



Research Article

Preparation of sea buckthorn-*Lactocaseibacillus paracasei* SMN-LBK mixture and optimization of its freeze-drying conditions

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Abstract: Sea buckthorn has good food medicine homology characteristics and is widely used in food processing and the development of functional foods. *Lactocaseibacillus paracasei* SMN-LBK (SMN-LBK) has been proven to have excellent probiotic properties. Preparing SMN-LBK-sea buckthorn mixture is a new strategy for jointly developing functional foods containing probiotics and food and medicine homologous raw materials. In this study, we screened the types of freeze-drying protectants for composite probiotic powder and optimized their freeze-drying process. The formulation of cryoprotectants was evaluated and optimized by response surface methodology using survival rate as an evaluation index. The results showed that the optimal formula was 10% sea buckthorn, 10% trehalose, 15% sucrose, 16% skim milk powder, and 4% sorbitol. On this basis, further optimizing the process conditions of freeze-drying was carried out. We found that the ratio of bacteria to the composite protective agent was 1:1, the thickness of the bacterial solution was 0.4 cm, and the pre-equilibration time was 30 min, and this resulted in the highest survival rate (88% ± 0.8%) of SMN-LBK. Additionally, the effects of freeze-drying on the antioxidant and enzyme activities of the mixture were evaluated. The DPPH radical scavenging rate was up to 72% at a concentration of 5 mg/mL, implying that the mixture had good antioxidant properties. The enzyme activities of LDH, HK, and ATPase of the mixture were significantly higher compared with those of the absence of protective agents, while PK showed no significant change, suggesting that PK may not be the key enzyme affecting the lyophilized activity of the mixture. This provides a theoretical basis for the development of food medicine homologous composite probiotic products.

Keywords: sea buckthorn; *L. paracasei* SMN-LBK; cryoprotectant; antioxidant properties; food and medicine homologous

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1 Introduction

Sea buckthorn (*Hippophae rhamnoides* L.) is a rich source of nutrients and bioactive compounds, including flavonoids, polyphenols, polysaccharides, lutein, organic acids, vitamin C, unsaturated fatty acids, and essential amino acids^[1]. Research has demonstrated that flavonoids and polyphenolic compounds can regulate immune system functions and influence gut microbiota and its metabolic processes. Flavonoids from sea buckthorn restored chronic unpredictable mild stress-induced depressive disorder and regulated the gut microbiota characterized by

decreasing the abundance of *Lactobacillaceae* and increasing the abundance of *Lachnospiraceae* at the family level^[2]. Additionally, sea buckthorn flavonoids mimicked neurotrophic functions in inducing neuronal cell differentiation via activating PI3K/Akt and ERK pathways^[3]. These results suggest the beneficial functions of sea buckthorn as a potential health food supplement in treating various brain disorders. Sea buckthorn polysaccharides have been shown to enhance liver health by modulating inflammatory responses, exhibiting antioxidant properties, and providing various protective effects^[4]. Supplementation with sea buckthorn polysaccharide ameliorated DSS-induced colitis in mice via

regulating Th17/Treg homeostasis through intestinal microbiota and their production of bile acid metabolites and short-chain fatty acids^[5]. As a raw material utilized in both food and medicinal applications, sea buckthorn possesses physiological functions that include the prevention of cardiovascular diseases, reduction of blood glucose levels, deceleration of aging processes, modulation of immune responses, and improvement of atherosclerosis^[6]. Consequently, sea buckthorn has been extensively employed in the production of functional foods^[7].

Probiotics are defined as microorganisms that provide health benefits to the host^[8]. They have garnered considerable attention within the food industry^[9], particularly due to their capacity to enhance immunity and modulate the balance of intestinal microbiota^[10]. A variety of freeze-dried probiotic products are commercially available for consumers of all age groups, primarily due to their convenience. Lactic acid bacteria (LAB) are employed as fermentation agents to enhance flavor and augment the nutritional value of food products^[11]. The use of freeze-dried LAB addresses the challenges associated with the preservation and transportation of traditional liquid ferments^[12]. SMN-LBK, a gram-positive bacterium found in koumiss, exhibits tolerance to both acid and bile salts. Furthermore, it demonstrates a strong ability to colonize the intestines and enhances the antioxidant activity of prune wine^[13,14]. The innovative combination of sea buckthorn and SMN-LBK in the formulation of composite probiotic powder not only regulates the flavor profile of the SMN-LBK powder but also represents a novel exploration of the functional synergy between sea buckthorn and SMN-LBK. The composite probiotic powder consists of food and medicine homology material and may possess significant potential for the regulation of human health, thereby facilitating broader applications.

It is vital to maintain the vitality and health-promoting properties of probiotics during production and storage^[15]. Liquid probiotic solutions have a rapidly decreasing live bacteria count, making them challenging to preserve. Therefore, a key challenge in creating viable probiotic products is finding ways to incorporate probiotics into foods without depleting their vitality. Probiotics are easier to store and transport when dried into a powder using the right preparation methods. Vacuum freeze-drying is often used to freeze-dry probiotics, which affords LAB a greater survival rate compared to spray drying or fluidized bed drying^[16]. However, low temperatures can harm the ability of probiotics to transfer nutrients across their membranes. Additionally, low temperatures can diminish the activity of enzymes involved in cell metabolism, leading to protein inactivation, DNA damage, and other processes that can further impact the survival rate of bacteria^[17]. Consequently, for the commercialization and application of probiotic powder, it is essential to carefully choose superior cryoprotectants, optimize freeze-drying conditions, and establish appropriate storage settings^[18]. Bacterial survival during freezing situations typically involves numerous important elements, such as cryoprotectants, cell characteristics, and freeze-thaw settings^[19].

In general, there are two types of cryoprotectants: low-molecular-weight molecules such as amino acids, sugar alcohols, and oligosaccharides, and macromolecular substances such as skim milk, polysaccharides, proteins, and other synthetic polymers. Low-molecular-weight protectants provide direct protection, while high-molecular-weight protectants promote the former^[20]. Selecting suitable cryoprotectants for bacterial cells is a major challenge due to variations in their size and shape^[21]. Because each protective agent has a unique effect on the microorganism. In addition, sea buckthorn is rich in

polysaccharides (e.g. fructose, glucose, sucrose), vitamins, amino acids, etc.^[22]. These active ingredients, which have the potential to be used as freeze-drying protectants, can work synergistically with other protectants to improve the protective effect and reduce the loss of strain by freeze-drying. There is no universal protective formulation available for probiotics^[23]. Therefore, it is necessary to choose cryoprotectants based on the characteristics of different strains to prevent cryoinjury.

This study focused on SMN-LBK and sea buckthorn, as well as the freeze-drying characteristics of their complexes. Based on the addition of sea buckthorn, a sea buckthorn probiotic composite powder was prepared using vacuum freeze-drying technology. The survival rate of bacteria was used as the indicator to explore the optimal combination of cryoprotectant, concentration, and freeze-drying conditions. Meanwhile, by analyzing the storage stability, antioxidant properties, enzyme activity, and related indicators of freeze-dried powder, the physiological damage caused by bacterial strains during freezing was studied, providing a theoretical basis for the development of SMN-LBK-sea buckthorn composite probiotic powder.

2 Materials and methods

2.1 Strain

SMN-LBK was a probiotic strain isolated from kumis in the Tacheng district of Xinjiang Uyghur Autonomous Region in China. It was obtained from the Collection of Characteristic Microorganisms from Xinjiang (CCMXJ) at Shihezi University.

2.2 Culture media and growth conditions

SMN-LBK was originally cultured in MRS broth medium (Haibo Biotechnology, China, Bioreagents), and by optimizing, it was finally cultured in an optimized medium consisting of 15 g/L oligomannose, 20 g/L soy peptone, 10 g/L yeast leach, 0.04 mol/L $\text{Na}_2\text{HPO}_4\text{-K}_2\text{HPO}_4$ phosphate buffer salt, 0.08 g/L manganese sulfate, and 0.6 g/L L-cysteine hydrochloride. MRS agar medium was used to calculate the number of viable bacteria.

All media were autoclaved (115 °C, 30 min) before use. The cells were statically incubated at 37 °C, and after inoculating with 3% (V/V) of the fermentation solution, the cells were activated for three generations of fermentation. Finally, cells were harvested after 16 h of culture. The fully activated strains were stored below 4 °C until for use.

2.3 Preparation of cryoprotectants

Based on the inclusion of 10% sea buckthorn (Jin Huashan Foods Co., Ltd., China), four major categories of cryoprotectants (all food-grade) including: 1) proteins: skimmed milk powder, peptone, and yeast extract powder; 2) sugars: glucose, trehalose, galactose, and sucrose; 3) amino acids: arginine, glycine, and glutamic sodium; and 4) sugar alcohols: sorbitol, mannitol, and glycerol. Each protectant was dissolved in deionized water, and sterile saline as the comparison group. All lyophilized protectants are autoclaved (115 °C, 15 min).

2.4 Bacterial suspension preparation and freeze-drying

SMN-LBK was concentrated by centrifugation at 4 °C, 8000 rpm/min for 10 min using a benchtop high-speed cryo-centrifuge 5417R (Eppendorf, Hamburg, Germany) and then resuspended in 0.85% sterile saline for washing. This process was

repeated twice. Bacterial suspension was prepared by combining bacterial precipitates and cryoprotectants in a 1:1 (W/V) ratio. After being equilibrated at 37 °C for 30 min, each solution was added to a lyophilized Celine vial, which was pre-frozen in a refrigerator at -80 °C for 10 h. They were lyophilized for 24 h in a SCIENTZ-18 N freeze dryer (Ningbo Xinzhi Biotechnology, China) with a condenser temperature of -55 °C and pressure less than 0.06 bar.

2.5 Determination of bacterial viability

Live cells were counted before and after lyophilization, and gradient dilutions were made in sterile saline. Next, 100 µL of the diluted solution was spread evenly on MRS solid agar medium using a spreading stick. The plates were then incubated at 37 °C for 48 h. The viability of SMN-LBK was determined by the standard counting method^[24]. To determine the number of bacteria per dilution, the average of three plates was used, with a bacterial count between 30–300 CFU. The formulas for calculating the number of viable bacteria and survival rate are as follows:

$$\text{Number of viable bacteria (CFU/mL)} = \frac{\text{(average number of colonies in 3 replicates of the same dilution/volume of the coated plate)} \times \text{dilution factor}}{1} \quad (1)$$

$$\text{Survival rate (\%)} = \frac{\text{viable cell number after freeze-drying}}{\text{viable cell number before freeze-drying}} \times 100 \quad (2)$$

2.6 Selection of sea buckthorn addition amount

A certain amount of sea buckthorn juice was centrifuged at 8000 rpm/min for 10 min to remove sea buckthorn pomace and sea buckthorn oil. The pH was adjusted to 6.0 with Na₂HCO₃ and different concentrations of sea buckthorn juice, 2%, 4%, 6%, 8%, 10%, and 12%. Freeze-drying survival was used as an indicator to determine the optimal amount of sea buckthorn to be added.

2.7 Effect of single cryoprotectants on SMN-LBK survival

Cryoprotectants including sugars (5%, 10%, 15%, 20%, 25%), proteins (5%, 10%, 15%, 20%, 25%), alcohols (2%, 4%, 6%, 8%, 10%), and amino acids (2%, 4%, 6%, 8%, 10%) were added individually to the bacterial suspensions at a 1:1 (V/V) ratio. Then the mixture was moved to the lyophilized Celine vial for pre-freezing in a refrigerator at -80 °C for 10 h. It was lyophilized for 24 h in the freeze dryer (Ningbo Xinzhi Biotechnology, China) with a condenser temperature of -55 °C and pressure less than 0.06 bar. After lyophilization, SMN-LBK survival was determined by referring to formulae (1) and (2).

2.8 Evaluating the effects of composite protectants on SMN-LBK survival using response surface methodology

Response surfaces are a common technique for analyzing experimental data and simplifying procedures. Each variable in this study had three levels: (-1) low, (0) middle, and (1) high. The independent variables that were used to identify many ideal combinations are displayed in Table 1 in both their actual and coded values.

The independent variables that affect strain survival were the levels of (A) trehalose, (B) sucrose, (C) skim milk powder, and (D) sorbitol. A total of 29 runs were made and the response value (Y)

Table 1 Response surface experimental results

Run	A	B	C	D	Survival rate (%)
	Sucrose (%)	Trehalose (%)	Skimmed milk powder (%)	Sorbitol (%)	
1	10	10	15	6	60.9
2	20	5	15	4	62.2
3	15	10	15	4	83.8
4	20	15	10	6	55.7
5	15	15	15	6	62.2
6	15	15	20	4	76.2
7	10	10	15	2	49.8
8	15	5	15	6	64.8
9	20	10	15	6	68.1
10	15	10	15	4	82.7
11	15	10	15	4	81.2
12	20	15	15	2	62.5
13	15	10	15	4	84.2
14	20	10	15	2	58.4
15	15	5	15	2	45.8
16	20	10	10	4	66.8
17	10	10	10	4	64.3
18	15	5	20	4	74.3
19	15	5	10	4	55.2
20	15	10	20	6	70.7
21	20	15	15	4	74.8
22	10	15	10	4	62.4
23	20	10	20	4	80.1
24	10	5	15	4	54.4
25	15	10	15	4	83.1
26	15	10	10	2	50.6
27	10	10	20	4	70.5
28	10	15	15	4	61.2
29	10	5	15	6	49.6

is the lyophilization survival rate, a quadratic polynomial regression model was assumed. The optimal values of the variables were determined by calculating regression equations and analyzing contour maps and three-dimensional surface maps^[25].

2.9 Optimization of lyophilization conditions

2.9.1 Selection of the ratio of bacteria to protective agent

The bacteria were collected by centrifuging the fermentation broth at 8000 rpm/min for 10 min. The bacterial solution was washed three times with equal volumes of 0.85% sterile saline. Then, the mixture was lyophilized using different ratios of bacterial slurry to protectant solution (1:0.5, 1:1, 1:1.5, 1:2, and 1:2.5, V/V). The optimal ratio was determined based on the lyophilized survival rate.

2.9.2 Selection of freeze-drying thicknesses of the mixture

Based on the pretreatment in the previous step, 0.3 cm, 0.4 cm, 0.5 cm, and 0.6 cm of the mixture were aspirated into lyophilized vials, respectively. The vials were then pre-frozen in a refrigerator at -80 °C for 10 h. Subsequently, the mixture was rapidly transferred to a vacuum freeze dryer and freeze-dried for 24 h.

The optimal thickness of the lyophilization was calculated by determining its lyophilized survival rate.

2.9.3 Selection of pre-equilibration time

Based on the formulation, the mixture was placed into lyophilized vials and equilibrated at different pre-equilibrium (0, 15, 30, 45, 60 min) at 37 °C. After equilibration, the vials were pre-freeze for 10 h under −80 °C. Then they were continuously freeze-dried for 24 h. The optimal equilibration time was screened by calculating the lyophilization survival rate.

2.10 Determination of antioxidant capacity of composite probiotic powder *in vitro*

Determination for DPPH, ABTS, hydroxyl radical scavenging rate (HRSA), and the reducing power of ferrous ions were referred to the reported methods with some modifications^[26-29].

The DPPH radical scavenging rate was calculated as follows:

$$\text{DPPH}(\%) = \left(1 - \frac{A - B}{C}\right) \times 100\% \quad (3)$$

Where A: sample mixed with DPPH solution, B: ethanol mixed with DPPH solution, C: sample mixed with ethanol.

The ABTS radical scavenging activity was calculated as follows:

$$\text{ABTS radical scavenging activity}(\%) = \left(1 - \frac{A - B}{C}\right) \times 100\% \quad (4)$$

Where: A was a sample plus 30 μL of PBS, B was a sample plus 30 μL of ABTS working solution, and C was 3.0 ml of ABTS⁺ working solution plus PBS.

The hydroxyl radical scavenging activity (HRSA) was calculated as follows:

$$\text{HRSA}(\%) = \left(1 - \frac{A - B}{A}\right) \times 100\% \quad (5)$$

Where: A was the mixture in which the sample was replaced by distilled water, and B was the sample.

2.11 Determination of enzyme activities after lyophilization

2.11.1 Preparation of cell-free extracts

Referring to the reported method^[30] with slight modifications, the bacterial solution was centrifuged at 10,000×g for 10 min at 4 °C, and the supernatant was used to determine the enzyme activity. The cells were washed twice with 0.85% sterile saline and then resuspended in an equal amount of the same buffer. Next, 3 mL of cell solution was taken and placed in an ice bath for cell fragmentation using sonication for 3 s with an interval of 9 s, for a total of 6 min. The resulting supernatant was used to determine enzyme activity and protein content.

2.11.2 Determination of lactate dehydrogenase (LDH), pyruvate kinase (PK), hexokinase (HK), and ATPase

Protein concentration was mainly determined by using the Thomas Brilliant Blue staining method^[31]. LDH, PK, HK, and ATPase of composite probiotic powder were determined using commercial kits (Nanjing Jiancheng Bioengineering Research Institute Co., Ltd., Nanjing, China) according to the manufacturer's instructions.

2.12 Stability of freeze-dried composite probiotic powder

The composite probiotic powder was prepared using an optimal combination of cryoprotectants. First, the SMN-LBK strain was mixed with the optimal combination for a mixture, and it was moved to the lyophilized Celine vial for pre-freezing in a refrigerator at −80 °C for 10 h. Then, it was lyophilized for 24 h in the freeze dryer (Ningbo Xinzhi Biotechnology, China) with a condenser temperature of −55 °C and pressure less than 0.06 bar. Secondly, the composite probiotic powder was stored for 60 days at −20 °C, 4 °C, and 25 °C. Finally, referring to formulae (1) and (2), cell viability was measured every 10 days for the first month, followed by one-month intervals.

2.13 Statistical analysis

Data were plotted and analyzed using Origin 9.1 (Originlab Guangzhou Office, Guangzhou, China), Design of Expert 8.0.6 (Stat-Ease, Minneapolis, USA), and SPSS 27.0 (International Business Machines Corporation, Armonk, New York, USA). Values were shown as the mean ± SD of three replicates. The one-way ANOVA was used to analyze the significance of the data. Means with different lowercase letters (a-e) were significantly different ($P < 0.05$).

3 Results and discussion

3.1 Sea buckthorn affected the survival of SMN-LBK

Sea buckthorn addition significantly affected the survival of SMN-LBK (Fig. 1A). The survival of SMN-LBK was increased with the increase in sea buckthorn addition in the range from 2% to 10%. However, the survival of SMN-LBK was significantly reduced in the 12% addition of the sea buckthorn group versus the 10% group. Sea buckthorn has the potential to serve as a carrier for the immobilization of probiotic cells, thereby facilitating a high survival rate of these cells in low-temperature environments^[32]. At lower concentrations, sea buckthorn enhances the proliferation of probiotics and exhibits prebiotic properties. Conversely, elevated concentrations of sea buckthorn have been observed to reduce the number of probiotics, a phenomenon that may be attributed to its acidic nature^[33]. Consequently, a concentration of 10% sea buckthorn was chosen for the preparation of the mixture with SMN-LBK, as it is preferable to the 12% concentration.

3.2 Protective agent improved the survival of SMN-LBK

All cryoprotectants enhanced the survival of the strains compared to sterile saline (0%) (Figs. 1B-1E). Sucrose had a higher survival rate (55.5%) at 15% concentration, followed by 10% glucose, 10% trehalose, 4% peptone, 6% skimmed milk powder, and 4% sorbitol (33%–47%, respectively). However, all others had a less protective effect on SMN-LBK (12%–31%).

Sucrose and trehalose are the most commonly used cryoprotectants, which can slow down the damage under adverse conditions, including low temperature and water loss. They keep the cell membrane at a lower temperature of the liquid crystal-gel phase transition, maintain the liquid crystalline state of the cell membrane, and reduce the likelihood of disrupting the structure and function of the phospholipid layer of the cell membrane from the outside^[34]. The bacteria had a high survival rate of 55.5% and 47.2% with the addition of 15% sucrose and 10% trehalose, respectively (Fig. 1B). It was higher than the optimal

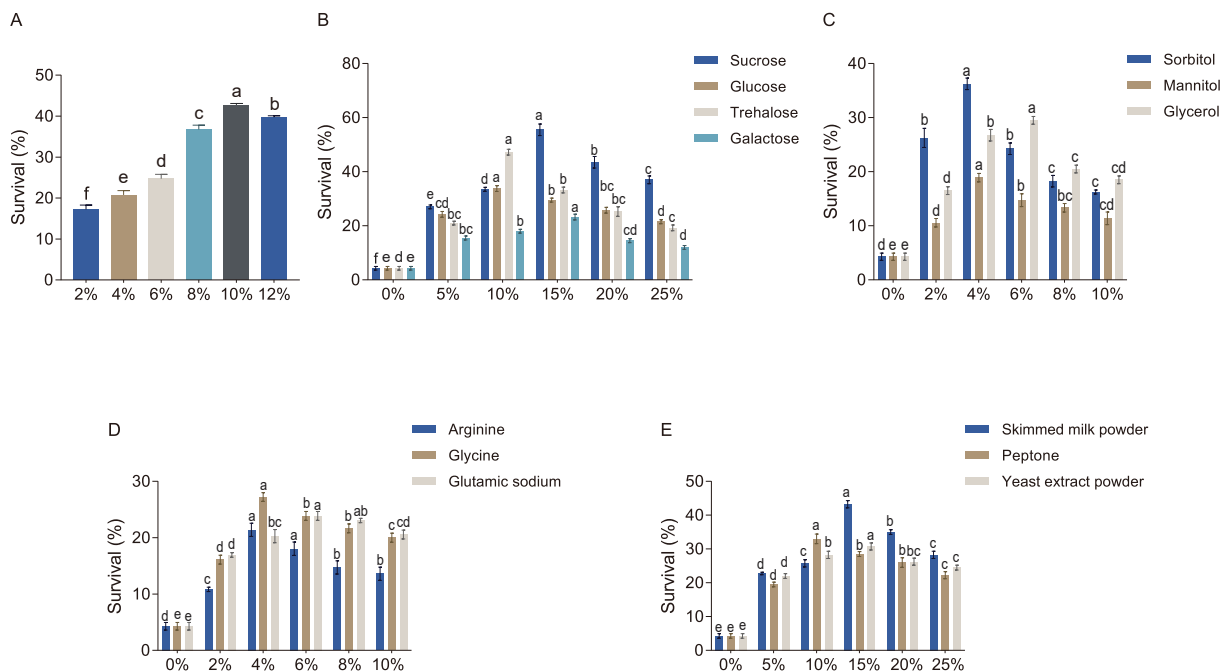


Figure 1 Effects of sea buckthorn addition and cryoprotectants on the survival rate of SMN-LBK. (A) Effects of sea buckthorn addition on the survival rate of SMN-LBK; and (B-E) Effects of four cryoprotectants on the survival rate of SMN-LBK. Different superscript letters on columns indicate significant differences between samples ($P < 0.05$).

concentration; the survival rate reduced with the increase in the concentration of sugars, which were 37% and 19%, respectively. Since the high concentration of sugar accelerates the polymerization process of proteins in the cells and produces a strong glassy structure, it makes the cells difficult to preserve. Hamoudi *et al.* reported that a 23% concentration of disaccharides (such as trehalose and sucrose) increased the survival rate of lyophilized *Geodesis* [35]. Miao *et al.* found that trehalose significantly improved the survival rate of *L. rhamnosus* during freeze-drying [36].

Hydrogen bonds between phosphate groups and water molecules can be effectively replaced by the hydroxyl groups of polyols forming hydrogen bonds with the phosphate groups of lipids. This substitution preserves proteins' and cell membranes' structural and functional integrity [37]. Sorbitol, mannitol, and glycerol are commonly used alcohol protectants. In Fig. 1C, the highest survival rate of 36.3% was observed with the addition of 4% sorbitol, which showed better protective properties than mannitol and glycerol, as well as good stability and antioxidant properties. Sorbitol may offer cell protection due to its ability to scavenge free radicals [38]. *L. plantarum* JH 287 cells that had been freeze-dried exhibited the best survival rate in a mixture containing 10% sorbitol and 10% sucrose [39].

Amino acids are suitable solutes that are essential for the lyophilization process [40]. Most amino acids are hydrophilic and can exist in aqueous solutions as ions or crystals. The amino and carboxyl can react with cooling water molecules to form hydrogen bonds, which raise the solution's viscosity and lessen cell damage [41]. In Fig. 1D, the highest survival rate of 27% was achieved with the addition of 4% glycine, and the protective effect was superior to that of arginine and monosodium glutamate. Amino acids can improve the growth of LAB strains, and the culture medium with aspartic acid significantly increased the lyophilized viability of *L. plantarum* strain [42]. Although amino acids had a protective effect on the lyophilization of SMN-LBK, the protective effect was slightly weaker compared to other

protective agents, and they were not considered to add to the complex protective agents.

Skim milk powder is a commonly used bacterial antifreeze. It produces a protective layer of proteins that protects cells from damage, insulates them from oxygen, and stabilizes the components of the cell membrane [43]. In Fig. 1E, the addition of 15% skim milk powder increased the survival rate to a maximum of 43.2%; however, the rate fell steadily with increasing addition amounts exceeding 10%, ultimately peaking at 28.3%. The addition amount went above 10%, and the survival percentage steadily dropped to 28.3%. Excessive amounts of skim milk powder caused denaturation of proteins, elevated systemic osmotic pressure, and decreased survival of probiotics [44].

Based on the above results, we selected 10% trehalose, 15% sucrose, 15% skimmed milk powder, and 4% sorbitol to prepare the composite cryoprotectant for future trials.

3.3 Response surface methodology to optimize composite protectants

Box-Behnken was created to maximize the mix of cryoprotectants. Table 1 displayed the actual values, and Table 2 showed the outcomes of the ANOVA. Ultimately, the relationship between the dependent variables and the three-dimensional response plots was described by equations with coefficient values. Fitting the quadratic regression equation to the data in Table 2 yields a quadratic polynomial regression equation for bacterial survival (Y) on (A) sucrose, (B) trehalose, (C) skimmed milk powder, and (D) sorbitol: $Y = 83.11 + 4.21A + 5.05B + 5.20C + 4.24D + 0.91AB + 2.18AC - 0.4AD - 4.33BC - 5.67BD - 4.93CD - 9.84A^2 - 9.90B^2 - 3.11C^2 - 14.05D^2$. The selected model has a P -value of < 0.05 , a model F -value of < 0.01 , and the misfit term is not significant ($P > 0.05$) (Table 2). This indicated that the model fits well, but the unknown term interfered little with the experimental results. The model was suitable for the optimization of the protective agent.

The model fully complied with all the design points. The main

Table 2 Variance analysis of response surface fitting regression equation

Source	Sum of squares	df	Mean square	F-value	P-value	
Model	3612.44	14	258.03	107.97	< 0.0001	significant
A: sucrose	251.86	1	251.86	105.39	< 0.0001	**
B: trehalose	312.67	1	312.67	130.83	< 0.0001	**
C: skimmed milk powder	265.03	1	265.03	110.9	< 0.0001	**
D: Sorbitol	183.52	1	183.52	76.79	< 0.0001	**
AB	4.73	1	4.73	1.98	0.1814	
AC	22.81	1	22.81	9.54	0.008	**
AD	0.8	1	0.8	0.34	0.5714	
BC	75.89	1	75.89	31.75	< 0.0001	**
BD	120.91	1	120.91	50.59	< 0.0001	**
CD	34.74	1	34.74	14.54	0.0019	**
A ²	493.34	1	493.34	206.43	< 0.0001	**
B ²	597.57	1	597.57	250.04	< 0.0001	**
C ²	52.31	1	52.31	21.89	0.0004	**
D ²	966.85	1	966.85	404.57	< 0.0001	**
Residual	33.46	14	2.39			
Lack of Fit	28.04	10	2.8	2.07	0.252	not significant
Pure Error	5.42	4	1.36			
Cor Total	3645.89	28				

items A, B, C, and D were highly significant ($P < 0.0001$). The interaction terms AC, BC, BD, and CD were highly significant ($P < 0.0001$) on strain survival, while the interaction terms AB and AD were not significant ($P > 0.05$). Comparing the F -values of the groups, the order of effect of different cryoprotectants on survival was: trehalose > skimmed milk powder > sucrose > sorbitol.

To illustrate the effects of the interactions between the four factors on the survival rate, three-dimensional surface plots and contour analyses between the factors and the response values were plotted using Design Expert 8.0.6 software (Fig. 2). On a surface that was steeper and more sloped, the interactions between the factors had a greater impact on the response values. The contour lines' sparsity and form can serve as indicators of how important the factor interactions are. Except for the interaction term AC, all other contour lines were widely distributed, signifying steeper surfaces.

The optimal cryoprotectants were verified using expectancy function analysis, which identified trehalose at 10.11%, sucrose at 15.34%, skim milk powder at 16.39%, and sorbitol at 3.81%. The optimum satisfaction was 84.5%. Considering the practical feasibility, the optimal process conditions were 10% trehalose, 15% sucrose, 16% skimmed milk powder, and 4% sorbitol. By repeating the experiment triple, the optimization results revealed an average survival rate of 83%, which was comparable to the model's theoretical value, showing that the model may be better employed for the optimization of cryoprotectants for SMN-LBK.

3.4 Optimization of lyophilization conditions for SMN-LBK-sea buckthorn mixture

The lyophilization survival of SMN-LBK showed a tendency to increase and then decrease in the whole range of ratios of the bacterial slurry to the protectant (Fig. 3A). The highest survival rate of 86% was observed at a ratio of 1:1 of bacterial suspension to protectant. It might be that a high concentration of the protective agent led to a decrease in cell permeability and retention of water, and a large number of ice crystals were formed, thus increasing

the rate of cell death. Furthermore, the density of the bacterium was reduced when there was too much protective agent, and the number of viable bacteria per unit volume of the powder was reduced^[45].

As the thickness of the freeze-dried material increased, the survival rate of the bacteria displayed a trend of increasing and then decreasing (Fig. 3B). The highest survival rate of 87.2% was observed when the thickness reached 0.4 cm. A sufficiently thick material reduces the degree to which ice crystals melt and lessens the damage that secondary freezing causes to bacteria. If the thickness is too low, frozen bacteria from the ultra-low temperature refrigerator will easily melt and re-freeze during the vacuum freeze dryer process, damaging the bacteria and lowering their survival rate. Overly thick layers make it harder for the cells to release the binding water during vacuuming, and the bacterial powder's moisture content rises, making it unsuitable for storing and freeze-drying^[46].

The mixing equilibrium time of the bacterial suspension and the composite lyophilized protective agent was in the range of 15 min to 60 min (Fig. 3C). The highest lyophilized cell survival of the strain was 88% at a mixed equilibrium time of 30 min. Before lyophilization, the mixture of bacterial cells and a protective agent was pre-equilibrated, allowing the protective agent to be fully mixed evenly. This allowed the protective agent to thoroughly penetrate the cells, increasing the cells' lyophilization survival rate. An excessively short mixing and equilibrium time between the bacterial slurry and the lyophilized protectant would prevent the bacterial slurry and protectant from fully mixing and from having an effective protective effect on the bacterial cells. An excessively long equilibrium time would also cause the bacterial cells to die at a faster rate due to prolonged exposure to the air.

3.5 Antioxidant and enzyme activities of SMN-LBK-sea buckthorn mixture

To analyze the antioxidant activity of the mixture, DPPH, ABTS+, HRSA, and total reducing capacity were determined. The

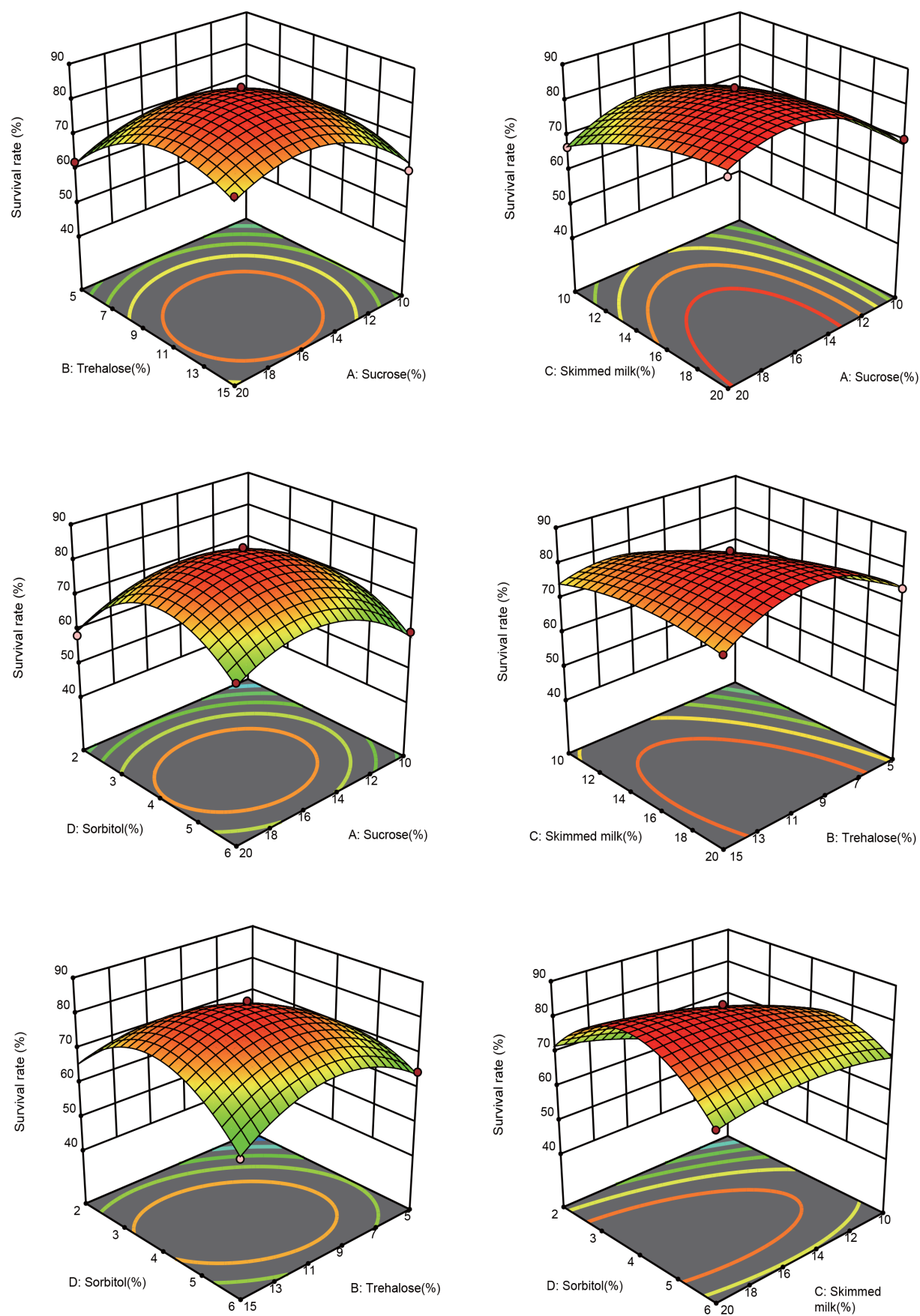


Figure 2 Response surface contour map and stereogram of protective agents.

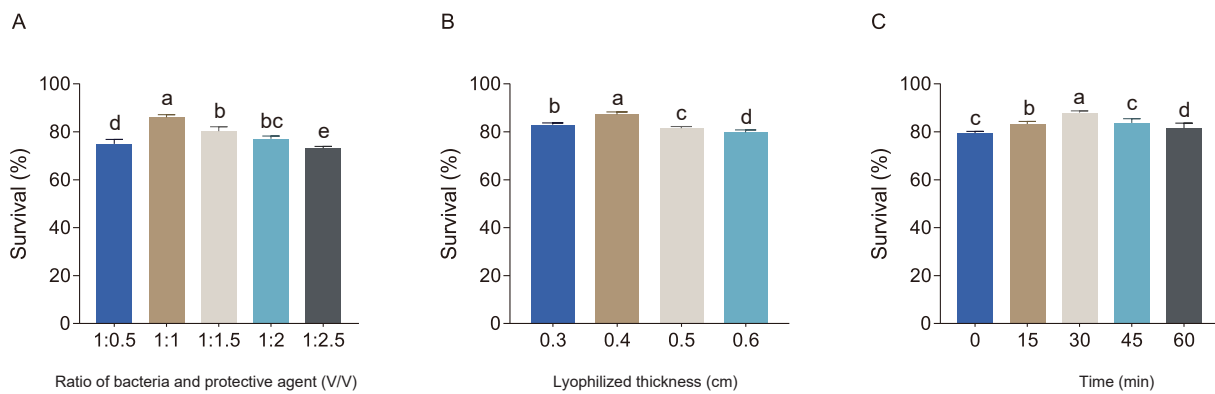


Figure 3 Selection of lyophilization conditions. (A) Bacterial suspension and protectant ratio; (B) Freeze-drying thickness of bacterial suspension; and (C) Pre-equilibration time. Different superscript letters on columns indicate significant differences between samples ($P < 0.05$).

results showed that the free radical scavenging ability of the mixture increases with the concentration of the powder in all four indicators, but the scavenging effect was different (Fig. 4). LAB is usually considered to have high antioxidant activity when the DPPH radical scavenging rate exceeds 30%^[47]. The DPPH radical scavenging rate was up to 72% at the mixture concentration of 5 mg/mL (Fig. 4A), which was significantly higher than the other three antioxidant properties. The content of ABTS+ cationic radicals was 18% (Fig. 4B), and HRSA was up to 28% with increasing concentrations of bacterial powder (Fig. 4C). The most significant free radicals that cause lipid peroxidation are hydroxyl radicals^[48]. Total reducing capacity and antioxidant activity showed a correlation that can be used as a gauge to assess possible antioxidant activity^[29], with an absorbance value of 0.8523 (Fig. 4D). Therefore, the mixture had good antioxidant properties for storage and application.

Changes in LDH, PK, HK, and ATPase activities of the mixture

during the freeze-drying process were determined to analyze the protective effects of composite protectants. The results showed that the mixture with protectants was better than that without protectants (Figs. 4E-4H). An essential enzyme LDH catalyzes the conversion of pyruvate to lactate and was widely present in human tissues, with the highest levels in the kidney, followed by cardiac and bone muscles, and was one of the most vital enzyme families for the anaerobic fermentation of sugar, as well as for gluconeogenesis, with the magnitude of the activity reflecting the energy metabolism and the ability of the strain to produce acid^[49]. The addition of protectants decreased the cell membrane's permeability and the amount of intracellular enzyme leakage but increased the viability of the lactic acid bacteria. The LDH activity was 0.13 U/mgprot without protectants, while the enzyme activity could reach 0.42 U/mgprot with protectants (Fig. 4E). This indicated that LDH was subjected to a certain degree of cellular damage during freeze-drying, which was correlated with the freeze-

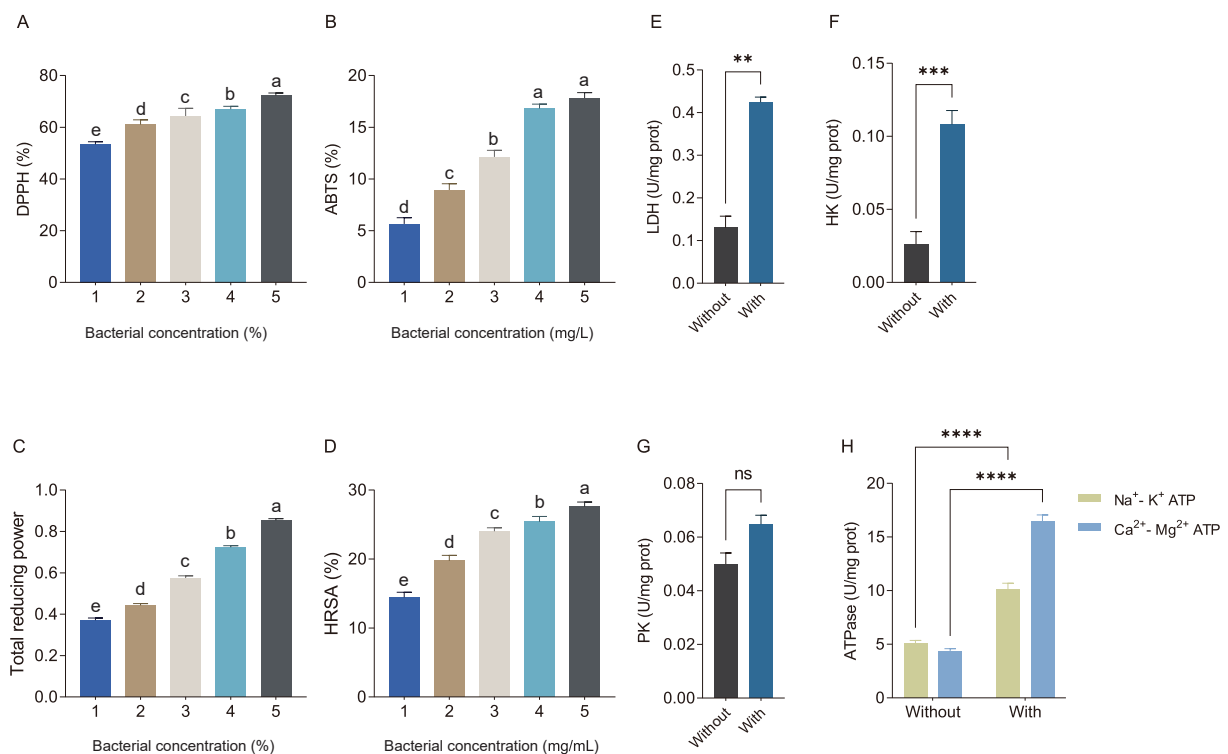


Figure 4 The determination of antioxidant and enzyme activities of the SMN-LBK-sea buckthorn mixture. (A-D) The determination of antioxidant activities of SMN-LBK-sea buckthorn mixture; (E-H) The determination of enzyme activities of SMN-LBK-sea buckthorn mixture. Different superscript letters on columns indicate significant differences between samples ($P < 0.05$).

drying survival rate of SMN-LBK.

HK is the rate-limiting enzyme in the first step of the glycolytic pathway, also known as the glucose sensor, which catalyzes the phosphorylation of hexose, a key entry step in many pathways. The magnitude of its activity correlates with the rate of glucose metabolism in lactic acid bacteria^[50]. The enzyme activity of HK without protectants was 0.03 U/mgprot, and increased to 0.11 U/mgprot with protectants (Fig. 4F). PK catalyzes the conversion of phosphoenolpyruvate to pyruvate in the final step of glycolysis. It is one of the major rate-limiting enzymes in the process of glycolysis and is essential for the synthesis of adenosine triphosphate in all organisms. Therefore, it plays a crucial role in the regulation of cellular metabolism^[51]. The cellular enzyme activity of the mixture after freeze-drying without and with protectants was 0.051 U/mgprot and 0.065 U/mgprot, respectively (Fig. 4G), but there was no significant correlation between the enzyme activities. This suggested that PK might not be a key enzyme for the lyophilization of bacterial strains in the freeze-drying process.

The living activities of biological cells are intimately correlated with the activity of ATPase. The cell-free extract with protectants had higher enzyme activity than that without protectants, and the Na⁺-K⁺-ATPase activity was 5.10 U/mgprot and 10.16 U/mgprot, respectively (Fig. 4H). Na⁺-K⁺-ATPase is a protein that is connected to both cell viability and membrane integrity and is present in the bilayer of the cytoplasmic membrane. It controls the balance of the concentration of ions in and out of the cell and acts as a carrier and enzyme. Moreover, it can catalyze ATP hydrolysis, maintain membrane potential on both sides of the cell membrane, regulate cell osmotic pressure, and provide energy for nutrient absorption^[52]. The activity of Ca²⁺-Mg²⁺-ATPase was 4.34 U/mgprot and 16.42 U/mgprot, respectively (Fig. 4H). Ca²⁺-Mg²⁺-ATPase can catalyze the hydrolysis of ATP in the inner part of the plasma membrane, release energy, drive the regulation of intracellular calcium ion transport, transmit cell signals, etc.^[53]. The results showed that the inactivation of ATPase would lead to a decrease in the activity of the freeze-dried mixture, but the cross-stress of protectants could protect the activity of ATPase and thus protect the activity of the freeze-dried mixture.

3.6 Storage stability of SMN-LBK-sea buckthorn mixture

To evaluate the storage stability of the mixture, we performed a continuous storage experiment for 60 days under -20 °C, 4 °C, and 25 °C. There was a declining trend at all three storage temperatures, with the decline being more pronounced at 25 °C (Fig. 5). This implied that highly elevated temperatures may inflict some damage on the bacteria, thereby drastically lowering their viability. At day 60, the viable bacterial counts were 2.4×10⁹ CFU/g and 1.7×10⁹ CFU/g at -20 °C and 4 °C, respectively, and both of them maintained high viable bacterial counts. After 60 days, the viable bacterial counts at -20 °C and 4 °C decreased, but the viable bacterial counts at -20 °C were more stable. Therefore, -20 °C is the ideal storage temperature for the mixture. Temperature during storage affects the viability of bacteria after freeze-drying, and a Maillard reaction occurs under high-temperature drying conditions. This reaction is a spontaneous reaction of carbonyl groups in amino acids and reducing sugars at low reactant concentration and low temperature^[20]. Additionally, high temperature promotes oxidation and accelerates the movement of water in and out of cells, both of which are detrimental to bacterial survival^[54, 55].

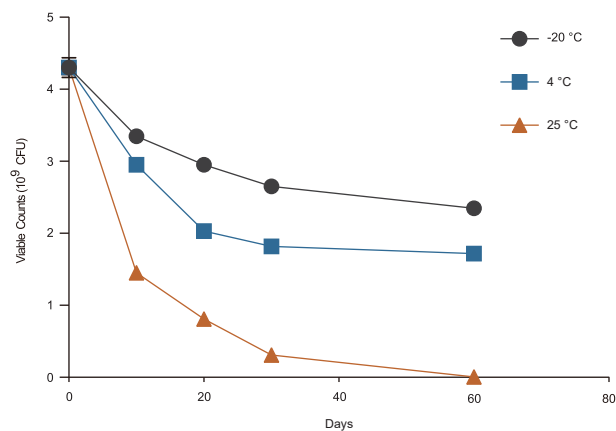


Figure 5 Effect of storage time and temperature on the viability and survival rate of the SMN-LBK-sea buckthorn mixture.

4 Conclusions

In this study, a lyophilized mixture of SMN-LBK and sea buckthorn was prepared, and its properties were evaluated through assessments of antioxidant activity, enzyme activity, and storage stability. The optimal protectants identified for the mixture included 10% trehalose, 15% sucrose, 16% skimmed milk powder, and 4% sorbitol. Under the conditions of a 1:1 ratio of bacterial suspension to protectants, a lyophilization thickness of 0.4 cm, and a pre-equilibration period of 30 minutes, the strains exhibited a maximum survival rate of 88%. These protectants enhanced both the antioxidant and enzyme activities of the lyophilized powder mixture, resulting in excellent storage stability. Looking ahead, there remains significant potential for the utilization of sea buckthorn in the food industry. The development of a diverse array of new products, including sea buckthorn jam, cookies, wine, beverages, dietary supplements, and bakery leavening agents, can be pursued to satisfy consumer demand for natural and nutrient-rich food products. Furthermore, sea buckthorn warrants further investigation in clinical trials to explore its potential applications in the treatment of chronic diseases, promotion of skin health, enhancement of eye health, and its prospective roles in drug development. Overall, this study offers valuable insights into the product development of sea buckthorn, emphasizing its properties that align with both food and medicinal applications and paving the way for innovative uses of probiotics. The findings provide a theoretical foundation for the freeze-drying process of the SMN-LBK-sea buckthorn mixture and its application in functional foods.

Data availability

Not applicable.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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Author contribution statement

Xingyu Huang: Investigation, Formal analysis, Writing-original draft, and Visualization. **Zhexin Fan:** Supervision and Resources. **Wenjing Wang:** Formal analysis, Visualization and Software. **Na Liu:** Investigation and Methodology. **Qingqing Yan:** Investigation and Visualization. **Xiushan Tan:** Resources. **Liusen Song:** Investigation and Methodology. **Baokun Li:** Supervision, Funding Acquisition and Resources. **Zhifeng Fang:** Supervision, Conceptualization, Funding Acquisition, Resources, and Review & editing.

Informed consent

Not applicable.

Ethics statement

Not applicable.

Use of AI statement

None.

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