milk powder, 24% lactose, and 4.8% sodium ascorbate yielded survival rates as high as 64.41% for Lactobacillus delbrueckii ssp. bulgaricus. These findings demonstrate that combinations of several protectants had better effects on the survival rates of Lactobacillus spp. when compared with single protectants. The existing complex protective agents mainly consist of small molecules with similar properties and therefore were subject to similar deficiencies as those of small molecules (Chen et al., 2019; Wang et al., 2019).

Therefore, this research was conducted to explore the effect of macromolecular cryoprotectants on the survival rate of L. plantarum during lyophilization and to identify the optimal combination of macromolecular and micromolecular cryoprotectants that would maintain the survival rate. Simultaneously, the protective mechanism of these cryoprotectants during lyophilization were investigated.

**MATERIALS AND METHODS**

**Microorganisms and Sample Preparations**

The L. plantarum strains AR113 and AR307 used in these experiments were provided by the Shanghai Engineering Research Center of Food Microbiology, University of Shanghai for Science and Technology (Shanghai, China). Lactiplantibacillus plantarum WCFS1 has been used extensively as a model strain. All of the strains were cultured in 50 mL of MRS broth and incubated at 37°C for 12 h. After culture, the bacterial cells were harvested by centrifugation at 7,378 x g for 5 min at 25°C (OD600 = 1) and washed twice in PBS (pH 6.5). The bacterial mass was collected from prepared and centrifuged aliquots (1.5 mL) of bacterial suspension.

**Preparation of Cryoprotectants**

Trehalose (99% purity), sorbitol (98% purity), mannose (99% purity), and mannitol (99% purity) were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Water-soluble soy polysaccharides, which had been extracted and refined from soybean, were provided by Pingdingshan JinJing Biological Technology Co. Ltd. (Anhui, China). These soy polysaccharides comprised 75% dietary fibers, but did not contain any starch or sugar. Eight types of protective media were used to optimize the bacterial cell survival rate, and each contained different concentrations of sugar or sugar alcohol: 10% PBS, 10% trehalose, 10% sorbitol, 10% mannose, 10% mannitol, 10% sucrose, 1% and 10% soy polysaccharides, and 1% gum arabic (wt/vol). The following complex protective agents were also added to bacterial suspensions: 1% soy polysaccharides and 10% mannitol; 1% soy polysaccharides and 10% sucrose; 1% soy polysaccharides and 10% trehalose; 1%
soy polysaccharides, 10% mannitol, and 10% sucrose; 1% soy polysaccharides, 10% mannitol and 10% trehalose; and 1% soy polysaccharides, 10% mannitol, 10% trehalose, and 10% sucrose. The bacterial masses were resuspended in sterilized cryoprotectants. Each suspension (~10^8 cfu/mL) was prepared and loaded into a penicillin bottle (5 mL).

**Freeze-Drying Process**

Cryotubes containing bacterial suspensions were desiccated in a CryoMed controlled-rate freezer (Kryo360–1.7, Planer PLC, Sunbury-on-Thames, UK) with a precooling temperature of −40°C for 3 h. Primary drying was carried out for 13 h at a shelf temperature of −30°C and under the maximum vacuum level (20 Pa) available with this system. Afterward, the shelf temperature was increased to 25°C within 1 h and was kept at the above vacuum level for 20 h. Once the freeze-drying procedure was finished, the cryotube was sealed with rubber plugs before transfer.

**Survival Rate Determination**

The number of viable bacteria at 3 stages, namely, before freezing, after frozen, and after freeze-drying, were measured using the plate-counting method. The dried bacterial powder was thawed in 0.9 mL of sterile saline (9% NaCl) and continuously diluted to 10^−5 in sterile saline by decimal dilutions. The diluted samples (10 μL) were inoculated on MRS agar plates and then cultured in a 37°C incubator. The cell counts were measured after 48 h of anaerobic culture at 37°C. The survival rates of *L. plantarum* during freeze-drying, after being frozen, and during drying under the effect of the cryoprotectant were calculated using the following equations:

\[
\text{Freeze-drying survival rate (\%) = \frac{N_2}{N_0} \times 100\%}, \quad [1]
\]

\[
\text{Frozen survival rate (\%) = \frac{N_1}{N_0} \times 100\%}, \quad [2]
\]

\[
\text{Drying survival rate (\%) = \frac{N_2}{N_0} \times 100\%}, \quad [3]
\]

where \(N_0\) and \(N_1\) represent the numbers of living cells before and after the freezing process, respectively, and \(N_2\) is the number of living cells after freeze-drying.

**Determination of Cell Membrane Properties**

The cell membrane integrities of *L. plantarum* AR113, AR307, and WCFS1 before and after freeze-drying were determined using the green-fluorescent nucleic acid stain carboxyfluorescein diacetate and the red-fluorescent nucleic acid stain propidium iodide. Each lyophilized sample was washed once and resuspended in 850 mL of PBS. Next, the bacterial suspension was stained with 50 μL of propidium iodide (concentration in suspension = 50 μg/mL) and 100 μL of fluorescein diacetate and stored in the dark at room temperature for 30 min. After staining, the bacterial suspension was rinsed with PBS 3 times. Finally, the bacterial pellet was resuspended in 500 μL of PBS, and the stained cells were observed using a fluorescence microscope (DM2500, Leica, Wetzlar, Germany) at 400× magnification.

**Assay of Enzyme Activity**

The cell pellet was washed once with PBS and resuspended in an equal volume of PBS. To remove debris, the cells were centrifuged (7,378 × g for 10 min at 4°C), and the cell-free extract was stored for enzymatic determination.

The activity of lactate dehydrogenase (LDH) was determined by monitoring the rate of absorbance reduction of NADH at 340 nm and 25°C (Cui et al., 2018). The measurement system contained 0.29 mL of pyruvic acid, 0.01 mL of cell-free extract, and 0.01 mL of NADH solution. The absorbance was recorded at 340 nm (UV-2102, UNICO) at exactly 1, 2, 3, 4, and 5 min after the reaction was initiated. The specific activity is expressed in milligrams of protein. The reduced absorption value per minute was calculated assuming that the 1.0 reduction in absorption value per minute represented 1 unit of enzyme activity, and this value is expressed as U. Specific kits were used to determine the specific activity of pyruvate kinase, which is expressed per milligram of protein. Both the LDH and pyruvate kinase (PK) assay kits were provided by Nanjing Ji-ancheng Bioengineering Institute (Nanjing, China).

**Data Analysis**

All the experiments were repeated 3 times. The statistical analysis was performed to assess differences between the experimental groups. The standard deviation (SD) was calculated from the parallel data of 3 samples and is expressed using error bars. The data are expressed as means ± SD. GraphPad Prism drawing,
SPSS single factor, and the multifactor method were used to analyze the significant differences between the data. A $P$-value of <0.05 was considered to indicate a significant difference.

RESULTS

Effects of Single Macromolecular Cryoprotectants on the Survival Rate of *L. plantarum*

Figure 1 presents the survival rates of *L. plantarum* treated with different single micromolecular and macromolecular cryoprotectants. As demonstrated by the control (PBS), the *L. plantarum* survival rate was dramatically reduced by freeze-drying. Among the micromolecular cryoprotectants, sucrose and trehalose yielded significantly higher survival rates compared with all the other evaluated agents. The freeze-drying survival rates ranged from 2.9-fold higher (40.93 ± 2.31% viability) with trehalose up to 5.5-fold higher (77.84 ± 2.85% viability) with sucrose, compared with the control group (14.14 ± 2.05% viability). In contrast, the effect of mannitol was similar to that of PBS. When trehalose and sucrose cryoprotectants were added separately to AR307, the freeze-drying survival rate was approximately 13.5 times higher than that in the control group, wherein the drying survival rate increased to 94.3% in the present of trehalose. Macromolecular cryoprotectants potentially have a high industrial potential. The freezing and drying survival rates of AR113 and WCFS1 strains treated with a macromolecular cryoprotectant were consistent with these resulting from exposure to sucrose, the most effective micromolecular cryoprotectant.

Soy polysaccharides, especially at a 10% concentration, significantly improved the frozen, drying, and freeze-drying survival rates of AR113. For WCFS1, the highest drying and freezing-drying survival rate were achieved with gum arabic, which had a similar effect as the best-performing micromolecules (Figure 1). These results indicated that the macromolecular cryoprotectants had a similar effect to the micromolecules in maintaining bacterial activity. Therefore, soy polysaccharides can be used as a new type of protective agent. This discovery broadens the range of available protective agents, and the prospects for application are huge. However, the same protective agent had different protective effects on the 3 strains, and the same strains with a different protective effect of different protective agents. Similar to micromolecules, the macromolecular cryoprotectants also exhibited some degree of the strain-specificity.

Effects of Composite Cryoprotectants on the Survival Rate of *L. plantarum*

To achieve better cryoprotection, we mixed either of the 2 best-performing micromolecular cryoprotectants...
[sucrose (77.84 ± 2.85% viability) or trehalose (70.21 ± 1.90% viability)] with the macromolecule cryoprotectant soy polysaccharide (60 ± 5.42% viability). We also combined the worst-performing micromolecular cryoprotectant, mannitol, with soy polysaccharide. Figure 2 illustrates the frozen, drying, and freeze-drying survival rates of *L. plantarum* in the present of the different composite cryoprotectants. Although the combination of mannitol and soy polysaccharide improved the survival rate relative to mannitol or soy polysaccharide alone, this effect was inferior to that of the other combinations. A high freeze-drying survival rate of 85.42 ± 6.05% was achieved for AR113 treated with the combination of soy polysaccharides and trehalose, and this rate represents increases in survival of 44.5% and 39.7%, respectively, compared with either component alone. The mixture of soy polysaccharides and trehalose also increased the freeze-drying survival rate of WCFS1 to 90.52 ± 2.91%, which represented increases in survival of 31.48 and 36.47% compared with either trehalose (59.04 ± 1.24%) or soy polysaccharide (54.05 ± 3.67%) alone, respectively. Similarly, the combination of soy polysaccharide and sucrose significantly improved the survival rate of the 3 strains (*P* < 0.05). More importantly, these composite cryoprotectants were efficient for all 3 strains studied, which was superior to the protective effect of single macromolecular cryoprotectants. Therefore, a combination of 2 cryoprotectants could effectively improve the cryoprotective and have the potential to reduce the differences between the strains.

We also evaluated the effects of combinations of 3 and 4 cryoprotectants. However, these did not provide better protection than the combinations of 2 cryoprotectants. Considering the economic factors, the data suggest that the combination of soy polysaccharide and trehalose or sucrose should be widely adopted.

**Mechanism by Which Cryoprotectants Affect the Survival Rate**

Next, the bacterial cell membrane properties were studied to determine the mechanism by which macromolecule cryoprotectants alone or in combination improved the survival of *L. plantarum* after freeze-drying. Figure 3 depicts fluorescence images of *L. plantarum* AR113, AR307, and WCFS1 after freeze-drying and staining with fluorescein diacetate and propidium iodide. Live cells are indicated by green fluorescence, whereas dead cells are indicated by red fluorescence. In the presence of PBS (control group), the field of vision was full of red fluorescence (Figure 3), indicating almost total damage to the membrane integrity after freeze-drying. In contrast, the membrane integrity was greatly improved in the presence of the compound cryoprotectants rather than other cryoprotectants during freeze-drying. These results demonstrate that cell membrane damage during the freeze-drying process may be the main cause of death. Additionally, among the 3 *Lactobacillus* species, the level of red fluorescence in AR307 was significantly higher than in the other 2 strains under the same cryoprotectants, indicating that AR307 had poorer freeze-drying resistance. This observation was consistent with the cell survival data (Figure 1 and Figure 2). In conclusion, cell membrane damage during freeze-drying is considered responsible for the loss of bacterial viability, and compound cryoprotectants can effectively maintain cell membrane integrity.
Lactate dehydrogenase is a key enzyme in the hydrolysis of pyruvic acid to lactic acid in glycolysis. The LDH activity reacts with acid production and regulates cell membrane fatty acid desaturation (Gupta and Bamezai, 2010). Lactate dehydrogenase has been reported to be responsible for a loss of bacterial viability (Li et al., 2011). Sucrose and soy polysaccharides can maintain the LDH activity, and significant increases in this coefficient were observed with the addition of composite cryoprotectants (Figure 4). This observation is consistent with the changes in the survival rates of strains treated with the same cryoprotectants (Figure 2). Taken together, these results suggest that macromolecule cryoprotectants, either alone or in combination, can increase the survival of *Lactobacilli* by maintaining the activity of LDH.

The enzyme PK catalyzes the formation of the second ATP reaction in glycolysis and is one of the main rate-limiting enzymes in this process. Specific PK enzyme activity was measured in the strains after freeze-drying, and the results showed that this coefficient was not significantly affected by different cryoprotectants. In contrast to other enzymes, the PK activity did not significantly increase in cells exposed to the optimal composite cryoprotectant (Figure 5). Previously, a similar result was observed in *L. reuteri* CICC6226, and PK was determined not to be the main factor in freezing-drying injury (Li et al., 2011).

**DISCUSSION**

Freeze-drying is a long-term LAB preservation technique used in industrial applications. However, this process reduces the viability of bacteria to some extent. Consequently, many methods have been tried to mitigate this loss of viability, including different cryoprotectants, culture conditions, and freeze-drying conditions. Among these, cryoprotectants have been the most widely adopted because of their functionality and convenience.

The addition of various sugars to improve the survival rates of desiccated microorganisms has been demonstrated in previous studies (Lee et al., 2016; Wang et al., 2019). In this study, soy polysaccharides and gum arabic both significantly increased the survival rate of...
L. plantarum strains, and the highest freeze-dried survival rate achieved with **soy polysaccharides** was 71.6% (Figure 1). According to a report by Lee et al. (2016), the highest **L. plantarum JH287** freeze-drying survival rate of 77.15% was achieved using sorbitol as a cryoprotectant. Dimitrellou et al. (2016) determined that trehalose yielded the highest **L. casei ATCC 393** viability rate of 66.2%. These data suggest that the ability of polysaccharides (soy polysaccharides and gum arabic) to protect cells is similar to that of commonly used protective agents. Additionally, soy polysaccharides are highly stable with a low viscosity in aqueous solution. Compared with other gums, soy polysaccharides can be dissolved in cold or warm water, and exhibit no gelation in a 10% solution. Similar to the research conducted by Maeda and Nakamura, (2009), we observed a significant increase in the viscosity of the liquid when the concentration of gum arabic reached 10%. Therefore, we selected soy polysaccharides for the composite protective agent.

Shu et al. (2018) have shown that the performance of composite cryoprotectants during freeze-drying was usually superior to that of single cryoprotectants. Our data demonstrate that a **L. plantarum WCFS1** survival rate of 90.52% was achieved using a mixture of soy polysaccharides and trehalose. Chen et al. (2015) reported a high survival rate of 64.41% when using a combination of skim milk, lactose, and sodium ascorbate. In another study, Chen et al. (2019) focused on improving the survival of **B. bifidum BBO1** by optimizing the composition of the composite cryoprotectants. In that study, the addition of the optimal compound protectants increased the survival rate by 90.37%, which was similar to our result. Cui et al. (2018) demonstrated that a combination of skim milk powder, sucrose, and trehalose led to significant differences in the protection of the 3 strains; here, the difference in survival rates between **L. plantarum CCFM8610** and **L. rhamnosus CCFM 1107** reached 30%. In our study, the compound protective agent was less strain-specific and thus effective for all 3 studied strains. Our results also show that the effectiveness of a 2-cryoprotectants combination was sufficiently better than the tested 3- and 4-cryoprotectant combinations. Soy polysaccharides, which offers another option to protect the cell during freeze-drying, can serve as a source of dietary fiber and thus can also be used as a functional ingredient in food applications (Maeda and Nakamura, 2009). Apart from their nutritional value, soy polysaccharides are derived from a safe source and have convenient physical properties, including high solubility and stability under different acid, heat, and salt levels (Nakamura et al., 2004). Overall, we demonstrated that combinations of macromolecular and micromolecular cryoprotectants led to high viability of **L. plantarum**, which is very important for industrial application.

Many studies have proven that lyoprotectants act to protect living cells from damage during lyophilization (Berny and Hennebert, 1991; Stephan et al., 2016). Although the exact mechanism by which cryoprotectants act on bacteria is unclear, evidence suggests that some cryoprotectants can act as membrane protectants (Martos et al., 2007). Li et al. (2010) demonstrated that indicators of cell membrane integrity can be considered as a measure of viability. In that study, an increase in the sucrose concentration led to a significant increase in the integrity of **L. casei Zhang**, which illustrates the protective effects of this micromolecular cryoprotectant on the cell membrane during freeze-drying. Another study demonstrated that some disaccharides and oligosaccharides can replace H-bonded water in membrane bilayers and reduce the water-binding capacities in samples to stabilize the membranes (Önneby et al., 2013). Soy polysaccharides may protect the membranes via a similar mechanism.

In addition to the above-described effects on the membranes, the protective properties of cryoprotectants were ascribed to their interactions with proteins (Crowe et al., 2001). Dextran can allow rapid water equilibration and prevent intracellular ice formation, thus maintaining a biological structure similar to the aqueous state and reducing cell damage during freeze-drying (Morgan and Vesey, 2009; Önneby et al., 2013). Another study by Li et al. (2011) demonstrated a marked improvement in LDH activity after freeze-drying when trehalose was

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**Figure 5.** Effects of cryoprotectants on the activity of pyruvate kinase in *Lactiplantibacillus plantarum* AR113 (A), WCFS1 (B), and AR307 (C) during freeze-drying. Data are presented as means ± SD (n = 3).
added as a cryoprotectant. Consistent with the above research, our results suggest that combinations of macromolecular and micromolecular cryoprotectants might exert a protective effect on L. plantarum viability by maintaining both the cell membrane integrity and LDH enzyme activity. However, the mechanisms by which these composite cryoprotectants maintain cell viability remain to be further explored.

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ORCIDs

Guangqiang Wang: https://orcid.org/0000-0002-3966-044X
Linyin Luo: https://orcid.org/0000-0003-0158-3028
Yongjun Xia: https://orcid.org/0000-0002-2727-2730
Lianzhong Ai: https://orcid.org/0000-0002-6681-9102