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The Impact of Formulation and Freeze Drying on the Properties and Performance of Freeze-Dried *Limosilactobacillus reuteri* R2LC

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Abstract: Freeze drying is a commonly used method for preserving probiotic bacteria and live biotherapeutic products. Before drying, the bacterial cells are formulated with a lyoprotectant, and the design of these two process steps are crucial to achieve a high-quality product. There are several factors that may affect the biological and physicochemical properties of the freeze-dried cells and we have used a Design of Experiment approach to investigate the effects of formulation and freeze-drying parameters on properties and performance of *Limosilactobacillus reuteri* R2LC. The biological characteristics of the dried bacteria were evaluated by measuring cell survival, metabolic activity and stability, and physicochemical characteristics were studied using visual inspection, differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and analysis of residual moisture content and bacterial aggregation. A comparison between the lyoprotectants trehalose and sucrose showed that the latter gave better freeze-drying survival, metabolic activity, and storage stability. We also want to highlight that there was a correlation between bacterial concentration, metabolic activity, and aggregation of bacteria, where a higher concentration (10^{10} CFU/mL) resulted in both higher metabolic activity and aggregation. Several other process and formulation factors affected both the biological and physicochemical properties of freeze-dried *L. reuteri* R2LC and it could be concluded that care must be taken to develop a production method that generates a product with high and consistent quality. These results may, or may not, be strain specific.

Keywords: probiotics; live biotherapeutics; *Limosilactobacillus reuteri* R2LC; formulation; freeze-drying; metabolic activity; viability; annealing; storage stability

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1. Introduction

With the growing interest in microorganisms with health-promoting properties in the pharmaceutical and food industries, robust production methods are in demand. Achieving high viability (number of live bacteria), storage stability, and metabolic activity (vitality) is required for high-quality probiotic and live biotherapeutic products (LBP). Commercially, probiotic strains are distributed either in the form of food/probiotic-based food products (such as yogurt and nutrition bars) or dried and formulated as capsules, such as tablets, chewing gums, oil suspensions, or powders, e.g., packaged in sachets [1].

Freeze drying is a widely used method to stabilize probiotics or LBPs, which removes water from the microorganisms to achieve preservation of their viability, metabolic activity, and improve the storage stability of the products. During the freeze-drying process, the cells are exposed to stressful conditions such as oxidative stress, osmotic stress, dehydration, and damage due to ice crystal formation [2,3]. Therefore, it is important to optimize

the process parameters with the aim of alleviating the stress and obtaining potent, reproducible, and stable products. The freeze-drying process consists of three steps: (1) Freezing; (2) Primary drying: removal of frozen water by sublimation; and (3) Secondary drying: removal of residual/unfrozen water by desorption. Several factors affect the properties of freeze-dried bacteria, such as the cultivation method (e.g., substrate, initial cell concentration, and time of harvest) [4–6], type and concentration of lyoprotectant [7,8], and the freeze-drying process [9,10]. The lyoprotectant plays an important role in protecting the properties (biological and physicochemical) of freeze-dried products. It is known that many anhydrobiotic plants [11] and animals [12,13] are protected from desiccation by accumulating disaccharides. Therefore, to mimic this strategy, disaccharides such as sucrose [7,14] and trehalose [15,16] are often used as lyoprotectants. These types of lyoprotectants protect the freeze-dried cells by forming an amorphous glassy matrix and high viscosity that prevent the cells from mechanical damage and degradation of proteins [17], and preserve the functionality/bioactivity and stability of the cells after freeze drying [18,19]. The effectiveness of the lyoprotectant is also dependent on the glass transition temperature of the sugars, a temperature at which material transition to a glassy or viscous state [20].

One way to promote the efficiency of freeze drying is to include an annealing step in the process, which involves keeping the product at a temperature above the glass transition temperature for about 15 min, resulting in increased ice crystal growth and ultimately lower water content in the dried product [21]. Previously, it has been shown that annealing with trehalose phosphate as the lyoprotectant is effective in achieving an efficient drying and increased stability of a strain of *Lactobacillus acidophilus* [22]. As a general goal, low water activity (<0.2) and residual water content (<5%) are required to obtain robust and stable freeze-dried probiotics [23].

The aim of this study was to investigate the influence of formulation and annealing on biological properties and physicochemical characteristics of freeze-dried *Limosilactobacillus reuteri* R2LC. This is a well-studied strain that has been shown to ameliorate acetic acid or DSS-induced colitis in rats [24] and mice [25]. In a recent paper, R2LC has been shown to mediate its anti-inflammatory effect and modulate the intestinal microbiota by transmitting probiotic signals to immune cells present in Peyer's patches [26]. The strain also has antimicrobial activity mediated by a polyketide [27]. We used a Design of Experiment (DOE) approach to evaluate the effects of different lyoprotectants (sucrose and trehalose) with concentrations between 10 and 20%, different bacterial concentrations (10^9 and 10^{10} CFU/mL), and annealing. The biological properties analysed included viability, metabolic activity, and stability, and the physicochemical characteristics analysed included water content, matrix structure, cake appearance, glass transition temperature, and aggregation of the bacteria.

2. Materials and Methods

2.1. Experimental Design

To investigate the impact of formulation and freeze drying on the biological and physicochemical properties of R2LC, a Design of Experiments (DoE) approach was taken. In this study, four different factors were investigated: type of lyoprotectant (sucrose and trehalose), lyoprotectant concentration (10, 15, and 20%), bacterial concentration (10^9 and 10^{10} CFU/mL), and the freeze-drying process (with and without annealing). MODDE version 13 (Sartorius Data Analytics, Umeå, Sweden) was used to generate a full factorial screening study with 24 independent runs (Table S1).

2.2. Cultivation of Limosilactobacillus reuteri R2LC

A glycerol stock (15%) working cell bank was prepared and kept at −80 °C. The main cultivation was performed in 8 L bioreactor (Belach Bioteknik, Stockholm, Sweden) which was inoculated to a starting OD_{600} of 0.2. Sterilization of the media component (yeast extract and yeast peptone) and bioreactor was conducted at 121 °C for 15 min; and then 5 L of growth medium (0.15 g $MnSO_4 \cdot H_2O$, 25 g $C_2H_3NaO_2$, 10 g K_2HPO_4, 5 mL Tween

80, 0.5 g L-cysteine hydrochloride monohydrate, 0.5 g $MgSO_4 \cdot 7H_2O$, 11.5 g sodium citrate tri-basic monohydrate, glucose (12.5 g/L), 50 g yeast extract (autoclaved) and 50 g yeast peptone (autoclaved)) was filtered sterilized into the bioreactor. The pH and temperature of the bioreactor was set to 5.7 and 37 °C, respectively, and a stirring speed of 200 rpm was used. Cultivation was carried out until the glucose concentration was not detectable (approximately 11–12 h). Glucose concentration was measured using a blood glucose tester (HemoCue AB, Ängelholm, Sweden).

2.3. Formulation/Sample Preparation

The cells were harvested and concentrated in two steps. First, the bacterial cells were concentrated 10× with a 750 kDa hollow fiber filter (GE Healthcare Biosciences Corp, Piscataway, NJ, USA). Secondly, cells were washed and concentrated sevenfold with 50 mM phosphate buffer and concentrated to around 10^{10} CFU/mL. One part of the concentrate was diluted to 10^9 CFU/mL in 50 mM phosphate buffer. The two cell suspensions were mixed 1:1 with different lyoprotectants (20–40% solutions of sucrose and trehalose). The final formulations were composed of 10^{10} or 10^9 CFU R2LC/mL in 10, 15, or 20% of sucrose or trehalose (in total 12 combinations). The bacterial suspensions were set on hold at 4 °C overnight and stirred at 200 rpm for 10 min before the filling process. The bacterial suspensions were kept under stirring during the filling process. A volume of 1 mL was filled into 2R glass vials (Schott Pharma, Lukacshaza, Hungary), and half stoppered in preparation for lyophilization.

2.4. Freeze-Drying

The formulated cells were freeze dried using a Christ, Epsilon 2-6D LSCplus (Martin Christ Gefriertrocknungsanlagen, Munich, Germany). Samples were divided in two sets: with and without an annealing step. Freezing took place by decreasing the temperature from 20 °C to −45 °C at a rate of 0.36 °C/min and holding it for 3 h. Annealing was introduced by increasing the temperature to −15 °C, hold for 3 h, and then decreased to −45 °C at a rate of 0.36 °C/min and holding it for 2 h. Primary drying was performed by increasing the temperature from −45 °C to −20 °C at a rate of 0.63 °C/min and decreasing the chamber pressure to 0.10 mBar and holding those settings for 45 h. The secondary drying was carried out by decreasing the pressure to 0.01 mBar and increasing the temperature to 20 °C at a rate of 0.17 °C/min and holding it for 25 h [28]. The vial stoppers were closed at 750 mBar. The vials were crimped with an aluminum cap and stored at −80 °C until used.

2.5. Visual Inspection

The cake appearance was evaluated on the basis of a scoring system (1–5). Score (1) signifies an intact and homogenous cake, (2) intact, heterogenous cake structure and dark yellow bottom, (3) shrinkage of the cake around the edges without change in the height, (4) partially collapsed cake (40–60%), and (5) a collapsed cake (>60%) (Table S2) [29].

2.6. Aggregation

The samples with lower (10^9 CFU/mL) and the highest (10^{10} CFU/mL) bacterial concentrations were diluted using saline solution (0.9% NaCl) 1:100 and 1:200 times respectively. The diluted samples were analysed using flow cytometry with a Cytoflex S (Beckman Coulter, Indianapolis, IN, USA). The setting parameters were as follows: gain was set to 1000 for side scatter (SSC) and forward scatter (FSC), and the threshold was set to 50,000 for FSC and 10,000 for SSC. For each sample, 10^5 events were recorded. Similar conditions were used for analysis of a control that consisted of standard diameter of 6 µm polystyrene beads (L34856) that had been diluted 1:10 in saline. The recorded data was then analysed using the FlowJo software version 10 (FlowJo, Ashland, OR, USA). After the selection of log scale, gating was selectively drawn around the 6 µm beads pattern and this gating was used for all samples. Particles above 6 µm were considered as aggregates. The

aggregation (%) was calculated as (no. of events counted in the bead region × no. of events in the bacterial region)/100. All measurements were completed in duplicates.

2.7. Water Content

The water content of the freeze-dried bacteria was measured using Karl Fischer Titration (Metrohm Ltd., Herisau, Switzerland) [30]. First, Hydranal-Methanol dry (blank) was measured in triplicates; the value was below 0.3%. The weight of empty and freeze-dried vials was measured, then bacterial cells were resuspended into dry Hydranal-methanol. Again, the weight was measured prior to incubation at room temperature for 1 h (to extract the water from the sample). Each sample was measured in triplicate.

2.8. Scanning Electron Microscopy (SEM)

Samples were collected by cutting out a vertical cross section of the freeze-dried cakes. These cross sections were positioned on circular stubs with double-sided adhesive carbon tape, and then placed in a Quanta 250 FEG ESEM Scanning Electron Microscope (FEI, Brno, Czech Republic). SEM images were collected with a Large Field Detector (LFD), using a 5 kV beam, under 60 Pa pressure. A minimum of two spots in the top and bottom parts of the freeze-dried cake were examined, employing magnifications of 100×, 500×, 1000×, and 2500× [28]. Porosity (%) of the material was evaluated by analysing the scanning electron microscopy images with ImageJ version 1.54e (National Institutes of Health, Bethesda, MD, USA) [31,32].

2.9. Differential Scanning Calorimetry

The glass transition temperature of freeze-dried *L. reuteri* R2LC-formulations were measured using differential scanning calorimetry (DSC; DSC 1 STARe system, Mettler Toledo, Columbus, OH, USA). The instrument was calibrated prior to the measurements; zinc and indium were used as reference materials. The reference material checks (T_m and ΔH) were within acceptable limits. The freeze-dried cake was cut horizontally and crushed; samples were taken in the range of 1–5 mg. The samples were weighed in aluminium pans, sealed with a lid, and an empty and sealed aluminium pan was used as a reference. To measure the glass transition temperature, samples were first kept at 20 °C and held for 1 min with a rate of 10 °C/min, then heated up to 90 °C at the heating rate of 10 °C/min. Secondly, the samples were again cooled to 20 °C and thereafter heated to 130 °C with a heating rate of 10 °C/min [28]. The measurements were completed under a nitrogen gas flow of 50 mL/min and each sample was analysed in duplicate.

2.10. Cell Viability

Freeze-dried bacteria were rehydrated in 1 mL MRS broth, tenfold serially diluted and plated on MRS (Sigma, Saint Louis, MO, USA) agar plates, which were incubated anaerobically at 37 °C for 48 h. The colonies were counted in an interval of 20–200 colonies per plate and survival % was calculated in terms of before and after freeze drying. The measurements were completed in duplicate.

$$Xv = \frac{N}{0.1}10^{y}$$

where *Xv* is cell viability, *N* is the number of colonies counted per plate, *y* is the tenfold dilution plated, and 0.1 represents 0.1 mL (100 µL) plated.

2.11. Metabolic Activity

L. reuteri R2LC produces lactic acid as a metabolic end product and this acidification was used to evaluate the metabolic activity of freeze-dried cells [33]. The freeze-dried cells were rehydrated with 1 mL of MRS broth and incubated at 37 °C without agitation for 2 h. To analyse the acidification rate, pH was measured at room temperature, at time points 0,

1, and 2 h. The high (10^{10} CFU/mL) R2LC concentration was diluted 10 times with MRS media before the cultivation.

2.12. Storage Stability

Accelerated storage stability of all freeze-dried variants was investigated by incubation of the vials at 37 °C for 4 weeks. Visual appearance, viability, metabolic activity, and residual water content were analysed at the timepoints 0, 2, and 4 weeks.

2.13. Statistical Analysis

The data were analysed statistically using analysis of variance (ANOVA) with post hoc Tukey HSD multiple comparisons test, Kruskal–Wallis test, and Pearson's correlation analysis in JASP version 0.17.2.1 (University of Amsterdam, Amsterdam, The Netherlands) [34]. To verify that the data complied with the assumptions of ANOVA, normal Q-Q plots were visually inspected and Shapiro–Wilk tests were performed. All tests were performed with $\alpha = 0.05$. Raincloud plots were used to visualize the results for individual analysis, probability distribution, and key summary statistics (mean and relevant confidence intervals) [35]. The effect size was described in terms of partial eta squared (η^2_p), which describes the relative proportion variance explained by each factor included in the analysis.

3. Results

Limosilactobacillus reuteri R2LC was grown in a bioreactor, whereafter it was formulated and lyophilized using a total of 24 different combinations of bacterial concentrations, lyoprotectants, and freeze-drying programs (Figure 1). The formulated and freeze-dried R2LC was subjected to evaluation of both physicochemical and biological outcomes.

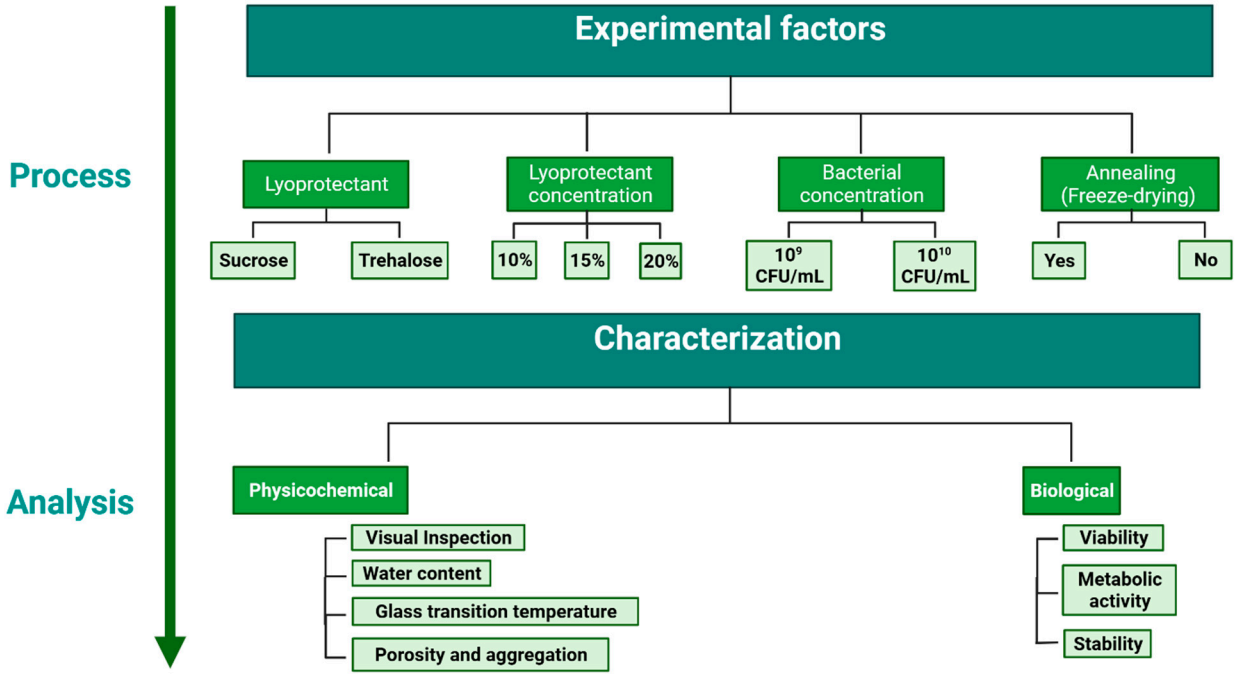

Figure 1. Outline of the study including tested variables and characterization of freeze-dried *L. reuteri* R2LC.

3.1. Effects of Experimental Factors on Physicochemical Properties of Lyophilized R2LC

The effects of varying the selected experimental factors (type and concentration of lyoprotectant, bacterial concentration, and freeze-drying program) on the measured physicochemical outcomes are summarized in Tables 1–5 and Figures 2–6. Statistically significant differences, including the associated effect sizes (η^2_p) are highlighted in the tables and detailed post hoc tests are included in the supplementary data sheets.

Table 1. Main effects and interactions of the experimental factors affecting water content. *p*-values in bold indicate statistically significant effects.

Factors	F	*p*	η^2_p
Lyoprotectant type	14.495	**0.004**	0.617
Annealing	6.035	**0.036**	0.401
Lyoprotectant concentration	33.819	**<0.001**	0.883
Bacterial concentration	27.711	**<0.001**	0.755
Lyoprotectant × Annealing	5.326	**0.046**	0.372
Lyoprotectant × Lyoprotectant concentration	9.511	**0.006**	0.679

All factors significantly affected the water content of freeze-dried R2LC, and in particular using the highest concentration of sucrose (20%) gave an elevated water content (t = 0.271, p_{tukey} = 0.004) (Figure 2 and Table S3). Also, the concentration of bacteria had an impact and 10^9 CFU/mL resulted in significantly higher water content than 10^{10} CFU/mL using sucrose (t = 4.933, p_{tukey} = 0.004) as lyoprotectants (Figure 2). Furthermore, annealing resulted in significantly elevated water content in the highest (20%) sucrose concentration (t = 10.829, p_{tukey} = 0.047).

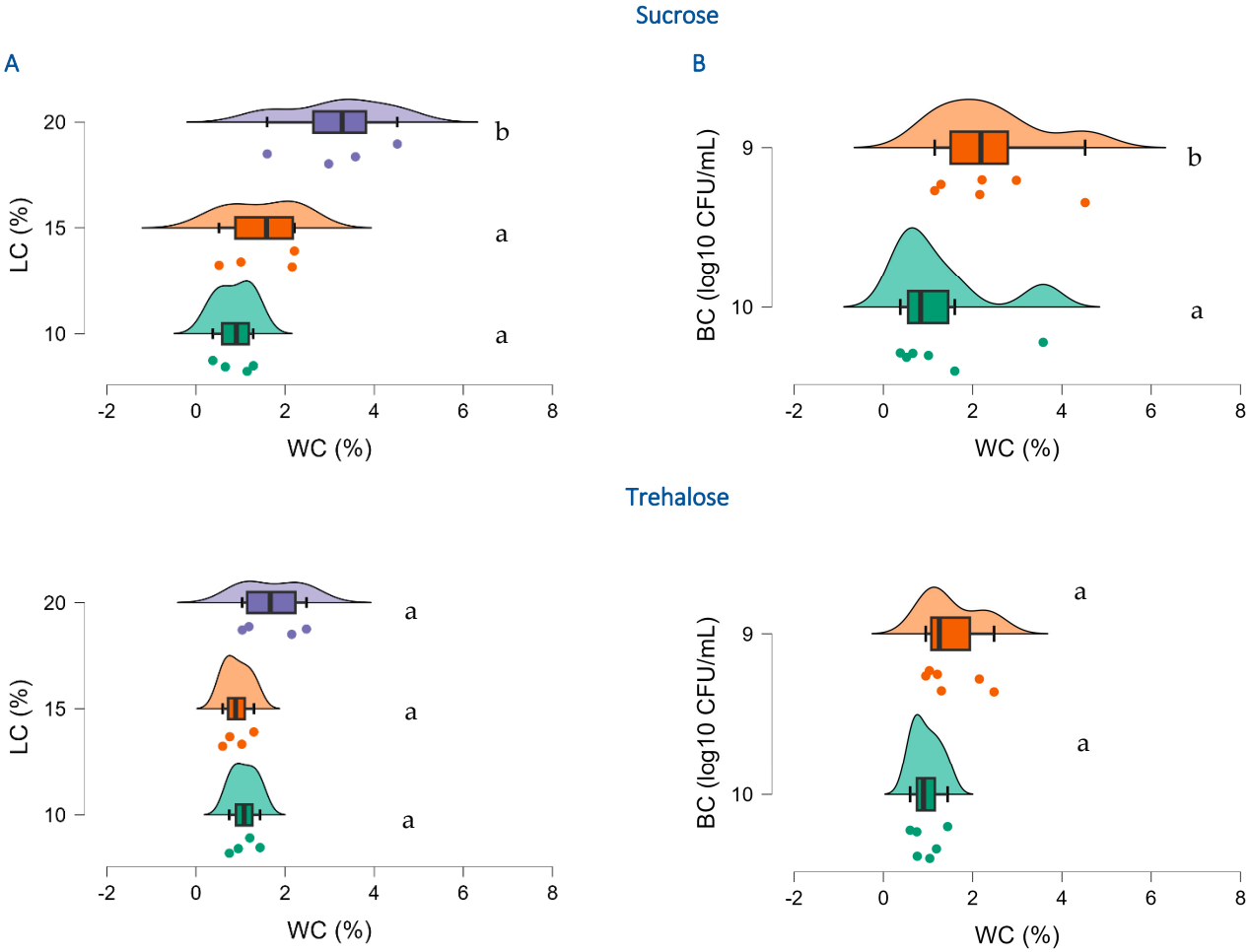

Figure 2. Effect of lyoprotectant concentration (**A**), bacterial concentration (**B**) and type of lyoprotectant (sucrose and trehalose) on water content of freeze-dried R2LC. LC: Lyoprotectant concentration; BC: Bacterial concentration, and WC: Water content. Datasets with different letters are significantly different ($p < 0.01$; data presented in **A**,**B** are not compared). (**A**): violet represents 20%, orange 15% and green 10% lyoprotectant concentration, respectively; (**B**): orange represents 10^9 CFU/mL and green 10^{10} CFU/mL R2LC.

ANOVA revealed significant effects of lyoprotectant type and bacterial concentration on glass transition temperature (Tg) (Tables 2 and S4). The Tg for the samples containing trehalose was around 30–40 °C higher than for samples with sucrose (Figure 3). Post hoc analysis showed that the type of lyoprotectant had a large impact on the glass transition temperature, where trehalose resulted in a significantly higher Tg than sucrose (t = 15.930, p_{tukey} < 0.001) (Figure 3). In addition, the concentration of bacteria had a significant (p_{tukey} = 0.010) effect on the glass transition temperature (Table 2 and Figure 3). Also, a significantly higher Tg was observed at high (10^{10} CFU/mL) concentration of both sucrose and trehalose (Figure 3B).

Table 2. Main effects and interactions of the experimental factors affecting glass transition temperature (Tg) of freeze-dried R2LC. *p*-values in bold indicate statistically significant effects.

Factors	F	*p*	η^2_p
Lyoprotectant type	253.768	**<0.001**	0.861
Annealing	0.462	0.500	0.011
Lyoprotectant concentration	1.467	0.243	0.067
Bacterial concentration	7.305	**0.010**	0.151
Annealing × Bacterial concentration	10.298	**0.003**	0.201
Lyoprotectant concentration × Bacterial concentration	4.422	**0.018**	0.177

Note. Type III Sum of Squares.

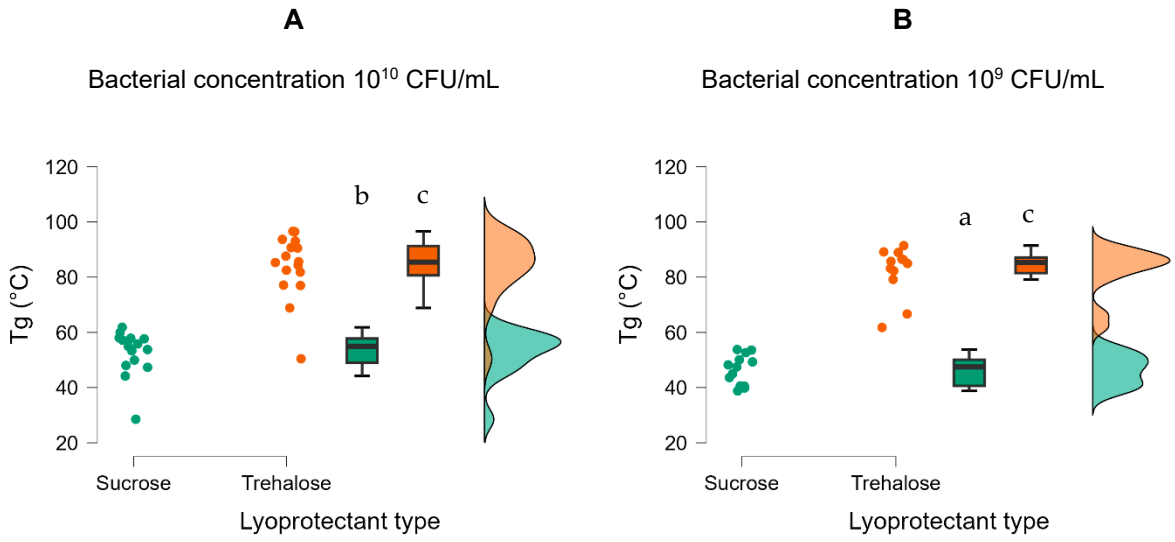

Figure 3. Effect of high (**A**) and low (**B**) bacterial concentration and type of lyoprotectant on glass transition temperature of freeze-dried R2LC. Datasets with different letters are significantly different ($p < 0.01$; data presented in **A**,**B** are compared). Green represents samples with sucrose and orange represents samples with trehalose as lyoprotectant.

The lyoprotectant type, lyoprotectant concentration, and bacterial concentration significantly impacted the aggregation of freeze-dried R2LC (Table 3). Post hoc analysis revealed that the difference between sucrose and trehalose was statistically significant t = −2.329, p_{tukey} = 0.022 (Table S5). Also, the higher concentration of bacteria (10^{10} CFU/mL) promoted aggregation and gave approximately 2 times more aggregates than for the lower concentration of bacteria, when using formulations with both sucrose (t = 19.757, p_{tukey} < 0.001) and trehalose (t = 15.634, p_{tukey} < 0.001) as a lyoprotectant (Figure 4 and Table S5).

Table 3. Main effects and interactions of the experimental factors affecting aggregation of freeze-dried R2LC. *p*-values in bold indicate statistically significant effects.

Factors	F	*P*	η^2_p
Lyoprotectant type	5.425	**0.022**	0.062
Annealing	2.250	0.137	0.027
Lyoprotectant concentration	6.418	**0.003**	0.135
Bacterial concentration	625.580	**<0.001**	0.884
Lyoprotectant × Annealing	13.163	**<0.001**	0.138
Lyoprotectant × Bacterial concentration	7.851	**0.006**	0.087

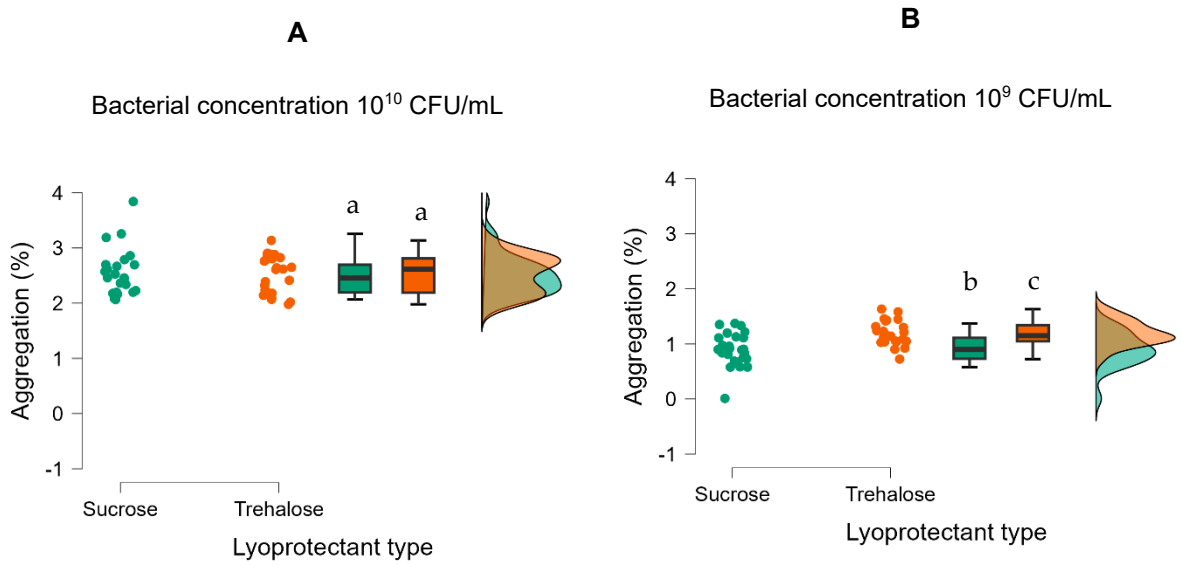

Figure 4. Effect of low (**A**) and high (**B**) bacterial concentration and type of lyoprotectant (sucrose and trehalose) on aggregation of freeze-dried R2LC. Datasets with different letters are significantly different ($p < 0.01$; data presented in **A**,**B** are compared). Green represents samples with sucrose and orange represents samples with trehalose as lyoprotectant.

Scanning electron microscopy was carried out for the characterization of the matrix structure and observation of bacterial structures. The matrices for all samples were amorphous (examples are seen in Figure 5). Bacterial cells were not visible in formulations with the lower bacterial concentration (Figure 5A,B) because they were well-covered by the matrix (Figure 5A), while for the higher bacterial concentration, the cells were noticeable under the surface of the matrix (Figure 5C,D; shown by a red arrow). In addition, matrix porosity was determined by analysing the SEM images, and an ANOVA showed that there were no significant differences between the samples (Supplementary Table S6).

The cake appearance, which was observed visually, was significantly affected by the lyoprotectant concentration and bacterial concentration ($p < 0.001$ and $p = 0.008$ respectively) according to a Kruskal–Wallis test (Table 4). In addition, Tukey's post hoc tests showed that lower (10^9 CFU/mL) R2LC concentration and the highest lyoprotectant concentration (20%) promoted a partial or fully collapsed cake using both sucrose and trehalose as lyoprotectants (Table S2).

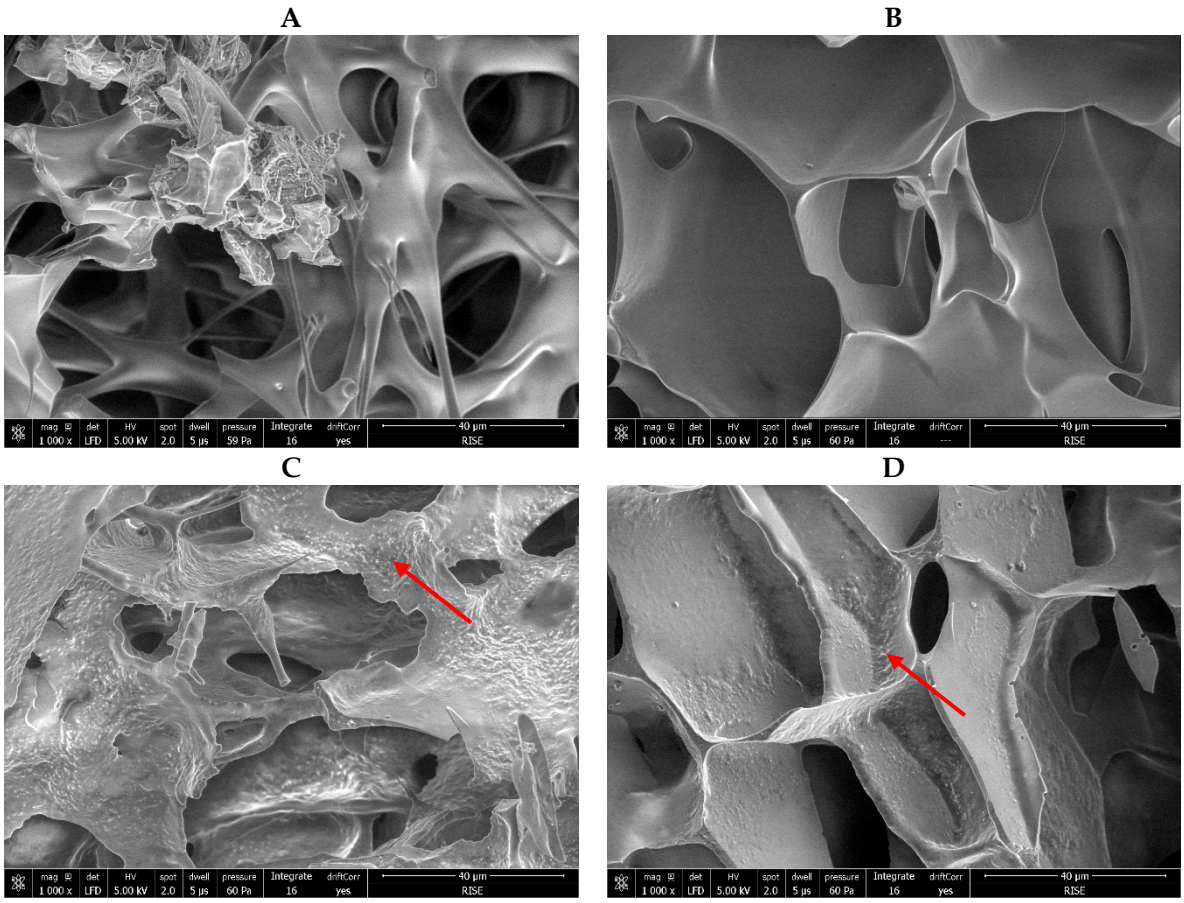

Figure 5. Examples of scanning electron microscopy (SEM) images of freeze-dried R2LC. All samples contain 10% lyoprotectant and were dried without an annealing step. (**A**) Sucrose with 10^9 CFU/mL; (**B**) Trehalose with 10^9 CFU/mL; (**C**) Sucrose with 10^{10} CFU/mL; (**D**) Trehalose with 10^{10} CFU/mL, bacterial cells embedded in the matrix shown by red arrows. All images have 1000× magnification.

Table 4. Main effects of the experimental factors affecting cake appearance. *p*-values in bold indicate statistically significant effects.

Factor	Statistic	df	*p*
Lyoprotectant type	0.069	1	0.792
Annealing	0.140	1	0.709
Lyoprotectant concentration	23.985	2	**<0.001**
Bacterial concentration	6.979	1	**0.008**

3.2. Effect of Accelerated Storage on Water Content of Freeze-Dried R2LC

To investigate the effect of accelerated storage on water content, the samples were stored at 37 °C and water content was measured after 2 weeks. We observed that samples with the highest lyoprotectant concentration (20%) and lower concentration of bacteria (10^9 CFU/mL) had increased water content after storage, when using both sucrose and trehalose as lyoprotectants ($p_{tukey} < 0.001$) (Table 5, Figure 6, and Table S8).

Table 5. Main effects and interactions of the experimental factors affecting water content of freeze-dried R2LC during accelerated storage. *p*-values in bold indicate statistically significant effects.

Factors	F	*p*	η^2_p
Lyoprotectant type	1.970	0.163	0.015
Annealing	0.005	0.943	<0.001
Lyoprotectant concentration (%)	38.179	**<0.001**	0.372
Lyoprotectant × Annealing	15.335	**<0.001**	0.106
Bacterial Concentration	63.277	**<0.001**	0.329
Annealing × Bacterial Concentration	3.450	0.066	0.026
Lyoprotectant concentration (%) × Bacterial Concentration	9.938	**<0.001**	0.134

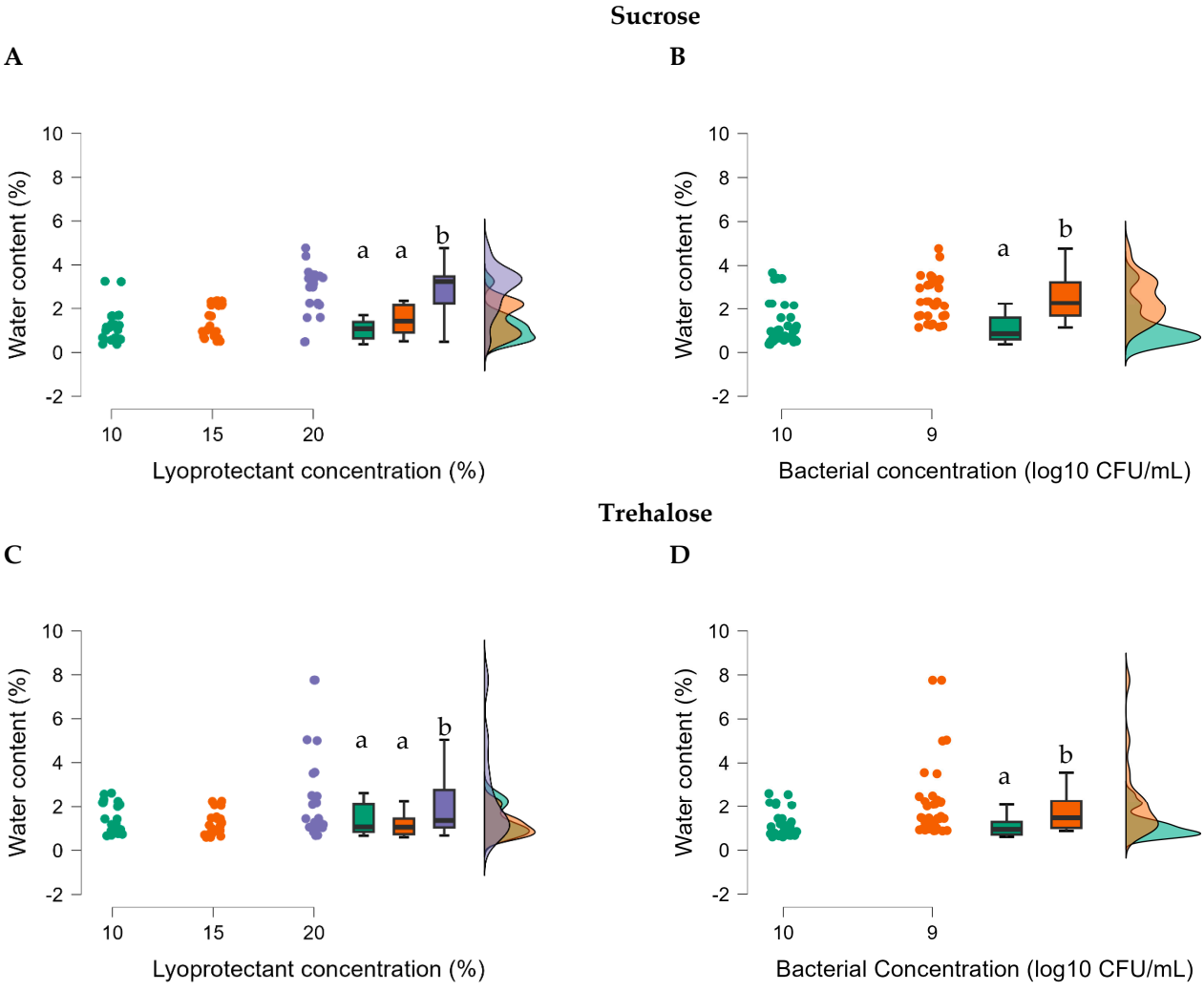

Figure 6. Accelerated storage: Effect of the different lyoprotectant types of sucrose (**A**,**B**) and trehalose (**C**,**D**) and interactions with lyoprotectant concentrations and bacterial concentrations on water content of freeze-dried R2LC. Datasets with different letters are significantly different ($p < 0.01$; data presented in **A**,**C** are compared and **B**,**D** are compared). (**A**,**C**), violet represents 20%, orange 15% and green 10% lyoprotectant concentration, respectively; (**B**,**D**), orange represents 10^9 CFU/mL and green represents 10^{10} CFU/mL R2LC.

3.3. Effects of Experimental Factors on Biological Properties of Lyophilized R2LC

The impact of the experimental factors on the biological outcomes of freeze-drying survival, metabolic activity, and storage stability was evaluated. We first investigated how well the bacteria survived the lyophilization process, and it was found that the type

and concentration of lyoprotectant and annealing had an impact. All factors apart from bacterial concentration significantly affected the freeze-drying survival ($p < 0.001$) (Table 6). Also, all factors except lyoprotectant concentration significantly affected the metabolic activity of freeze-dried R2LC ($p < 0.001$) (Table 7). Post hoc analysis showed that sucrose resulted in significantly higher survival than trehalose (t = 7.007, $p_{tukey} < 0.001$) (Figure 7 and Table S9). In addition, annealing had a positive effect when using sucrose but not trehalose as lyoprotectant (t = 8.292, $p_{tukey} < 0.001$) (Figure 8E,F). A final observation was that the concentration of lyoprotectant had an impact on the survival, with 15% being most efficient for sucrose and 20% for trehalose (t = 4.133, $p_{tukey} = 0.003$) (Figure 7A,B).

Table 6. Main effects and interactions of the experimental factors affecting freeze-drying survival of freeze-dried R2LC. *p*-values in bold indicate statistically significant effects.

Factors	F	*p*	η^2_p
Lyoprotectant type	49.097	**<0.001**	0.584
Annealing	12.986	**<0.001**	0.271
Lyoprotectant concentration	3.568	**0.039**	0.169
Bacterial concentration	1.279×10^{-4}	0.991	3.654×10^{-6}
Lyoprotectant × Annealing	22.274	**<0.001**	0.389
Lyoprotectant × Lyoprotectant concentration	5.383	**0.009**	0.235
Lyoprotectant × Bacterial concentration	3.188	0.083	0.083
Annealing × Bacterial concentration	3.838	0.058	0.099

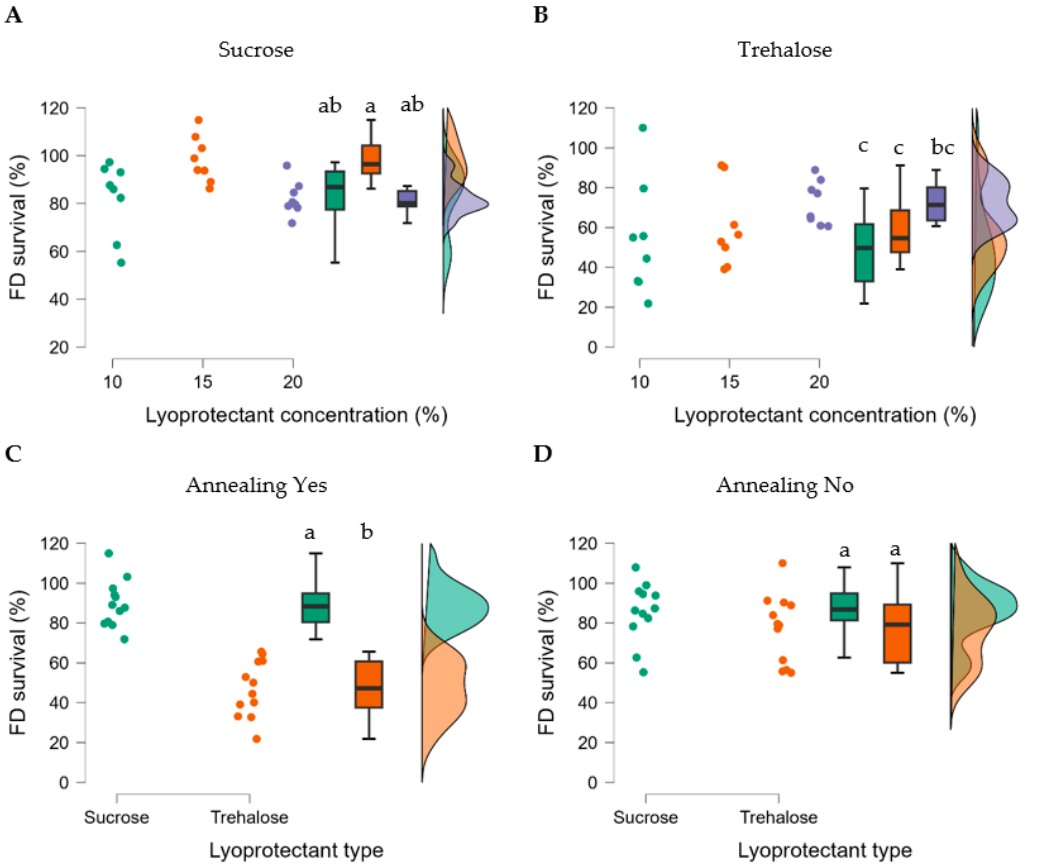

Figure 7. Effect of lyoprotectant sucrose (**A**) and trehalose (**B**), with annealing (**C**), and without annealing (**D**) on freeze-drying survival. Datasets with different letters are significantly different ($p < 0.01$; data presented in **A**,**B** are compared and **C**,**D** are compared). (**A**,**B**), violet represents 20%, orange 15% and green 10% lyoprotectant concentration, respectively; (**C**,**D**), orange represents samples with sucrose and green represents trehalose as lyoprotectant.

Furthermore, the metabolic activity of the freeze-dried R2LC was investigated and it was shown that sucrose gave a higher metabolic activity than trehalose (t = 43.608, $p_{tukey} < 0.001$) (Figure 8, Table 7, and Table S10). In addition, using an annealing step had a positive impact when using sucrose (t = 16.428, $p_{tukey} < 0.001$) but not trehalose (t = 0.159, $p_{tukey} = 0.999$) as the lyoprotectant (Figure 8B,D). Additionally, a higher bacterial concentration gave an increased metabolic activity (t = 34.811, $p_{tukey} < 0.001$) (Figure 8A,C).

Table 7. Main effects and interactions of the experimental factors affecting freeze-drying metabolic activity of freeze-dried R2LC. *p*-values in bold indicate statistically significant effects.

Factors	F	*p*	η^2_p
Lyoprotectant type	1901.639	**<0.001**	0.982
Annealing	137.569	**<0.001**	0.797
Lyoprotectant concentration	2.150	0.132	0.109
Bacterial concentration	1211.809	**<0.001**	0.972
Lyoprotectant × Annealing	132.329	**<0.001**	0.791
Annealing × Bacterial concentration	47.846	**<0.001**	0.578
Lyoprotectant concentration × Bacterial concentration	5.708	**0.007**	0.246

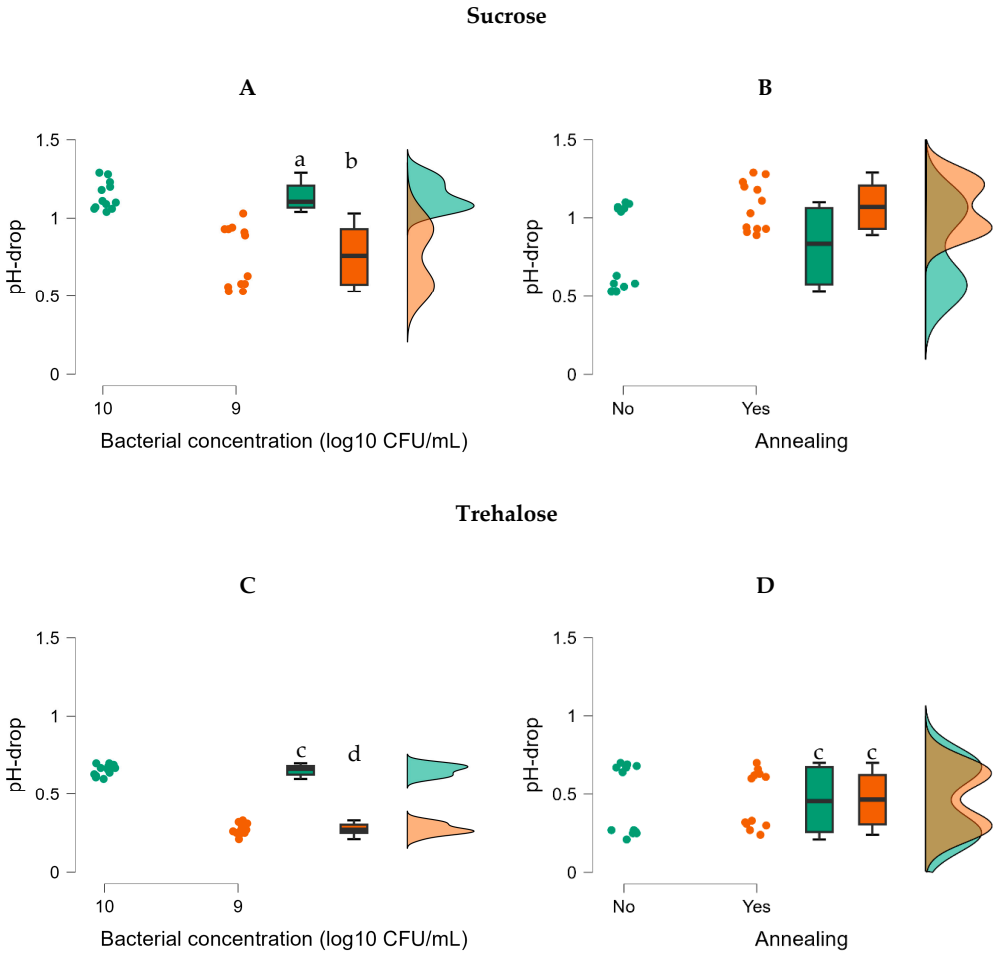

Figure 8. Effect of lyoprotectant, annealing, and bacterial concentration on metabolic activity. Datasets with different letters are significantly different ($p < 0.01$; data presented in **A**,**C** are compared and **B**,**D** are compared). (**A**,**C**), orange represents 10^9 CFU/mL and green represents 10^{10} CFU/mL R2LC; Figure 6B,D, orange represents samples with annealing step and green represents samples without annealing step.

3.4. Effect of Accelerated Storage Stability on Biological Properties

Storage stability of the freeze-dried R2LC samples was investigated after incubation at 37 °C (a temperature resulting in an accelerated decline in activity) for 2 weeks. As shown in Table 8, Table S11 and S12, both types of lyoprotectant and bacterial concentration had a significant impact on survival and metabolic activity. Post hoc Tukey tests revealed that sucrose gave significantly better survival (t = 4.412, $p_{tukey} < 0.001$) and often more than 2 times higher metabolic activity (t = 2.444, $p_{tukey} = 0.033$) than trehalose (Figure 9). Interestingly, 10–15% sucrose gave the best stability for the lower bacterial concentration, while 15–20% sucrose was better for the higher bacterial concentration (Figure 9A,C). Trehalose gave better stability for the highest bacterial concentration than for the lower concentration (Figure 9B,D). Furthermore, the survival was significantly lower for the trehalose-containing formulations compared to the formulations with sucrose.

Table 8. Main effects and interactions of the experimental factors affecting biological properties (A) storage survival and (B) metabolic activity of freeze-dried R2LC storage stability at week 2. *p*-values in bold indicate statistically significant effects.

A			
Factors	**F**	***p***	**η^2_p**
Lyoprotectant type	19.470	**<0.001**	0.357
Annealing	3.154	0.084	0.083
Lyoprotectant concentration	2.324	0.113	0.117
Bacterial concentration	5.445	**0.025**	0.135
Lyoprotectant × Annealing	7.925	**0.008**	0.185
Lyoprotectant × Lyoprotectant concentration	3.609	**0.038**	0.171
Lyoprotectant × Bacterial concentration	6.463	**0.016**	0.156
Annealing × Bacterial concentration	3.086	0.088	0.081
Lyoprotectant concentration × Bacterial concentration	9.728	**<0.001**	0.357
B			
Factors	**F**	***p***	**η^2_p**
Lyoprotectant type	5.971	**0.033**	0.352
Annealing	1.097	0.317	0.091
Lyoprotectant concentration	2.675	0.113	0.327
Bacterial concentration	45.088	**<0.001**	0.804
Lyoprotectant × Bacterial concentration	4.133	0.067	0.273
Lyoprotectant concentration × Bacterial concentration	11.184	**0.002**	0.670

3.5. Correlation between Physicochemical and Biological Outcomes

Finally, potential correlations between the physicochemical and biological outcomes were investigated. This analysis was exploratory and should be regarded as such. There was a positive correlation between freeze-drying survival and metabolic activity (Pearson's r = 0.463, $p < 0.001$), as well as the physicochemical parameter aggregation correlating positively with metabolic activity (Pearson's r = 0.503, $p < 0.001$) (Table 9, whilst Tg correlated negatively with both freeze-drying survival (Pearson's r = −0.602, $p = 0.002$), and metabolic activity (Pearson's r = −0.551, $p = 0.005$). In addition, correlations between physicochemical parameters were seen (Table 9).

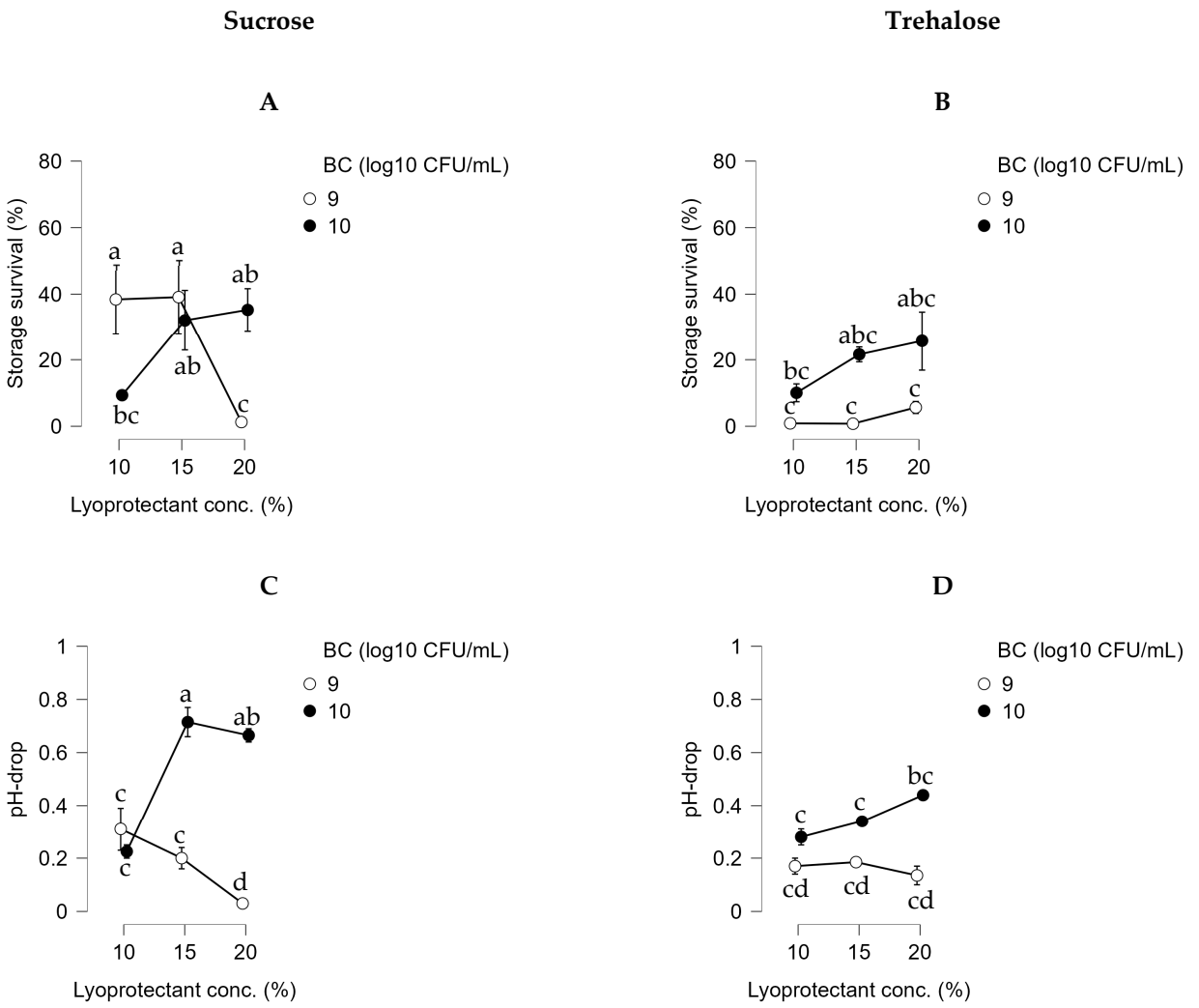

Figure 9. Evaluation of accelerated stability after 2 weeks. Effects of different factors and their interactions on survival (**A**,**B**) and metabolic activity (**C**,**D**) of freeze-dried R2LC. Datasets with different letters are significantly different ($p < 0.01$; data presented in **A**,**B** compared and **C**,**D** are compared).

Table 9. Pearson's correlations between physicochemical and biological outcomes. Statistically significant values ($p < 0.05$) are highlighted with bold figures.

Pearson's Correlations			
		Pearson's r	***p***
Metabolic activity	FD survival	0.463	**<0.001**
Metabolic activity	Aggregation	0.503	**<0.001**
Metabolic activity	Porosity	0.282	0.181
Metabolic activity	Water content	0.063	0.770
Metabolic activity	Glass transition temperature	−0.551	**0.005**
Metabolic activity	Cake appearance	−0.118	0.426
FD survival	Aggregation	0.056	0.707
FD survival	Porosity	0.352	0.091
FD survival	Water content	0.203	0.341
FD survival	Glass transition temperature	−0.602	**0.002**
FD survival	Cake appearance	0.045	0.759
Aggregation	Porosity	0.233	0.273
Aggregation	Water content	−0.432	**0.035**
Aggregation	Glass transition temperature	0.254	0.232
Aggregation	Cake appearance	−0.306	**0.035**
Porosity	Water content	−0.214	0.316

Table 9. *Cont.*

Pearson's Correlations			
		Pearson's r	***p***
Porosity	Glass transition temperature	−0.142	0.508
Porosity	Cake appearance	−0.088	0.682
Water content	Glass transition temperature	−0.537	**0.007**
Water content	Cake appearance	0.613	**0.001**
Glass transition temperature	Cake appearance	−0.124	0.564

Evaluation of the results from the stability study showed that survival and metabolic activity had a positive correlation (Pearson's r = 0.798, $p < 0.001$), but there was no direct correlation between metabolic activity and survival with residual water content (%) (Table 10).

Table 10. Pearson's correlations between physicochemical and biological properties after the accelerated stability study. Statistically significant values ($p < 0.05$) are highlighted with bold figures.

		Pearson's r	***p***
Metabolic activity	Water content	−0.174	0.143
Metabolic activity	Survival	0.798	**<0.001**
Water content	Survival	−0.054	0.522

4. Discussion

Formulation and process parameters are known to play an important role in achieving freeze-dried probiotics with high viable counts, stability, and metabolic activity (vitality). Many studies of the impact of different lyoprotectants and process parameters have previously been carried out [6,8,36–38], but mostly they have focused on the evaluation of either biological or physicochemical properties of the freeze-dried product. We therefore decided to carry out a comprehensive investigation of the effects of various production factors on both physicochemical and biological properties of freeze-dried *Limosilactobacillus reuteri* R2LC. The freeze-drying process includes a wide range of variables and our goal was to use a standard method and evaluate a limited number of factors representing different aspects of the process. These included (i) the lyoprotectant for which both the type of protecting sugar and its concentration were evaluated; (ii) the concentration of bacteria; and (iii) an annealing step in the freeze-drying program (Figure 1).

The biological properties, i.e., freeze-drying survival, metabolic activity, and storage stability, are the main attributes that contribute to the quality and potency of the probiotic product. Type of sugar was the only factor that had a significant effect on all three quality attributes (Tables 6–8) and sucrose performed better overall than trehalose (Figures 7–9). Contrary to this, there are several publications that describe that trehalose provides a better protection of freeze-dried organisms and proteins than sucrose [39–43]. The reason for this discrepancy is not known, but it may be an effect specific to *L. reuteri* or the strain used (R2LC). The composition of lyoprotectants could be more complex than the pure sugar formulations used in the present study and many researchers have achieved good results using combinations of different protectants such as gelatine, skim milk, glucose, sucrose, and trehalose [44,45].

Lyoprotectant concentration also affected the biological properties, but the only significant effect was seen on freeze-drying survival (Tables 6–8). However, the effect of sucrose concentration varied greatly and interacted with the bacterial concentration (Figure 9). Using 10% sucrose gave a poor stability of the highest concentration of bacteria while 20% sucrose worked well for this bacterial concentration but gave very poor stability and collapsed cakes when used in combination with the low concentration of bacteria (Figure 9, Table S7). Overall, 15 or 20% sucrose combined with high bacterial concentration gave the best stability. This indicates that a high concentration of sucrose is needed to protect a

high concentration of bacteria, but also that a high concentration of bacteria can stabilize the cake with a high concentration of sucrose. It has previously been shown that different concentrations and types of lyoprotectants affect the biological parameters. Jawan et al. (2022) investigated the effect of different types (monosaccharides, disaccharides, sugar alcohol, complex media) and concentrations (5, 10, and 20%) of lyoprotectants on cell viability and antimicrobial activity of freeze-dried *Lactococcus lactis* Gh1, and they observed that galactose resulted in the highest freeze-drying survival followed by trehalose and peptone. Storage stability was also investigated and viability varied significantly between different lyoprotectant types and concentrations [46].

The products with high bacterial concentration generally had higher metabolic activity than the products with low concentration (diluted to the same concentration in the assay). Interestingly, both a high concentration of bacteria and high metabolic activity correlated with aggregation of the bacteria and, to the best of our knowledge, this type of correlation has not previously been reported. We also observed a positive effect of annealing on the biological outcomes when using sucrose but not trehalose as a lyoprotectant. This is in accordance with the effect of annealing on freeze-dried *Lactobacillus acidophilus* previously described by Ekdawi-Sever et al. [22].

This work highlights the importance of optimizing the lyoprotectant, its balance with the bacterial concentration, and an evaluation of annealing as part of the freeze-drying process. Several factors affect the quality of the freeze-dried bacteria and many of these effects can also be strain-specific. To achieve a product with sufficient and consistent quality, knowledge about the strain selected for the product concept is therefore key in developing a production method that is specifically optimized for the intended product as well as the intended application and indication.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol3040092/s1, Table S1: DOE: Full factorial screening with 24 independent runs; Table S2: Description of the cake appearance and their corresponding scoring numbers; Table S3. Tukey post-hoc tests: effects and interactions of the factors on water content of freeze-dried R2LC; Table S4. Tukey post-hoc tests: effects and interactions of the factors on Tg of freeze-dried R2LC; Table S5. Tukey post-hoc tests: effects and interactions of the factors on aggregation of freeze-dried R2LC; Table S6. Porosity of freeze-dried R2LC and an ANOVA and Tukey post-hoc tests: effects and interactions of the factors on porosity of freeze-dried R2LC; Table S7. Tukey post-hoc tests: effects and interactions of the factors on cake appearance of freeze-dried R2LC; Table S8. Accelerated stability- Tukey post-hoc tests: effects and interactions of the factors on water content of freeze-dried R2LC; Table S9. Post-Hoc test to evaluate the effect of all variables on freeze-drying survival of freeze-dried R2LC; Table S10. Post-Hoc test to evaluate the effect of all variables on freeze-drying metabolic activity of freeze-dried R2LC; Table S11. Post-Hoc test to evaluate the effect of accelerated storage stability (week 2) on survival; Table S12. Post-Hoc test to evaluate the effect of accelerated storage stability (week 2) on metabolic activity.

Author Contributions: Conceptualization, N.T., L.R., A.M.F. and S.R.; Methodology, N.T., E.S.C., S.K., A.M.F. and S.R.; Formal analysis, N.T., D.T.O. and E.S.C.; Investigation, N.T., Z.G., D.T.O. and S.K.; Resources, A.M.F. and S.R.; Data curation, N.T., E.S.C. and S.R.; Writing—original draft, N.T.; Writing—review & editing, D.T.O., E.S.C., L.R., A.M.F. and S.R.; Visualization, N.T.; Supervision, L.R. and S.R.; Project administration, N.T.; Funding acquisition, L.R., A.M.F. and S.R. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: SR is shareholder of Ilya Pharma. NT, LR and SK have stock options and are employees of Ilya Pharma.

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