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Optimally lyophilized *Lactobacillus plantarum* SNUG 12071 survival under storage and gastrointestinal conditions

보관 및 장관 조건 하에서의 최적 동결건조한 *Lactobacillus plantarum* SNUG 12071의 생존

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Abstract

Optimally lyophilized *Lactobacillus plantarum* SNUG 12071 survival under storage and gastrointestinal conditions

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A sufficient amount of viable probiotic bacteria must be delivered to the gut in order to exert beneficial health effects. Lyophilization in protective carrier materials can enhance probiotic viability in a strain-specific manner. We investigated the effects and interactions of three cryoprotective agents (CPA) (skim milk, sucrose, and sorbitol) in their ability to improve survival of a novel probiotic strain, *Lactobacillus plantarum* SNUG 12071, during the harsh lyophilization process using a

3-factor rotatable central composite design (CCD) and response surface methodology (RSM). After CCD-RSM analysis and validation, the stationary point of the response surface, 11.0% skim milk, 4.3% sucrose, and 5.7% sorbitol, was determined to be the optimal CPA formulation to maintain *L. plantarum*. The stationary point CPA formulation achieved 81.9% *L. plantarum* survival during 48 h lyophilization. In contrast, cells lyophilized with no CPA maintained only 22.3% survival, leading to an almost 60% increase in lyophilization survival. Storage stability of optimally lyophilized *L. plantarum* was assessed for 6 months at room temperature (25°C) and refrigerated (4°C) conditions. Viability decreased by 0.0 and 0.4-log cfu (colony forming units)·mL^{-1} in refrigerated and room temperature conditions, respectively, after 6-months of storage, showing successful stability in both storage conditions. Lyophilization with protective agents also showed significant promise in improving *L. plantarum* survival in gastrointestinal conditions. Lyophilization mitigated 86.2% of the 1.6-log cfu·mL^{-1} viability decrease observed in unprotected cells in 2.0% bile acid conditions. Lyophilized cells also minimized 98.4% of the dramatic 3.4-log cfu·mL^{-1} viability decrease experienced by naked *L. plantarum* in pH 2.0 conditions. The results of this study provided a novel CPA

formulation and demonstrated the benefits of optimizing lyophilization conditions in order to improve probiotic viability during lyophilization, storage, and gastrointestinal conditions.

Key words: Lyophilization, cryoprotectant, *Lactobacillus* spp., central composite design, response surface methodology.

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Analysis of Variance (ANOVA)

Bile acid (BA)

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Generally regarded as safe (GRAS)

Man, Rogosa and Sharpe (MRS)

Optical density (OD)

Phosphate buffer solution (PBS)

Relative humidity (R.H.)

Response Surface Methodology (RSM)

Standard error of the mean (SEM)

Stationary point (SP)

I. Introduction

Probiotics are living microorganisms that can exert a beneficial effect on their host [1, 2]. In order for probiotics to confer a health benefit on human hosts, a sufficient amount of viable bacteria must reach the gut. Optimal probiotic dosage varies between bacterial species and desired health effect, however 10^6–10^7 colony forming units (cfu) daily is recognized as the general minimum dosage [3, 4].

However, two main challenges hinder the viability of ingested bacteria: maintenance of viability during storage and harsh acidic conditions and high bile acid (BA) concentrations in the gastrointestinal (GI) tract. The human stomach is highly acidic (pH 1.5–4.0) and though the small intestine is neutral (pH 6.5–7.5), high concentrations of BA are present (0.3%) [5, 6]. These harsh GI tract conditions in combination with long periods of storage can result in reduced probiotic viability [4].

A promising solution to maintain probiotic viability is encapsulation in protective materials. Encapsulation involves the entrapment of active agents, such as bacteria, in generally recognized as safe (GRAS) carrier materials. A commonly used technique for bacterial encapsulation is lyophilization, a process involving the freezing and vacuum-drying of probiotics encapsulated in a protective carrier material in order to

dehydrate the solution to a powder form [7]. However, due to the prolonged exposure of bacteria to freezing temperatures and dehydrating conditions, bacterial viability can significantly decrease [8].

Process viability can be maintained through optimizing a variety of process parameters [9]. Early stationary phase bacteria have been shown to be better fit to survive harsh lyophilization processing and the final moisture content has also been shown to affect bacterial survival [10]. Combinations of cryprotective agents (CPA) at varying concentrations can be used as carrier materials to protect and maintain bacterial viability during processing and storage in lyophilization conditions [9, 11]. Skim milk is traditionally used as the formula base for *Lactobacillus* spp. cryoprotection due to its ability to alter cell membrane fluidity and calcium stabilization of cellular enzymes [12]. Polysaccharides such as sucrose have also been shown to have membrane and enzyme stabilizing effects during lyophilization, which can contribute to their protective ability [13]. The inclusion of prebiotics, non-digestible nutrients which enrich commensal gut bacteria, in the encapsulating matrix can also improve lyophilized bacterial survival [14].

Maintaining the viability of lyophilized bacteria is crucial to effective gut delivery and increased health benefits of probiotic strains.

Therefore, optimization of critical process parameters should be performed prior to testing encapsulated probiotic survival in storage and gastrointestinal conditions. As optimal lyophilization process conditions vary by species and strain, a systematic process should be followed to develop a strain-specific optimized methodology [4]. Central Composite Design (CCD) is a practical and useful Design of Experiment (DOE) technique used in a wide range of optimization studies [14-16]. The number of factors "k", experimental parameters to be optimized, determines the number of experimental conditions "n" to be tested ($n= 2^k+2k+1$). Full factorial points are tested in addition to central and star points, which are coded based on the desired range of experimental parameters to be tested. Response surface methodology (RSM) is then performed on the CCD experimental data to determine the stationary point of all factors, which can result in optimal parameter conditions [17].

The aim of this study was to optimize the survival of a novel candidate probiotic strain, *Lactobacillus plantarum* SNUG 10271, after lyophilization through growth curve analysis, CCD-RSM analysis of CPA combinations, and moisture content assessment. The optimized lyophilization method was then assessed in its ability to protect and maintain bacterial viability in storage and gastrointestinal conditions.

II. Materials and Methods

1. Bacterial strain and growth conditions

Lactobacillus plantarum subsp. *plantarum* SNUG10271 isolated from fecal samples of a healthy Korean adult and stored at -80°C in 25% glycerol was used for this study. *L. plantarum* was cultured in Man, Rogosa and Sharpe (MRS) broth medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 0.05% L-cysteine-hydrochloride (Sigma-Aldrich, St Louis, MO, USA) to promote anaerobic growth conditions. MRS agar containing 0.05% L-Cysteine was used for cell viability quantification. *L. plantarum* was culture activated three times from frozen stock and the final culture conducted for 18 h to early stationary growth as determined by growth curve analysis (**Fig. 1**). All broth and agar cultures were incubated at 37°C in anaerobic conditions.

2. Bacterial enumeration and survival

Bacterial samples were prepared for enumeration based on their encapsulation condition. Naked bacterial samples were directly enumerated from broth culture. Lyophilized bacterial samples were suspended with 1 mL of 1× phosphate-buffered saline (PBS; pH 7.4, Biosesang, Seongnam-si, South Korea) to their original volume and reactivated by 30 min incubation (37°C, CO_2 5%). All prepared bacterial samples were enumerated by the viable plate method [18] and expressed as cfu·mL^{-1}. Ten-fold serial dilutions in MRS broth supplemented with 0.05% L-cysteine were performed and then bacterial suspensions plated on MRS agar supplemented with 0.05% L-cysteine and incubated for 48 h at 37°C. Bacterial cell concentration was measured as optical density at an absorbance of 600 nm (OD_{600}) using a microplate reader.

Bacterial survival was calculated using the following equation:

$$Survival\ \ (\%) = \left(\frac{N}{N_0}\right) \times 100\%$$

N_0 = Pre-lyophilization cfu·mL^{-1}; N = Post-lyophilization cfu·mL^{-1}

3. Bacterial lyophilization

Skim milk, sucrose, and sorbitol were weighed to the appropriate concentrations for the given experimental conditions and mixed to 25 mL with 1× PBS. CPA combinations were then autoclaved (110°C, 5 min) twice to sterilize the solution without caramelizing the skim milk [19]. *L. plantarum* cultured to early stationary growth was then transferred to 5 mL aliquots per CPA combination and centrifuged (4,000 rpm, 5 min, 4°C) and supernatant removed. Cell pellets were then re-suspended in sterile 1× PBS, centrifuged (4,000 rpm, 5 min, 4°C), and remaining supernatant removed. Washed cell pellets were then thoroughly suspended in 5 mL of the appropriate CPA combination or PBS (negative control) by vortex-mixing samples for approximately 30 s. One mL aliquots of each suspension were then distributed to sterile glass vials in triplicate. All samples were frozen overnight at -80°C and then lyophilized under vacuum (<-50°C, 0.05 mTorr) in a freeze-drier for 24, 48, or 72 h. Bacteria were enumerated as previously described.

4. Central Composite Design Experimental Procedure

A 3-factor rotatable central composite design (CCD) experiment was performed in order to determine the optimal concentrations of three CPA materials, skim milk, sucrose, and sorbitol, to maintain *L. plantarum* viability during harsh freeze-drying conditions. The CCD design is structured to test an experimental response to a range of 5 coded levels of each factor, including a center point (0), factorial points (±1), and star points (±α) to estimate for curvature. The alpha value to determine the distance between the star point and center point in a full factorial design is determined by the following equation in which k is the number of factors:

$$\alpha = [2^k]^{1/4}$$

In this three-factor experimental design, α was 1.7 based on the above equation. The concentration ranges of CPA's were determined based on previous studies [16, 20-22] and coded following central, factorial, and star points as shown in Table 1. Twenty experimental runs were required for the 3-factor rotatable CCD, including 6 center point replicates. The CCD conditions (**Table 2**) were tested with a PBS negative control in three independent experiments following the lyophilization and bacterial enumeration method previously described.

ıble 1. Lyophilization 3-factor rotatable CCD coded levels and factors

	Factor values at 3-factor rotatable CCD coded levels				
'actor (%)	**-1.7**	**-1.0**	**0.0**	**+1.0**	**+1.7**
Skim milk	5.0	8.7	14.0	19.4	24.0
Sucrose	0.0	2.0	5.0	8.0	10.0
Sorbitol	0.0	2.0	5.0	8.0	10.0

Table 2. Lyophilization 3-factor rotatable CCD experimental design

	Cryoprotective Agent (CPA) Combination		
No.	**Skim Milk (%)**	**Sucrose (%)**	**Sorbitol (%)**
C1	8.7	8.0	8.0
C2	14.0	5.0	5.0
C3	8.7	2.0	8.0
C4	14.0	5.0	5.0
C5	14.0	5.0	5.0
C6	19.4	2.0	2.0
C7	19.4	8.0	8.0
C8	19.4	8.0	2.0
C9	8.7	8.0	2.0
C10	19.4	2.0	8.0
C11	8.7	2.0	2.0
C12	14.0	5.0	5.0
C13	14.0	5.0	10.0
C14	14.0	5.0	5.0
C15	14.0	10.0	5.0
C16	14.0	5.0	0.0
C17	5.0	5.0	5.0
C18	14.0	5.0	5.0
C19	23.0	5.0	5.0
C20	14.0	0.0	5.0
PBS	0.0	0.0	0.0

5. Moisture content analysis

Lyophilized bacterial powder samples were produced through the previously described methods and lyophilized for 24, 48, or 72 h. Moisture content was immediately measured with Karl Fischer Moisture Titrator MKV-710 and Evaporator ADP-611 (KEM Kyoto Electronics Manufacturing Co. Ltd., Tokyo, Japan). Lyophilized *L. plantarum* samples were weighed to approximately 0.2 g and inserted in the evaporation chamber. Moisture titration was then conducted through sample evaporation at 150°C chamber temperature and a gas flow of 120 $mL \cdot min^{-1}$. The titration parameters were as follows: T(stir): 0 s, T(wait): 30 s, T(Max): 300 s, End Time: 15 s, End Level: 75 mV, and Data Sampling Time: 10 s. Moisture content is expressed as the percent moisture (%) of samples and measurements of each lyophilization time point conducted in quadruplet.

6. Storage stability assessment

One milliliter of *L. plantarum* suspended in the appropriate CPA mixture was lyophilized as previously described and sealed in a sterile environment. Sealed vials were then wrapped in foil to prevent light exposure and stored in refrigerated (4°C), room temperature (25°C), and accelerated (40°C, 75% relative humidity [R.H.]) storage conditions for 6 months. Triplicate samples were enumerated at various time points to monitor viability throughout the storage period as previously described.

7. Acid and bile resistance assay

Naked and lyophilized *L. plantarum* were tested in triplicate in their ability to survive acidic and bile conditions. Naked bacteria were cultured as previously described, 1 mL bacterial aliquots centrifuged (13,000× g, 3 min), washed twice with PBS, and then suspended in 1 mL of MRS + 0.05% L-cysteine broth media. Naked and lyophilized *L. plantarum* were enumerated initially at 0 h to establish the initial cfu·mL^{-1} of the bacterial samples. For the acid resistance assay, the media was acidified to pH 6.5, 4.0, and 2.0, autoclaved (121°C, 15 psi, 15 min) and cell suspensions incubated anaerobically at 37°C for 2 h. For the bile resistance assay, media was maintained at a pH of 6.5 with oxgall BA (Sigma Aldrich, St Louis, MO, USA) included at concentrations of 0.0, 0.3, and 2% and cell suspensions incubated anaerobically at 37°C for 6 h. After the 2 and 6 h incubation periods, cell suspensions were centrifuged (13,000× g, 3 min), supernatant removed, washed twice with PBS to remove any remaining acidic or bile-containing media and then enumerated as previously described.

8**. Statistical analysis**

Data are expressed as mean ± standard error of the mean (SEM). CCD-RSM analysis and plot visualization was performed using the rsm package in R version 3.2.4 (R Foundation for Statistical Computing, Vienna, Austria) and RStudio 0.99.893 (RStudio, Boston, MA, USA) [17, 23]. GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA) was used to visualize and analyze all other data using Kruskal-Wallis test followed by Dunn's multiple comparison test or two-way analysis of variance (ANOVA) and Bonferroni posttest. Statistical significance was given as '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$.

III. Results

1. Growth curve analysis of *L. plantarum* SNUG 10271

The growth curve of naked *L. plantarum* SNUG 10271 was analyzed in order to determine the incubation time necessary to achieve early stationary phase growth. Periodic cell concentration measurement (OD) and viable bacteria enumeration ($cfu \cdot mL^{-1}$) was conducted over a 24 h incubation period (**Fig. 1**). Exponential growth phase was shown to last until approximately 16 to 18 h incubation. Stationary growth was maintained from 18 to 24 h incubation, as the concentration of viable bacterial cells ($\log cfu \cdot mL^{-1}$) remained stable while the total cell concentration (OD_{600}) increased.

These results indicate increasing concentrations of dead cells in the growth culture during stationary phase (18–24 h). Therefore, 18 h was selected as the upper incubation time limit for early stationary growth culture time of *L. plantarum* SNUG 10271.

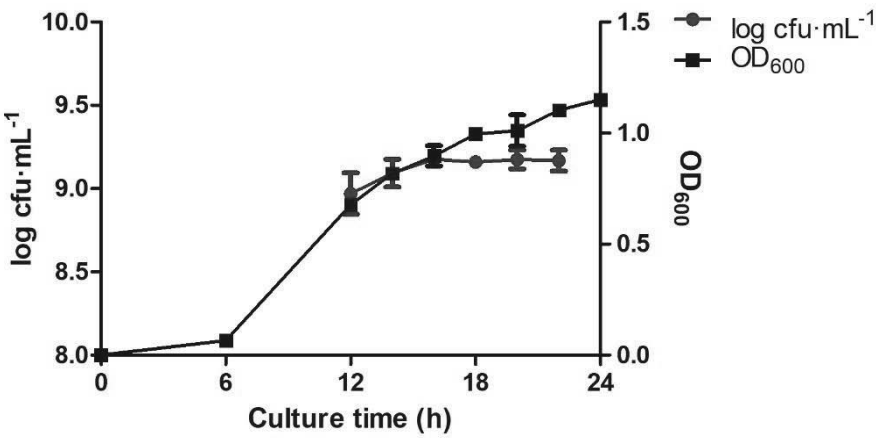


Figure 1. *L. plantarum* SNUG 10271 growth curve

L. plantarum SNUG 10271 was cultured in MRS broth supplemented with 0.05% L-cysteine in anaerobic conditions for 24 h and OD_{600} measured periodically. Duplicated results are expressed as the mean viability (log cfu·mL^{-1}) and optical density at 600 nm (OD_{600}) ± SEM.

2. Effect of 3-factor rotatable CCD CPA combinations on *L. plantarum* lyophilization process survival

Twenty combinations of 3 CPA's, skim milk, sucrose, and sorbitol, with varying concentrations were used as carrier materials to protect *L. plantarum* during lyophilization. All 20 CPA combinations tested following a 3-factor CCD (**Tables 1 and 2**) were assessed in their ability to maintain *L. plantarum* viability during the lyophilization process (<50°C, 0.05 mTorr, 48 h) as a measure of survival (%).

All 20 combinations of the varying CPA concentrations were shown to have higher lyophilization process survival than the PBS negative control of 22.3% (**Fig. 2**). C11 (8.7% skim milk, 2.0% sucrose, 2.0% sorbitol) was shown to have the overall lowest survival at 65.8% and C8 (19.4% skim milk, 8.0% sucrose, 2.0% sorbitol) was shown to have the overall highest survival at 89.5%.

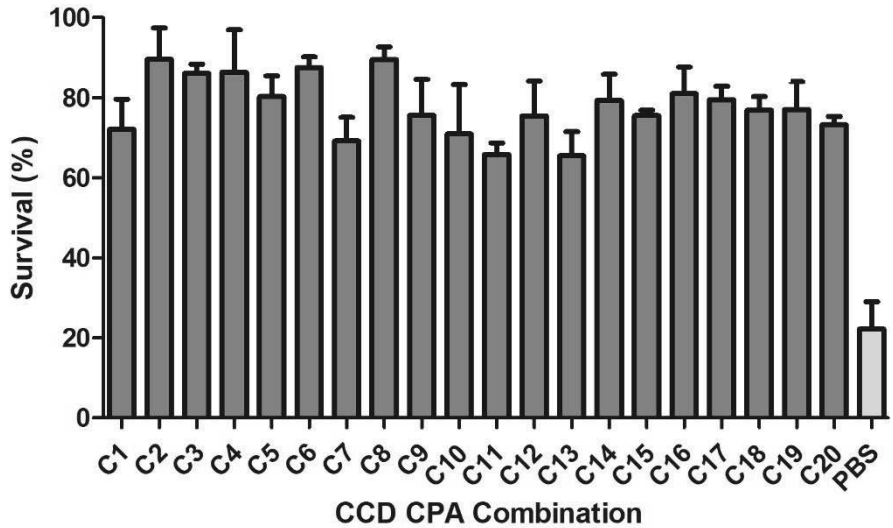


Figure 2. *L. plantarum* lyophilization process survival in CCD CPA combination conditions

L. plantarum was lyophilized (<-50°C, 0.05 mTorr, 48 h) in CCD CPA combination conditions and a PBS negative control. Survival (%) was calculated and triplicate results expressed as the mean (%) ± SEM.

3. 3-factor CCD-RSM stationary point analysis

The interactions of each individual CPA with each other were analyzed and visualized through response surface plotting of the coded factor values (**Table 1**) and the response of *L. plantarum* survival, log survival (%), to interaction between the CPA's (**Fig. 3**). The interaction between sucrose and skim milk showed the stationary point to be a maximum point (**Fig. 3a**), with *L. plantarum* survival slightly decreasing outward from the central stationary point (**Fig. 3d**).

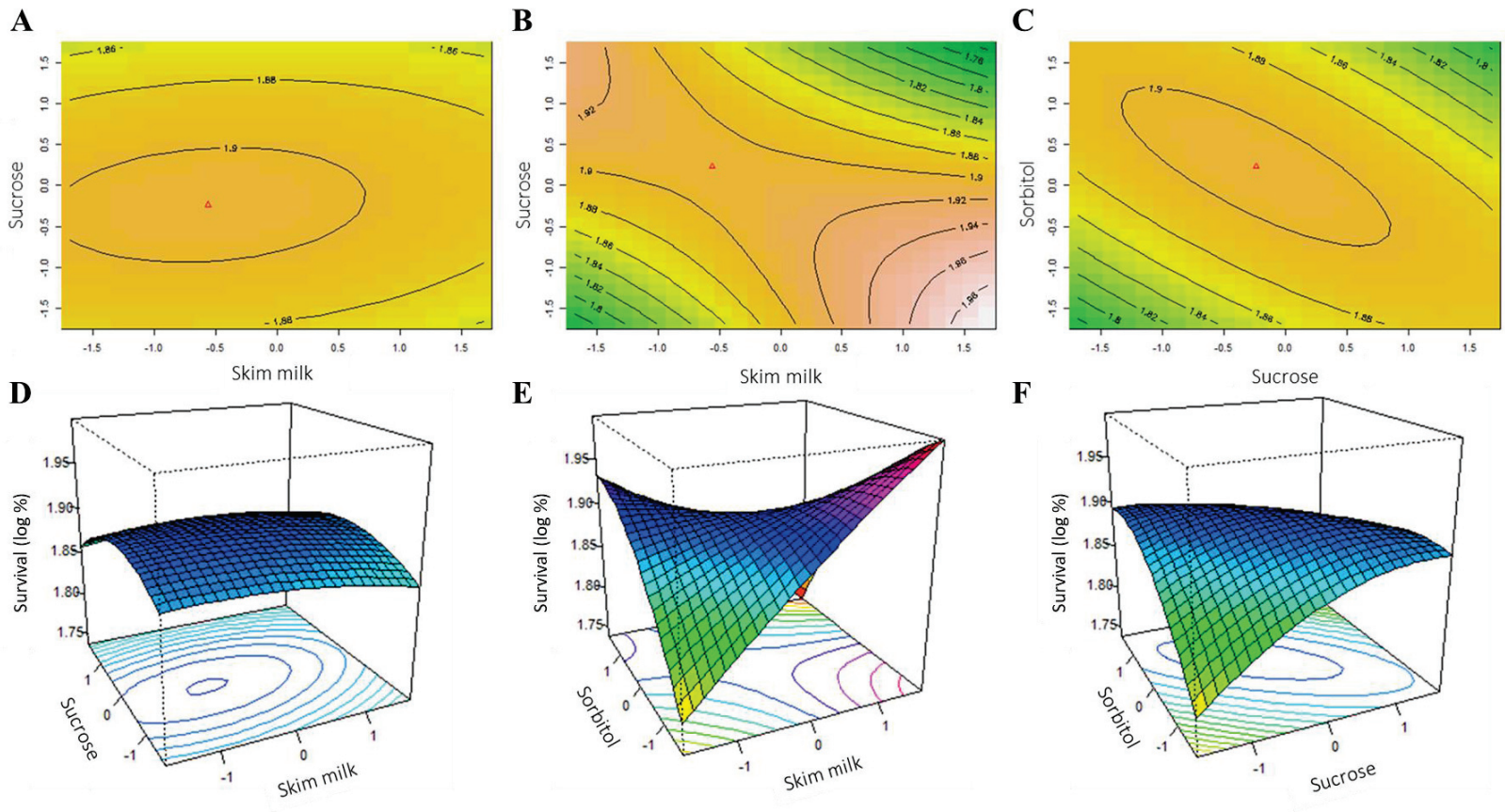
Similarly, the interaction between sucrose and sorbitol yielded a maximum stationary point (**Fig. 3c and 3f).** In the interaction between sucrose and sorbitol (**Fig. 3c**), the lowest coded concentrations of sucrose and sorbitol (coded value -1.7: 0.0% sucrose, 5.0% sorbitol) showed a sharper decrease in survival from the stationary point, approximately 1.9 to 1.8-log % survival, than the lowest coded concentrations of sucrose and skim milk (**Fig**. **3f**). This suggests that the absence of both sucrose and sorbitol from the base skim milk mixture has a stronger negative impact on lyophilization survival than the absence of sucrose alone.

The interaction between sorbitol and skim milk yielded a saddle point (**Fig. 3b** and **3e**), in which the stationary point was neither a clear minimum nor maximum. Interaction between the lowest concentration

of sorbitol (coded value -1.7: 0.0% sorbitol) with the lowest concentration of skim milk (coded value -1.7: 5.0% skim milk) yielded a steep decrease in survival from the stationary point, approximately 1.9- to 1.8-log % (**Fig. 3c**).

The sharpest decrease in survival from the stationary point of all of the CPA interactions was observed at the lowest concentration of sorbitol and the highest concentration of skim milk (coded value 1.7: 23.0% skim milk), from approximately 1.9- to 1.8-log % (**Fig. 3c**). In contrast, the highest increase in survival from the stationary point was observed in the interaction between the highest skim milk coded concentration value and the lowest sorbitol coded concentration value, from approximately 1.9- to 1.2-log % (**Fig. 3c**).

Through RSM analysis, the stationary point of the response surface of all three CPA combinations was determined to be 11.0% skim milk, 4.3% sucrose, and 5.7% sorbitol after decoding and converting the stationary point values.


A
B
C
Sucrose
Skim milk
Sucrose
Skim milk
Sorbitol
Sucrose
D
E
F
Survival (log %)
Sucrose
Skim milk
Survival (log %)
Sorbitol
Skim milk
Survival (log %)
Sorbitol
Sucrose

ɔhical response surface of CPA interactions in *L. plantarum* lyophilization survival

ɔlantarum lyophilized (<-50°C, 0.05 mTorr, 48 h) following the 20 CCD CPA combinations was measured and RSM analysis :d on the log survival. Contour (A, B, C) and perspective plots (D, E, F) were constructed to visualize the interactions between n milk, sucrose, and sorbitol, and their ability to maintain *L. plantarum* survival after lyophilization at their respective coded :y points of each CPA interaction are shown as red triangles in each respective contour plot (A, B, C).

4. CCD-RSM stationary point validation

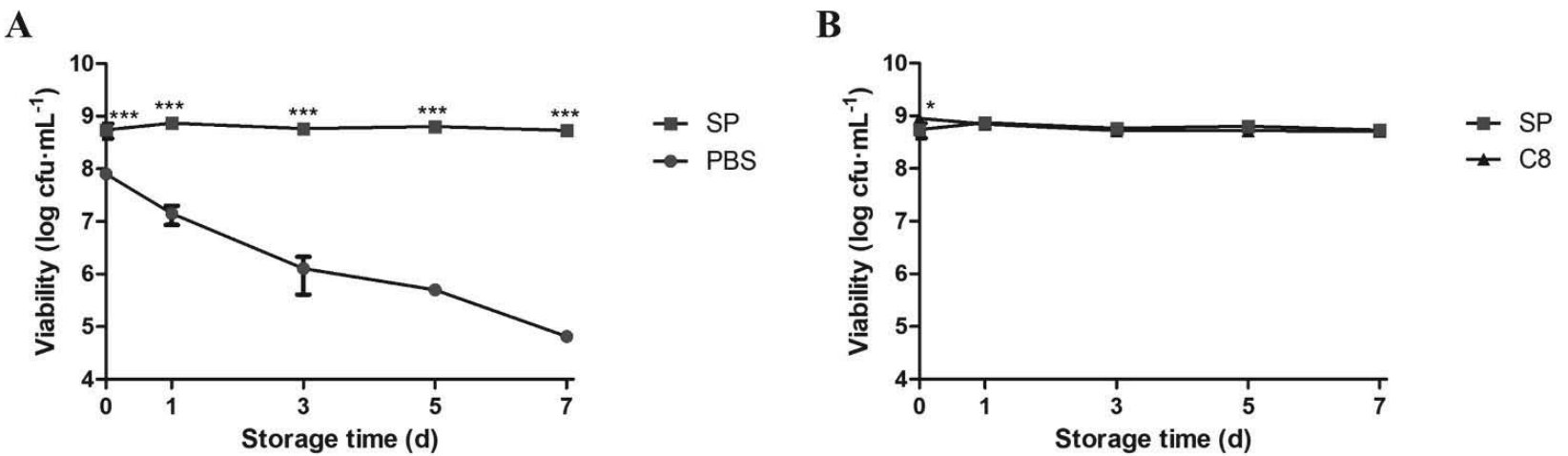
To investigate whether the stationary point determined through response surface analysis of the CCD experimental data (11.0% skim milk, 4.3% sucrose, 5.7% sorbitol) was effective at maintaining *L. plantarum* viability post-lyophilization, a 7-day accelerated storage study was conducted. The stationary point (SP) was validated by comparison with a PBS negative control and the best performing CCD CPA combination, C8 (19.4% skim milk, 8.0% sucrose, 2.0% sorbitol). *L. plantarum* lyophilized in the three different carrier material conditions were stored at 40°C with 75% R.H. for 7 days immediately after lyophilization (<50°C, 0.05 mTorr, 48 h) (**Fig. 4**).

The PBS negative control showed a significantly lower viability than the SP conditions at every time point of the 7-day storage period, including day 0 through two-way ANOVA and Bonferroni posttest ($p<0.001$). PBS showed a steady decrease in survival with a total decrease of 3.1-log cfu·mL^{-1} after 7 days in accelerated storage conditions (**Fig. 4a**).

Both SP and C8 CPA combinations showed steady maintenance of *L. plantarum* viability over the 7-day storage period, despite the accelerated storage conditions. After 7-day storage, both samples maintained 8.7-log

viable *L. plantarum* cells (cfu·mL^{-1}). Two-way ANOVA and Bonferroni posttest analysis of the SP and C8 CPA combinations resulted in no significant difference between the two formulations in their ability to preserve *L. plantarum* viability at any time point during the 7-day storage except day 0 ($p<0.05$) (**Fig. 4b**).

Based on the validation study, SP was selected as the CPA formulation for all proceeding lyophilization experiments. Both the C8 and SP formulations maintained comparable *L. plantarum* viability during 7-day storage in accelerated conditions despite the differing concentrations of all 3 CPA's in both combinations.



ɔphilization CCD-RSM stationary point validation

was lyophilized (<-50°C, 0.05 mTorr, 48 h) in the CCD stationary point CPA combination (SP) with (A) a negative) and (B) a positive control (C8). Lyophilized bacteria were immediately stored in accelerated conditions (40°C, 75% umerated after 0, 1, 3, 5, and 7 days of storage. Triplicate results are expressed as the mean log cfu·mL^{-1} ± SEM. ysis was conducted with two-way ANOVA and Bonferroni posttest ('*' $p<0.05$, '***' $p<0.001$).

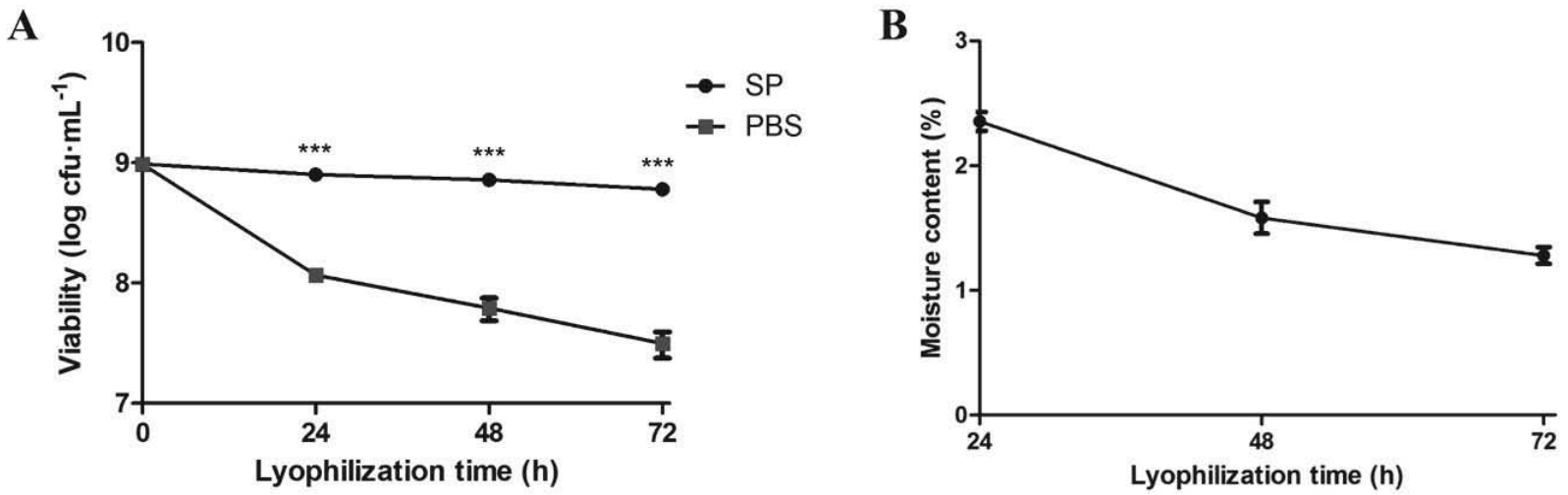
5. Effect of lyophilization time on moisture content and *L. plantarum* survival

After the selection of the SP CPA combination as the carrier material for *L. plantarum* during lyophilization, the effect of lyophilization time on the moisture content and survival of lyophilized *L. plantarum* was assessed after 24, 48, and 72 h lyophilization (<50°C, 0.05 mTorr) (**Fig. 5**).

L. plantarum showed an overall trend of slightly decreasing viability as lyophilization time increased, though the decrease was not statistically significant. The viability (log cfu·mL^{-1}) of *L. plantarum* lyophilized in the SP CPA combination for 24, 48, and 72 h decreased by 0.1-, 0.1-, and 0.2-log cfu·mL^{-1} respectively from *L. plantarum* viability pre-lyophilization (0 d). This trend was heightened in *L. plantarum* lyophilized in PBS as a negative control for 24, 48, and 72 h decreased by 1.0-, 1.2-, and 1.5-log cfu·mL^{-1} respectively. Two-way ANOVA and Bonferroni posttest analysis revealed that *L. plantarum* lyophilized in PBS control had significantly lower viability than *L. plantarum* lyophilized in SP CPA combination conditions after 24, 48, and 72 h lyophilization ($p<0.001$) (**Fig. 5a**).

L. plantarum lyophilized for 24, 48, and 72 h showed a slight trend

of decreasing moisture content (%) as lyophilization time increased. After 24, 48, and 72 h of lyophilization, a moisture content of 2.4, 1.6, 1.3% was yielded respectively (**Fig. 5b**).



ct of lyophilization time on lyophilized *L. plantarum* moisture content and survival

was lyophilized (<-50°C, 0.05 mTorr) in SP CPA combination or PBS negative control conditions for 24, 48, and 72 ated (log cfu·mL^{-1}) and (B) moisture content (%) measured. Triplicate results are expressed as the mean log cfu·mL^{-1} ntent (%) ± SEM. Statistical analysis was conducted using two-way ANOVA and Bonferroni posttest ('***' $p<0.001$).

6. Survival of optimally lyophilized *L. plantarum* in storage conditions

To determine the ability of the optimized lyophilization method to protect *L. plantarum*, storage stability was assessed in three storage conditions. Lyophilized *L. plantarum* were stored at room (25°C) and refrigerator (4°C) temperatures for 6 months in addition to accelerated conditions (40°C, R.H. 75%) for 2 months (**Fig. 6**).

Accelerated storage conditions are commonly used in stability testing in order to provide a preliminary indication of bacterial stability as well as test stability in harsh storage conditions [24]. *L. plantarum* in accelerated storage conditions showed a total decrease of 2.9-log cfu·mL^{-1} over the 60-day storage period (**Fig. 6a**).

Lyophilized *L. plantarum* stored at 4°C experienced a decrease of 0.0-log cfu·mL^{-1} over the course of 6 months of storage, while 6 months at 25°C yielded a decrease of 0.4-log cfu·mL^{-1}. Two-way ANOVA and Bonferroni post-test conducted between 4°C and 25°C storage revealed a significant decrease of viability in room temperature in comparison to refrigerated conditions after 2 months of storage ($p<0.05$) (**Fig. 6b**).

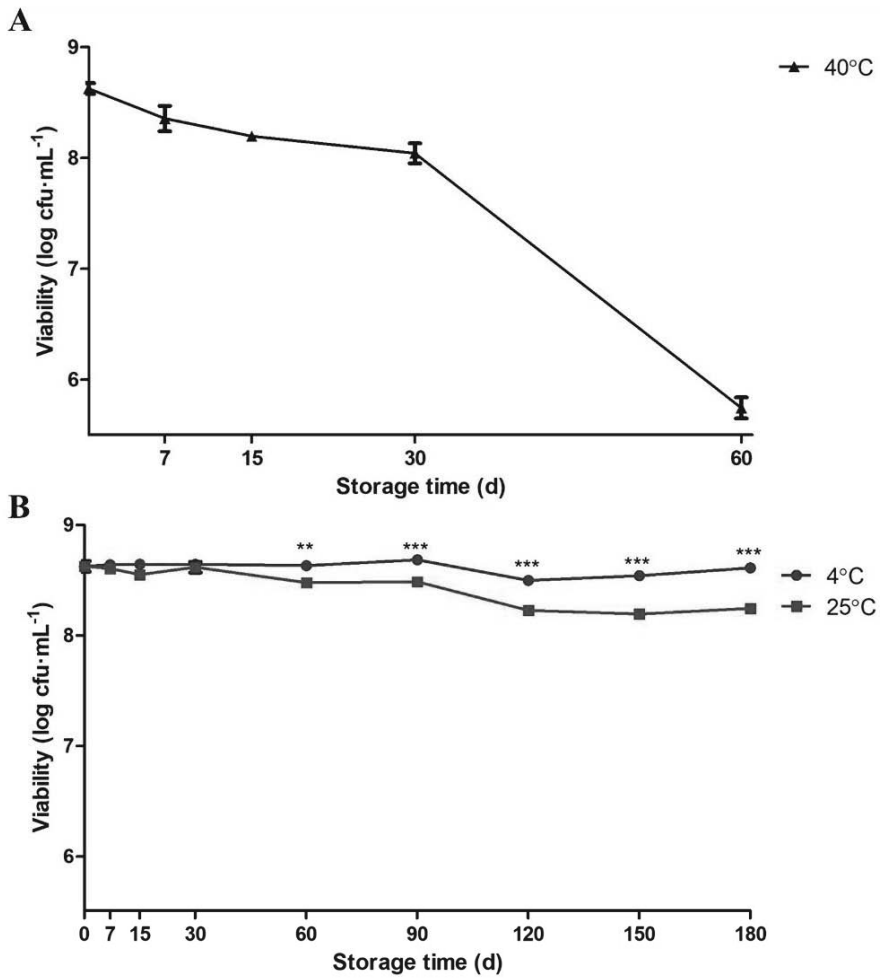


Figure 6. Storage survival of optimally lyophilized *L. plantarum*

L. plantarum was lyophilized under optimized conditions and (A) stored for 2 months in accelerated storage conditions (40°C, R.H. 75%) and (B) refrigerated (4°C) and room temperature (25°C) conditions for 6 months and periodically enumerated. Triplicate results are expressed as the mean log cfu·mL^{-1} ± SEM. Statistical analysis was conducted by two-way ANOVA and Bonferroni posttest ('**' $p<0.01$, '***' $p<0.001$).

7. Survival of naked and optimally lyophilized *L. plantarum* in *in vitro* gastrointestinal conditions

Optimally lyophilized and naked *L. plantarum* were assessed *in vitro* in their ability to maintain viability in acidic and BA conditions similar to those found in the stomach and small intestine (**Fig. 7**).

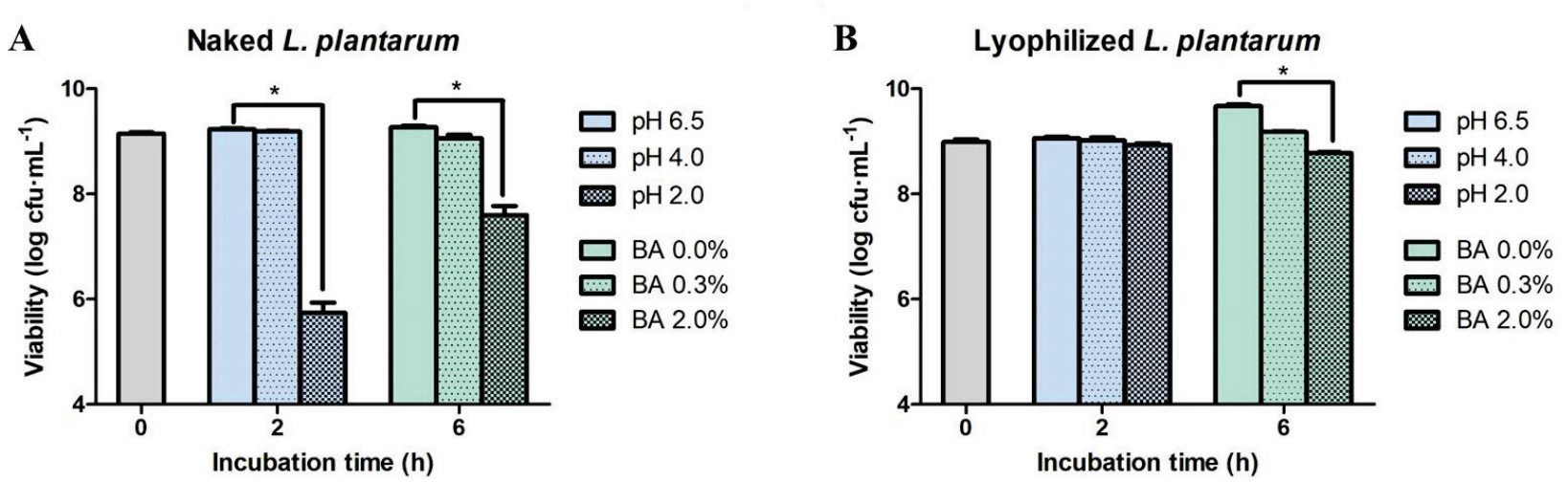
Naked *L. plantarum* had an initial viability of approximately 10^9 cfu·mL^{-1} at 0 h incubation. After 2 h of incubation in pH 6.5, 4.0, and 2.0 conditions with 0.0% BA, naked *L. plantarum* showed an increase of 0.1-log cfu·mL^{-1}, an increase of 0.0-log cfu·mL^{-1}, and a decrease of 3.4-log cfu·mL^{-1} respectively. Kruskal-Wallis test followed by Dunn's multiple comparison test revealed a significant decrease in viability of naked *L. plantarum* incubated in pH 2.0, but not 4.0 conditions for 2 h compared to pH 6.5 conditions for 2 h ($p<0.05$) (**Fig. 7a**).

After 6 h of incubation in 0.0%, 0.3%, and 2.0% BA conditions with pH 6.5, naked *L. plantarum* showed an increase of 0.1-log cfu·mL^{-1}, a decrease of 0.1-log cfu·mL^{-1}, and a decrease of 1.2-log cfu·mL^{-1}. Kruskal-Wallis test followed by Dunn's multiple comparison test revealed a significant decrease in viability of naked *L. plantarum* incubated in 2.0% BA conditions for 6 h compared to 0.0% BA conditions for 6 h ($p<0.05$) and 0.3% BA conditions showed no

significant decrease. *L. plantarum* in unprotected conditions was therefore determined to be more sensitive to harsh acidic conditions (pH 2.0) than harsh bile acid exposure (2.0%), but not very sensitive to lower range acidic conditions (pH 4.0) nor BA conditions (0.3%) (**Fig. 7a**).

After 2 h of incubation in pH 6.5, 4.0, and 2.0 conditions with 0.0% BA, lyophilized *L. plantarum* showed an increase of 0.1-log cfu·mL^{-1}, no change (0.0-log cfu·mL^{-1}), and a decrease of 0.1-log cfu·mL^{-1} respectively. Kruskal-Wallis test followed by Dunn's multiple comparison test revealed no significant decrease in viability of lyophilized *L. plantarum* incubated in pH 2.0 or 4.0 conditions compared to pH 6.5 control conditions after 2 h incubation ($p<0.05$) (**Fig. 7b**).

After 6 h of incubation in 0.0%, 0.3%, and 2.0% BA conditions with pH 6.5, lyophilized *L. plantarum* showed a 0.7-log cfu·mL^{-1} increase, an increase of 0.2-log cfu·mL^{-1}, and a decrease of 0.2-log cfu·mL^{-1}. Kruskal-Wallis test followed by Dunn's multiple comparison test revealed a significant decrease in viability of lyophilized *L. plantarum* incubated in 2.0% BA conditions, but not 0.3% BA for 6 h compared to 0.0% BA conditions for 6 h ($p<0.05$) (**Fig. 7b**). Lyophilized *L. plantarum* was therefore determined to be slightly more sensitive to BA conditions than acidic conditions.



ɔlantarum survival in acid and bile conditions

on-encapsulated) and (B) lyophilized *L. plantarum* were incubated at 37°C for 2 h at pH 6.5, 4.0, and 2.0 (0.0% BA) 0.0, 0.3, and 2.0% BA (pH 6.5) and enumerated at 0, 2, and 6 h. Triplicated results are expressed as the mean log EM. Kruskal-Wallis test followed by Dunn's multiple comparison test were performed ('*' $p<0.05$).

IV. Discussion

The optimization process used in this study focused primarily on maximizing the immediate survival of *L. plantarum* by optimizing the concentrations of cryoprotectants shown in previous studies to significantly improve lyophilization survival of other *L. plantarum* strains through a thorough Design of Experiment (DOE) central composite design (CCD). This part of the optimization process was made a priority due to the fact that the composition of media used to suspend bacteria is widely considered to be the most important factor for post-lyophilization survival [8].

Cell death during lyophilization is principally caused by extracellular ice crystal formation leading to cell membrane damage, however addition of cryoprotectant can reduce ice formation by increasing solute concentrations [25-27]. A multitude of studies have demonstrated increased lyophilized *Lactobacillus* spp. survival during freeze-drying and subsequent storage when cells are suspended in a wide range of different cryoprotectant solutions [9, 21, 22], which was also demonstrated in this study. The formula tested in this study containing only 5% skim milk showed considerably higher survival during after 48 h lyophilization than the PBS control, demonstrating the ability of skim

milk to increase lyophilization process viability even at relatively low concentrations. In this study based on response surface plotting of interactions between skim milk, sucrose, and sorbitol, lower or higher concentrations of sucrose had a relatively moderate impact on *L. plantarum* survival in its interactions with skim milk and sorbitol.

The interaction between the prebiotic sorbitol and skim milk resulted in a saddle stationary point. This interaction confounds the direct interpretation of the stationary point, however a variety of factors led us to determine the RSM stationary point to be the optimal CPA formulation. First, while the CCD-RSM study focused on process survival, the protective effect of sorbitol on *Lactobacillus* spp. survival in other lyophilization studies was shown to be more significant in increasing storage stability than lyophilization survival [28]. In this study, despite a lower initial survival of *L. plantarum* lyophilized in the SP CPA formulation (11.0% skim milk, 4.3% sucrose, and 5.7% sorbitol) than the C8 CPA formulation (19.4% skim milk, 8.0% sucrose, 2.0% sorbitol), the SP CPA formula with a higher concentration of sorbitol and reduced concentrations of skim milk and sucrose showed no significant difference in viability during a 7-day accelerated storage period and was slightly higher at day 1, 3, 5, and 7 than the C8 CPA formulation.

Second, prebiotics as well as the combination of prebiotics with probiotics, synbiotics, are known to have a favorable influence on the intestinal microflora, which in turn can elicit a beneficial health effect on the human host [29]. Sorbitol is a naturally-occurring GRAS polyol isomer and has been shown to selectively enrich intestinal *Lactobacillus* spp. *in vivo*, further demonstrating the positive health effects of sorbitol inclusion in the suspending material [30]. Third, high consumption of sucrose, a disaccharide sugar, has been shown to have a negative impact on gut microbiota health [31]. Therefore, the SP CPA formulation containing higher sorbitol and minimized sucrose concentrations was evaluated to be more optimal for overall *L. plantarum* viability and potentially higher beneficial health effects in later *in vivo* applications than the C8 CPA formulation.

Growth phase and moisture content were also optimized in this study. Eighteen hours of growth was determined to be the maximum growth culture time to achieve early stationary phase growth of *L. plantarum* SNUG 12071, the optimal growth condition to lyophilize bacteria, which is consistent with the early stationary phase culture time of other *L. plantarum* strains [32, 33]. For moisture content optimization, generally the target moisture content of lyophilized biological materials is 1–3%

for optimal storage stability and it is understood that less residual moisture leads to higher storage stability [34]. However, in the case of bacterial lyophilization, enough residual moisture must be retained in the lyophilized material to support necessary cellular processes [35, 36]. Therefore, in this study, 24 h of lyophilization resulting in 2.4% moisture content was selected as the optimal lyophilization time due to the higher moisture content level within the 1–3% range as well as cost and energy-saving concerns of prolonging drying times up to 48 or 72 h.

Storage temperature was shown to have an influence on the rate of viability decline of lyophilized *L. plantarum*, which is consistent with previous studies [16, 21, 36]. Lower storage temperature was associated with higher viability maintenance, with refrigerated temperature showing 99.8% survival after 6 months of storage. While room temperature conditions showed a statistically significant decrease in viability after 2 months of storage in comparison to refrigerated conditions, lyophilized *L. plantarum* retained 95.6% survival after 6 months of storage. This places its survival well above the generally recommended amount, 10^6–10^7 cfu, demonstrating adequate storage stability at room temperature which is a more economic storage option than refrigerated conditions. Accelerated storage conditions were

conducted for 2 months until viable bacteria decreased below the generally recommended amount of probiotic bacteria required for a probiotic effect, 10^6 cfu. This demonstrated the ability of optimally lyophilized *L. plantarum* to survive harsh storage temperatures for certain periods of time.

This study also sought to illuminate the capability of optimally lyophilized *L. plantarum* to survive *in vitro* gastrointestinal conditions through acid and BA resistance assays. Here, a protective effect was conferred to optimally lyophilized *L. plantarum* in high concentrations of BA (2.0%) in comparison to the naked negative control. BA in the small intestine has been shown to have an antimicrobial effect of gut microbes through detergent-like properties and hydrophobic interactions which can damage bacterial cell membranes leading to cell lysis and death [37]. Therefore, the increased survival of lyophilized *L. plantarum* could possibly be attributed to the membrane stabilizing effect of skim milk and sucrose.

Fatty acid composition of bacterial cell membranes has also been shown to affect bile resistance, with higher membrane unsaturated fatty acid concentrations showing a relationship to higher bile resistance [38]. Cell membranes are also known to adapt to freezing temperatures and

freeze-drying conditions by altering fatty membrane composition to increase the ratio of unsaturated fatty acids [39], an effect which is further enhanced by the inclusion of polyols such as sorbitol in some lactic acid bacteria such as *L. plantarum* [40]. A similar change to membrane fatty acid composition of *L. plantarum* lyophilized in the presence of sorbitol could have occurred in this study, conferring an enhanced ability to resist bile-mediated bacterial cell lysis.

In highly acidic conditions, pH 2.0 for 2 h, naked *L. plantarum* experienced an over 3-log cfu·mL^{-1} decrease from the starting concentration, while lyophilized *L. plantarum* experienced a less than 0.1-log cfu·mL^{-1} decrease. This could be attributed to the buffering effect of skim milk on hydrogen ions, which has been shown in other studies to offer a protective effect to other probiotic species in functional food and encapsulation applications [41, 42]. However, reports have also indicated that the same proteins in skim milk that provide the buffering effect, mainly casein [43], can also stimulate increased acid production in the gastric tract [44]. Therefore, in order to further verify the successful gastric survival of skim milk-based lyophilized probiotics, *in vivo* testing should also be conducted to account for *in vivo* homeostatic adjustments that could reduce the protective buffering effect.

In conclusion, optimizing the culture time, CPA (skim milk, sucrose, and sorbitol) concentrations, and moisture content of lyophilized *L. plantarum* SNUG 12071 cells resulted in a novel CPA formulation which improved process, storage, and gastrointestinal condition survival. The methods used in this study can be applied to determine optimal experimental conditions to improve viability of other target probiotic strains in order to foster gastrointestinal tract colonization to exert therapeutic effects.

VI. References

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국문초록

보관 및 장관 조건 하에서의 최적 동결건조한 *Lactobacillus plantarum* SNUG 12071의 생존

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유익한 건강 영향을 미치기 위해서는 충분한 양의 살아있는 프로바이오틱 박테리아가 장에 전달 되어야한다. 보호된 수송 물질에서의 동결 건조는 균주 특이적 방법으로 생존력을 향상 시킬 수 있다. 우리는 가혹한 동결 건조 공정 동안 새로운 프로바이오틱 균주인 *Lactobacillus plantarum* SNUG 12071의 생존율을 향상시키는 능력에 있어 세 종류의 동결보호제 (cryoprotective agents, CPA)인 탈지유, 자당, 그리고 소르비톨의 효과에 대한 상호 작용을 3-factor rotatable central composite design (CCD) 및 응답 표면 방법론(response surface methodology, RSM)을 통하여 조사하였다. CCD-RSM 분석 및 검증 후, *L. plantarum* 생존율을 유지하기위한 최적의 CPA 공식으로 11.0%의 탈지유, 4.3%의 자당 및 5.7%의 소르비톨이 반응 표면의 정지 지점으로 결정되었다. CPA공식에서 정저점은 동결 건조 48시간 동안 81.9% *L. plantarum* 생존율을 나타냈다. 대조적으로, CPA가 없이 동결 건조 된 세포는 22.3%의 생존만을 유지하여 동결 건조 생존율이 약 60% 증가함을 보였다. 최적의 조건에서 동결 건조된 *L. plantarum*의 저장 안정성은 실온(25℃) 및

냉장(4℃) 조건에서 6개월 동안 진행되었다. 생존율은 6개월 저장 후 냉장 및 상온 조건에서 각각 0.0 및 0.4-log cfu (colony forming units) mL^{-1}로 감소하여 두 저장 조건 모두에서 안정성을 보였다. 동결보호제를 이용한 동결 건조는 위장관 조건에서 *L. plantarum* 생존율을 향상시키는 유의한 결과를 보여 주었다. 동결 건조는 2.0% 담즙산 조건에서 비보호된 세포에서 관찰된 1.6-log cfu·mL^{-1} 생존율 감소의 86.2%를 완화시켰다. 동결 건조된 세포는 또한 pH 2.0 조건에서 비보호된 *L. plantarum*이 겪은 극적 3.4-log cfu·mL^{-1} 생존 능력 감소의 98.4%를 최소화했다. 본 연구의 결과는 새로운 CPA 공식을 제공하고 동결 건조, 저장 및 위장 조건 동안 생균 생존 능력을 향상시키기 위해 동결 건조 조건을 최적화하는 이점을 제시하였다.

주요 단어: 동결건조, 동결보호제, 젖산균 종, 중심합성계획법, 반응 표면 방법론

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