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Freeze-Dried Preservation of *Carnobacterium maltaromaticum* CNCM I-3298 and Investigation of the Underlying Mechanisms

Fernanda Fonseca *, Pascale Lieben, Xavier Wood †, Stéphanie Cenard and Stéphanie Passot

INRAE, AgroParisTech, UMR SayFood, Université Paris-Saclay, 91120 Palaiseau, France; pascale.lieben@inrae.fr (P.L.); xavier.wood@gmail.com (X.W.); scenard@icloud.com (S.C.); stephanie.passot@inrae.fr (S.P.)

* Correspondence: fernanda.fonseca@inrae.fr; Tel.: +33-18-910-1112

† Current address: Beiersdorf, Beiersdorfstrasse 3, 22529 Hamburg, Germany.

Featured Application

This study outlines the production conditions of stable freeze-dried *Carnobacterium maltaromaticum* concentrates, intended for use in food control, probiotic supplementation, and other applications. It also provides a better understanding of the degradation mechanisms and cellular components most affected by the stabilisation process, as well as the appropriate formulation conditions.

Abstract

Carnobacterium maltaromaticum is a lactic acid bacterium with growing applications in food biopreservation, probiotics, and microbial time-temperature integrators for tracking food quality throughout the cold chain. To develop efficient, ready-to-use dried concentrates for these applications, this study evaluated the impact of different protective formulations on the freeze-drying and storage stability of *C. maltaromaticum* CNCM I-3298 and explored the underlying molecular mechanisms. The formulations included sucrose, trehalose, and trehalose combined with maltodextrins. Sodium ascorbate was added to the most promising formulations for its potential antioxidant effect. Specific acidifying activity measurements before and after freezing, after freeze-drying and during storage at 25 °C revealed a gradual loss of bacterial activity following freeze-drying and storage. Sucrose, and trehalose with sodium ascorbate provided the best and outstanding protection. Increasing the glass transition temperature using trehalose or trehalose–maltodextrin matrices did not improve stability at 25 °C. Based on prior studies of proteins, we hypothesized that fast relaxation dynamics contribute to the degradation of cells in the glassy state. Fourier transform infrared micro-spectroscopy revealed that freeze-drying primarily affected nucleic acids, proteins (amide I and II), and cell wall components. Storage caused minor additional changes. First results relating to sodium ascorbate's positive effect when added to trehalose and some spectral features in the fingerprint region need further investigation.

Keywords: freeze-drying; lactic acid bacteria; food protection; formulation; cell injury; FTIR spectroscopy; glassy state; glass transition temperature

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1. Introduction

Carnobacterium maltaromaticum (formerly *Carnobacterium piscicola*) is a lactic acid bacterium (LAB) that has been isolated from various sources, including fish intestines [1],

chilled meat packed under vacuum or in a modified atmosphere [2], seafood [3,4], poultry [5] and dairy products [3,6]. It is a psychrotrophic, freeze-tolerant microorganism that produces lactic acid like other LAB, as well as bacteriocins and organic acids (formate and acetate) under vacuum-packed storage conditions relevant to food [7]. Its ability to produce these antimicrobial compounds explains *C. maltaromaticum*'s effectiveness as a biopreservation agent [8,9]. It is also relevant from a technological perspective in terms of probiotic supplementation of fish [10] and farm feeds [11], as well as the fermentation of vegetables (radish) [12]. Furthermore, due to its responses to temperature variations in terms of growth and metabolic activity being like those of spoilage and pathogenic bacteria, *C. maltaromaticum* has also been used as the active core of LAB-based Time-Temperature Integrators (TTIs) to track food quality throughout the cold chain [13].

Using *C. maltaromaticum* in various applications (fermentation, TTI, bioprotection and probiotics) requires producing ready-to-use bacterial concentrates, preferably in a dehydrated form [14]. Previous studies have investigated *C. maltaromaticum* fermentation conditions to maximize cell concentrations and acidifying activity [15,16]. *C. maltaromaticum* exhibits high resistance to freeze-thawing, a trait that has been attributed to its high content of unsaturated fatty acids [17,18]. However, little research has concerned the dehydration response of Carnobacteria. Available data suggest that *C. divergens* is sensitive to spray drying [19], and our preliminary results on *C. maltaromaticum* indicated that it is also susceptible to freeze-drying and storage (poster at a conference, [20]).

Freeze-drying and subsequent storage in a dry state expose cells to environmental stresses (osmotic, mechanical, and oxidative), which can damage cellular components and lead to loss of function and cell death. To help bacteria overcome these stresses and improve their dehydration resistance, the addition of protective molecules to cell concentrates before freeze-drying is a cost-effective approach. An efficient combination of molecules must preserve the cells' biological activity through dehydration and storage while providing an acceptable physical structure (i.e., a non-collapsed matrix) [21]. Protective molecules preserve cells in one or more of the following ways: by replacing water lost during drying (e.g., sugars and polyols); by minimizing molecular mobility through increased matrix viscosity (e.g., polymers and disaccharides with high glass transition temperatures); and by limiting oxidation reactions (e.g., antioxidants) [21–23]. However, the efficacy of a protective molecule may vary with the microorganism, formulation composition, process step, and process operating conditions (e.g., the thermal history of the product) [24,25].

This study aimed to investigate the efficiency of the commonly used molecules in protecting lactic acid bacteria (LAB) against freeze-drying and storage in *C. maltaromaticum*. Concentrates were produced under industrial-scale conditions, with controlled pH, temperature and harvest time, and freeze-drying was conducted on a pilot scale. The formulations were selected based on the protective mechanisms of each molecule, covering the three main mechanisms: sucrose and trehalose (two disaccharides that replace water and vitrify); two maltodextrins with high glass transition temperatures (to enhance vitrification); and sodium ascorbate (an antioxidant). Fourier transform infrared micro-spectroscopy was employed to gain insight into injury and protection sites within the cells.

2. Materials and Methods

The experimental approach used in this study is presented in Figure 1.

2.1. Production and Storage Conditions of Freeze-Dried Carnobacterium maltaromaticum CNCM I-3298 Concentrates

Carnobacterium maltaromaticum CNCM I-3298 (Institut Pasteur, Paris, France) was used in this study, and the stock cultures were stored at −80 °C. Inocula were precultured twice

at 30 °C before being inoculated into a 2.2 L bioreactor (LSL-Biolafitte, Saint-Germain-en-Laye, France) containing 1.9 L of culture medium. The preculture and culture medium were composed of 15 g·L^{-1} trehalose (TrehaTM, Hayashibara, Tokyo, Japan), 10 g·L^{-1} proteose peptone (Oxoid, Basingtoke, Hampshire, UK), 5 g·L^{-1} yeast extract (Oxoid, Basingtoke, Hampshire, UK), 5 g·L^{-1} Tween 80 (VWR, Leuven, Belgium), and sterilized at 120 °C for 15 min.

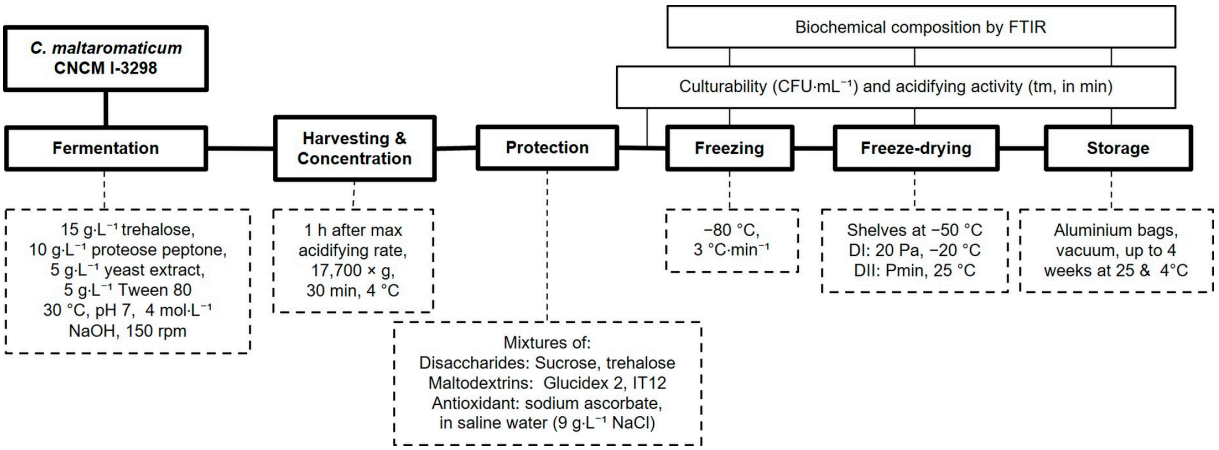


Figure 1. The experimental approach used in this study.

Fermentation was conducted at 30 °C with the pH maintained at 7 by the automatic addition of 4 M NaOH and constant agitation at 150 rpm to ensure homogeneous conditions. The consumption of NaOH was recorded throughout the process and used to calculate the acidification rate. One hour after the maximum acidification rate was attained, cells were collected by centrifugation (Avanti J2-21M/E centrifuge; Beckman Coulter, Fullerton, CA, USA) at 17,700× g for 30 min at 4 °C. The harvested cell pellets were then resuspended at 4 °C in a sterile protective aqueous solution at a ratio of 2:1 (w/w, protective solution to cell pellet).

Different molecules were tested as protective agents: two disaccharides: trehalose (TrehaTM, Hayashibara, Tokyo, Japan), and sucrose (VWR, Leuven, Belgium) largely used for preserving cells and their biological constituents against process stress, maltodextrin Glucidex® IT 12 (Dextrose equivalent (DE) of 12; Roquette, Lestrem, France) and maltodextrin Glucidex® 2 (DE of 2; Roquette, Lestrem, France), which are two food polymers; sodium ascorbate (Royal DSM N.V., Limburg, The Netherland), a water-soluble antioxidant that has long been used in the food industry. The protective solutions were prepared in saline solution (9 g·L^{-1} of NaCl) and sterilized. The compositions of the protective solutions used in this study are presented in Table A1.

The protected cell concentrates were aliquoted into 4 mL vials (1 mL filled volume) and frozen at −80 °C. The vials were then transferred to pre-cooled shelves at −50 °C in pilot-scale freeze-dryers (SMH 15 and SMH 90 (Usifroid, Elancourt, France)). Following a 1 h holding period at −50 °C, the chamber pressure was reduced to 20 Pa, while the shelf temperature was raised to −20 °C at a rate of 0.2 °C·min^{-1} to initiate the sublimation stage. Sublimation was maintained for 50 h, after which the shelf temperature was increased to 25 °C at 0.20 °C·min^{-1} to begin the desorption stage, which continued for 10 h. Product temperature was monitored throughout the freeze-drying process using thermocouples. Upon completion of the freeze-drying cycle, the vacuum was released by air injection, and the vials were immediately sealed with caps. The freeze-dried samples were subsequently vacuum-packed in aluminium bags and stored at −80 °C until analysis, or at 25 °C or 4 °C

for stability studies. In addition, aliquots of the protected cell concentrates were frozen and preserved at −80 °C for subsequent characterization.

To cover the whole range of experiments discussed in this paper, eight batch cultures (biological replicates) were carried out, and the sucrose protective solution was tested in each batch as a reference to make relevant comparisons. The fermentation and freeze-drying processes were rigorously controlled and monitored to ensure reproducibility. A range of fermentation descriptors was calculated to confirm the absence of experimental deviations. The number of technical replicates (measurements on the same sample) is indicated for each characterization method.

2.2. Biological Activity of C. maltaromaticum

2.2.1. Culturability

Before analysis, frozen samples were thawed at room temperature for 3 min and powder samples were rehydrated by adding 1 mL of sterilized culture medium, vortexed for 5 min, and then left to stand for 5 min at ambient temperature.

Cell culturability was assessed using the agar plate count method on fresh (pre-freezing), thawed, and rehydrated cell suspensions. Serial dilutions were prepared in saline solution (9 $g \cdot L^{-1}$ NaCl) and inoculated into molten sterile culture medium supplemented with 15 $g \cdot L^{-1}$ bacteriological agar (Biokar Diagnostic, Beauvais, France). The plates were incubated under anaerobic conditions (GENbox anaer; bioMérieux, Marcy l'Etoile, France) at 30 °C for 48 h, after which colonies were counted. Cell concentration was expressed as colony-forming units per millilitre ($CFU \cdot mL^{-1}$). All plate count determinations were carried out in triplicate.

2.2.2. Acidifying Activity

The acidifying activity was measured using the CINAC system [26]. The measurements were performed at 30 °C, in a sterilized medium of the same composition as the culture medium. Each sample (thawed or rehydrated) was diluted 10 times (100 µL sample in 900 µL saline solution), and 100 µL of this dilution was inoculated into three flasks containing 150 mL of culture medium (triplicate). The time necessary to reach the maximum acidifying rate (tm, in minutes) was used to characterize the acidifying activity of the bacterial suspensions. The longer the time (the higher the tm), the lower the acidifying activity.

The acidifying activity data were then weighed by the culturability results to obtain the specific acidifying activity descriptor tspe (in $min \cdot (\log(CFU \cdot mL^{-1})^{-1})$). It was calculated as the ratio of tm to the corresponding log of cell concentration (Equation (1)) [25]:

$$tspe = \frac{tm}{\log\left(CFU \cdot mL^{-1}\right)} \quad (1)$$

The tspe descriptor measures the biological activity of *C. maltaromaticum* CNCM I-3298, including its physiological state and culturability. It was measured before freezing (tspe_BF), after freeze-thawing (tspe_F), after freeze-drying (tspe_FD), and after storage (tspe_S) at 25 °C or 4 °C.

The losses of specific acidifying activity following freezing and freeze-drying were calculated as follows:

$$\Delta tspe_F_ = tspe_F - tspe_BF \quad (2)$$

$$\Delta tspe_FD = tspe_FD - tspe_F \quad (3)$$

$$\Delta tspe_S = tspe_S - tspe_FD \quad (4)$$

Storage stability was evaluated from the tspe evolution during four weeks of storage at 25 °C.

The tspe value (min·(log(CFU·mL^{-1})$^{-1}$)) was plotted as a function of the storage time (t, in days). The experimental values were fitted using the following equation (Equation (5)):

$$tspe = -K_{tspe} \times \mathrm{t(days)} + \mathrm{b} \quad (5)$$

where the specific acidifying activity loss rate constant K_{tspe} (in min·(log(CFU·mL^{-1})$^{-1}$)·day^{-1}) refers to the absolute value of the slope of the linear regression. The lower the absolute value of the slope K_{tspe}, the higher the storage stability was. This equation was used to predict storage stability.

2.3. Glass Transition Temperature and Water Activity of Freeze-Dried C. maltaromaticum Concentrates

The glass transition temperature (Tg, °C) of the most resistant freeze-dried protected cell concentrates (Suc and TreA15) was determined by differential scanning calorimetry (DSC Pyris1, Perkin Elmer LLC, Norwalk, CT, USA) according to the procedure described by Guerrero Sanchez et al. 2023 [27]. Briefly, approximately 25 mg of each sample was sealed in aluminium pans and analyzed using cooling and heating rates of 10 °C·min^{-1}. Samples were first heated from 5 to 120–150 °C, subsequently cooled to 5 °C and then reheated to 120–150 °C. The glass transition temperature was calculated as the maximal value of the peak observed in the first derivative curve of the heat flow. All measurements were performed in triplicate.

The water activity (a_w) of the freeze-dried samples Suc and TreA15 was measured at 25 °C using a LabMaster-aw water activity meter (Novasina, Precisa, Poissy, France) on freeze-dried samples.

2.4. Biochemical Characterization of C. maltaromaticum Cells

The biochemical characterization of *C. maltaromaticum* protected concentrates in an aqueous environment was performed at several stagescess, including after freezing, after freeze-drying, and during storage for up to four weeks of storage at 25 °C and 4 °C. Analysis were conducted using an FTIR microscope (Nicolet iN10, Thermo Fisher Scientific; Madison, WI, USA), following the methodology described by Girardeau et al. [17] and Guerrero Sanchez et al. [27]. Briefly, a small quantity of freeze-thawed or rehydrated protected cell concentrates, previously washed three times with saline solution, was placed in a CaF_2 microchamber and separated from the saline solution side (diluent) by a thin Mylar® strip (polyethylene terephthalate film from GoodFellow, Lille, France). Spectral acquisition was carried out using Omnic software (version 8.1, Thermo Fisher Scientific; Madison, WI, USA). Spectra were collected from 128 co-added scans between 4000 and 975 cm^{-1} at a resolution of 4 cm^{-1}. For each bacterial sample, three categories of spectra were recorded: background spectra sample spectra, and diluent spectra. At minimum of 65 spectra were acquired per each protective condition, process stage and biological replicate. Spectral pre-processing was conducted according to the procedure described by Meneghel et al. [28]. Water contributions were removed from sample spectra using an in-house MATLAB script (version 8.3.0.532, Mathworks, Natick, MA, USA) as previously reported [17,25,27–29]. Following water subtraction, spectra were processed using Unscrambler® X software (version 10.2, CAMO Software AS, Oslo, Norway). Analyses focused on three spectral regions: 3050–2800 cm^{-1}, 1800–1370 cm^{-1}, and 1370–975 cm^{-1}. An extended multiplicative scatter correction (EMSC) procedure was applied to normalize and correct baseline variations within each region of interest. The resulting spectra were then statistically analyzed using principal component analysis (PCA) to reveal variance in the data (score plots) and spectral features (wavenumbers) of interest (loading plots). Peak assignments for the principal absorption bands were established based on previously published data on bacterial infrared

spectra (Table S1, Supplementary Materials) [17,24,30–34]. Further analysis was conducted within the three selected spectral regions. For each protective condition, process step, and biological replicate, second-order derivative spectra were calculated using the Savitzky–Golay algorithm (third-degree polynomial, 9-point smoothing factor) and subsequently averaged (Figure S1, Supplementary Materials). For each wavenumber (*vi*) corresponding to peaks identified in the second-order derivative spectra, the relative variation ($\Delta_{v\,FTIR}$) in peak height (*h*) was calculated after freeze-drying (FD) and after four weeks of storage at 25 °C (S4w), relative to either the freezing or freeze-dried condition, according to Equations (6)–(8):

$$\Delta_{vFTIR_FD} = h_{viFreeze-drying} - h_{viFreezing} \quad (6)$$

$$\Delta_{vFTIR_S4w} = h_{vi4\ weeks\ of\ storage} - h_{viFreeze-drying} \quad (7)$$

$$\Delta_{vFTIR_FD+S4w} = h_{vi4\ weeks\ of\ storage} - h_{viFreezing} \quad (8)$$

The relative variation in the peak's height ($\Delta_{v\,FTIR}$) was also used to compare two protective formulations (*Px* and *Py*) after freeze-drying (FD) and 4 weeks of storage at 25 °C (S4w) as follows (Equations (9) and (10)).

$$\Delta_{vFTIR_FD_Px\text{-}Py} = h_{vi_FD_Px} - h_{vi_FD_Py} \quad (9)$$

$$\Delta_{vFTIR_FD+S4w_Px\text{-}Py} = h_{vi_{FD}+S4w_Px} - h_{vi_{FD}+S4w_Py} \quad (10)$$

A positive $\Delta_{v\,FTIR}$ value indicates a decrease in the peak's height and vice versa.

FTIR microspectroscopic analysis was conducted on only one or two biological replicates. Consequently, variability in the calculation of the relative variation in peak height ($\Delta_{v\,FTIR}$) could not be assessed, resulting in a predominantly qualitative interpretation of the data.

2.5. Statistical Analyses

Specific acidifying activity and storage stability data were compared using the non-parametric Kruskal–Wallis test, the Conover Iman post hoc test and a significance level of 95% (p-value < 0.05) (XLSTAT v19.6, Addinsoft, Paris, France).

3. Results and Discussion

3.1. Protective Formulations' Selection According to Putative Stabilisation Mechanisms

Adding protective molecules to cell concentrates before freeze-drying is a strategy currently used to limit the degradation of lactic acid bacteria (LAB) functionalities, such as culturability and acidifying activity, resulting from the stresses cells face during dehydration and subsequent storage.

The first part of this study compared the efficiency of trehalose alone and in combination with two maltodextrins exhibiting high glass transition temperatures (Table A2) with that of sucrose alone in improving the freeze-drying and storage resistance of *Carnobacterium maltaromaticum* CNCM I-3298. Sucrose, trehalose and maltodextrins have previously been used alone and in mixtures to preserve LAB during freeze-drying [22,24,27,35–37]. However, their effectiveness varies among the microorganisms and production conditions. The protective effect of disaccharides and polymers on cell components during drying and subsequent storage is usually ascribed to two main mechanisms: (i) the water substitution theory and (ii) the vitrification theory [38,39]. The former is based on the ability of disaccharides, such as sucrose and trehalose, to protect cells by forming hydrogen bonds with polar groups of cell components as water is removed during drying, thus maintaining the native structure of membrane proteins and lipids. The vitrification mechanism involves reducing

molecular mobility in a glassy matrix to limit diffusion-controlled degradation reactions such as non-enzymatic browning [40–42]. A matrix exhibiting a higher glass transition temperature (Tg) is generally associated with higher viscosity, lower free volume, and lower molecular mobility, resulting in a more stable glassy matrix, particularly at elevated storage temperatures [43].

The second part of the study examined the effect of incorporating an antioxidant into the most promising formulations identified in the first part on the storage stability *of C. maltaromaticum* I-3298. Sodium ascorbate (vitamin C), often used to limit oxidation in dried LAB [24,27,44,45], was chosen as the antioxidant for this study.

3.2. Resistance of C. maltaromaticum CNCM I-3298 to the Stabilisation Process Protected with Disaccharides and Maltodextrins

The impact of protective formulations on the resistance of *C. maltaromaticum* CNCM I-3298 to each step of the stabilization process was assessed by measuring the specific acidifying activity (tspe) of protected bacterial concentrates before and after freezing, after freeze-drying, and after one, two and four weeks of storage at 25 °C (tspe, tm, and log(CFU·mL^{-1}), Figure S2; Supplementary Materials).

Regardless of the formulation used, *C. maltaromaticum* showed no sensitivity to freezing (Figures 2 and S2, Supplementary Materials), consistent with previous studies [15] and its psychrophilic properties. After freeze-drying (FD), *C. maltaromaticum* cells exhibited a slight increase in the loss of specific acidifying activity (dtspe (<6 min·(log (CFU·mL^{-1})$^{-1}$); Figure 2). In contrast, storage at 25 °C led to a gradual increase in dtspe values (Figure 2).

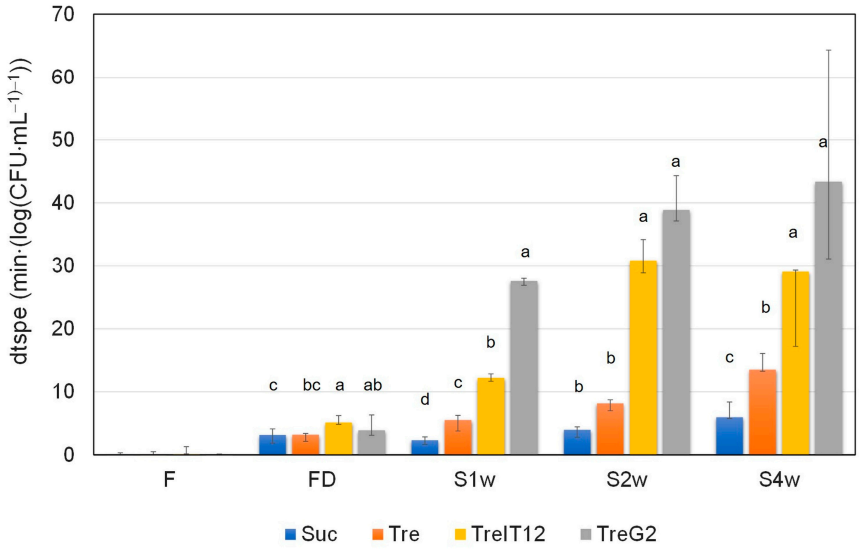


Figure 2. Specific acidifying activity losses (dtspe, in min·(log(CFU·mL^{-1})$^{-1}$)) of *C. maltaromaticum* CNCM I-3298 at each step of the process: freezing (F), freeze-drying (FD) and following one (S1w), two (S2w) and four (S4w) weeks of storage at 25 °C. Data are medians of 5–7 biological replicates; error bars represent interquartile ranges. For each step of the process, letters (a, b, c, d) represent statistical differences between samples at a 95% confidence level. Formulation compositions are detailed in Table A1.

Differences observed among protective formulations after freeze-drying became more pronounced as early as the first week of storage (S1w), with sucrose providing the highest level of protection after four weeks at 25 °C, followed by trehalose alone. The lowest activity losses after 1 month of storage (S4w) are comparable to the values observed for a resistant LAB such as *L. plantarum* WCFS1 [29].

Formulations combining trehalose with maltodextrins exhibited the poorest protection (TreG2 and TreIT12, Figure 2). Among the two maltodextrins, Glucidex® 2 appeared slightly less effective than Glucidex® IT12, although no significant differences were observed after 2 or four weeks of storage. The positive effect of maltodextrin on the resistance of *L. salivarius* CECT5713 to freeze-drying and accelerated storage at 37 °C [24] was not confirmed in this study. However, the superior protective effect of sucrose over trehalose during the storage of *C. maltaromaticum* CNCM I-3298 is consistent with previous studies conducted on other lactic acid bacteria, such as *Limosilactibacillus reuteri* [37] and *Lacticaseibacillus rhamnosus* GG [46].

To get insight into the mechanisms by which sucrose outperformed trehalose, we examined the properties of these two molecules reported in the literature. Despite their similar molecular structures, sucrose and trehalose exhibit distinct physical properties, such as the affinity to establish hydrogen bonds (i.e., between sugars and water, between sugars and polar groups from the cells) and the glass transition temperature, which may account for their differing efficiencies [47–50]. For example, trehalose is less efficient at replacing sugar–protein hydrogen bonds [51]. It also exhibits a destructuring effect on the water structure [48], which may promote phase separation during freezing and eventually lead to alterations in protein secondary structure [52,53].

Both sugars replace water and interact with membranes by binding lipid polar headgroups and proteins, thus protecting them during drying [38,39,54]. Some studies on proteins [53,55] and liposomes [56] indicate a higher protective effect of trehalose than sucrose. However, membranes may also be stabilized in the dry state with sucrose, and interestingly, three times less sucrose than trehalose is required [57]. Moreover, similar protective effects of sucrose and trehalose on membrane lipids and cellular proteins in Gram-positive and Gram-negative bacteria [58], and on the membrane integrity of LAB [59], were observed following freeze-drying. Studies on sugar interactions with the polar headgroups of LAB membranes' lipids are scarce [25,27], and do not involve the same formulations.

Regarding the vitrification theory, increasing the glass transition temperature (Tg) of the glassy matrix by using trehalose or trehalose–maltodextrin mixtures instead of sucrose (Table A2) was ineffective in improving *C. maltaromaticum* storage stability. Increasing the difference between the storage temperature and the glass transition temperature (T–Tg) below Tg has been correlated with reduced molecular mobility, and consequently, improved storage stability for different LAB (*Ligilactobacillus salivarius* CECT5713 [24,27], *Lactobacillus delbrueckii* subsp. *bulgaricus* [29,60], and *Lactiplantibacillus plantarum* WCFS1 [29], but did not appear to be a critical stability determinant of *C. maltaromaticum* CNCM I-3298 under the tested conditions. This result, however, is consistent with previous observations on proteins [61–63]. It has been proposed that fast dynamics (β relaxations occurring on the nanosecond timescale) are better predictors of protein stability than Tg alone [61,64]. Investigations into molecular mobility in sucrose- and trehalose-based matrices have demonstrated that sucrose is more effective at reducing local molecular mobility, which drives chemical reactions such as oxidation, deamidation, and potentially protein aggregation at storage temperatures below Tg and at low relative humidity (RH < 22%) [61]. As the bacterial concentrates were stored at 25 °C, which is well below the Tg of the sucrose formulation (32 °C), the fast-dynamics hypothesis proposed by studies on proteins [61,64] may help explain the observed stability differences at least in part.

3.3. Sites of Injury and Protection Mechanisms During Freeze-Drying and Storage by FTIR Microspectroscopy

To identify cellular components altered by freeze-drying, and storage for 4 weeks at 25 °C, as well as the protective effects of the four formulations, FTIR microspectroscopy analyses were performed on two biological replicates of *C. maltaromaticum* cell concentrates.

Principal component analyses (PCA) were conducted on normalized and EMSC-corrected spectra in three spectral regions: 3050–2800 cm^{-1}, 1800–1370 cm^{-1}, and 1370–975 cm^{-1}. The principal absorption bands identified in the loading plots were assigned to cellular components based on published literature (Table S1, Supplementary Materials).

No clustering was observed in the 3050–2800 cm^{-1} region, regardless of formulation or processing step (Figure S3, Supplementary Materials). These findings indicate a limited impact of freeze-drying and storage on membrane lipids, consistent with previous studies on lactic acid bacteria [24,25,27,29].

In contrast, PCA of the 1800–1370 cm^{-1} and 1370–975 cm^{-1} regions revealed that protective formulation and freeze-drying were the main drivers of spectral variation, whereas storage had only a minor effect (Figure 3). This suggests that the loss of biological activity during storage mainly originates from biochemical alterations induced by freeze-drying, as previously reported for other LAB [24].



Figure 3. Principal component analysis (PCA) of FTIR normalized and corrected spectra of *C. maltaromaticum* CNCM I-3298 frozen (F), freeze-dried (FD) and after 4 weeks of storage at 25 °C (S4w), in the spectral regions 1370–975 cm^{-1}: (**a**,**b**) 1800–1370 cm^{-1} (**b**,**c**). Biological replicates are plotted independently in the score plots (**a**,**c**, circles and triangles symbols). The discriminating bands according to PC1 (black lines) and PC2 (grey dotted lines) are indicated in the loading plots (**b**,**d**). Formulation compositions are detailed in Table A1.

In the 1370–975 cm^{-1} region, which provides information on proteins (amide III), nucleic acids, phosphorylated compounds, and cell wall polysaccharides, a clear separation along PC1 (51% variance) was observed (Figure 3a). Cells protected with trehalose-based formulations (Tre, TreIT12, TreG2) exhibited positive PC1 scores and were associated with bands at 1240–1230 cm^{-1} and 1090–1080 cm^{-1}, mainly attributed to phosphodiesters, phospholipids, nucleic acids, and teichoic acids (Figure 3b). Sucrose-protected cells showed negative PC1 scores and were associated with bands at 1147–1128 cm^{-1}, 1074–1047 cm^{-1}, and 1007–1003 cm^{-1}, mainly corresponding to sugar rings of the cell wall and nucleic acids (Figure 3a,b).

PC2 (15% variance) reflected process effects (Figure 3a). Freeze-dried and stored cells were characterized by two negative bands at 1033 (cell wall) and 995 cm^{-1} (nucleic acids) (Figure 3b).

In the 1800–1370 cm^{-1} region, related mainly to protein structure, PC1 and PC2 explained 86% of the total variance (Figure 3c). Frozen samples exhibited negative PC1 scores corresponding to protein amide I bands and cell wall carbohydrates. Freeze-dried and stored cells exhibited positive PC1 scores associated with nucleic acids, amide II structures of proteins, and lipids bands (Figure 3d). PC2 accounted for sample heterogeneity with no significant discrimination among formulations.

FTIR analysis was performed on two biological replicates, which are shown independently in Figure 3 (circles representing one replicate and triangles the other) and in Figure S4, Supplementary Materials. The variability observed between the two replicates makes it difficult to establish a quantitative relationship between FTIR spectral changes and biological activity. However, qualitatively, it can be observed that the trehalose and trehalose–maltodextrin formulations, which exhibited the lowest storage stability, induced greater biochemical (spectral) alterations after freeze-drying than sucrose, particularly in cell wall components and proteins (Figure S4, Supplementary Materials).

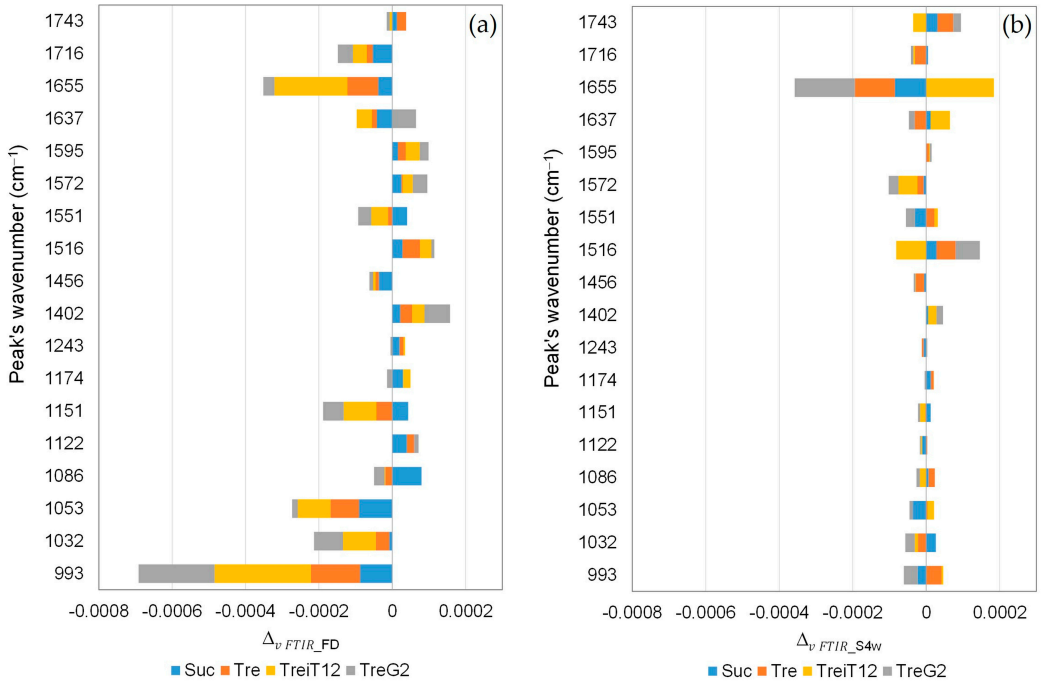


Figure 4. Changes in the peak's height ($\Delta_{v\,FTIR}$) of the mean second derivative of the FTIR bands after freeze-drying (FD) (**a**) and 4 weeks of storage at 25 °C (S4w) (**b**) compared to the corresponding frozen sample. $\Delta_{v\,FTIR}$ values are the means of two biological replicates. Only peaks that exhibited $\Delta_{v\,FTIR}$ values larger than 10% of the highest $\Delta_{v\,FTIR}$ value (absolute value of 3×10^{-5} in this study) for at least one of the samples are displayed.

3.4. Protection Efficiency of an Antioxidant on the Storage Stability of C. maltaromaticum

To further enhance storage stability, the impact of oxidation was evaluated by incorporating sodium ascorbate into the formulations (Figure 5). Supplementing the trehalose formulation with 15 g·L^{-1} of sodium ascorbate significantly reduced the loss of specific acidifying activity after freeze-drying and four weeks of storage at 25 °C. No significant effect was observed for sucrose.

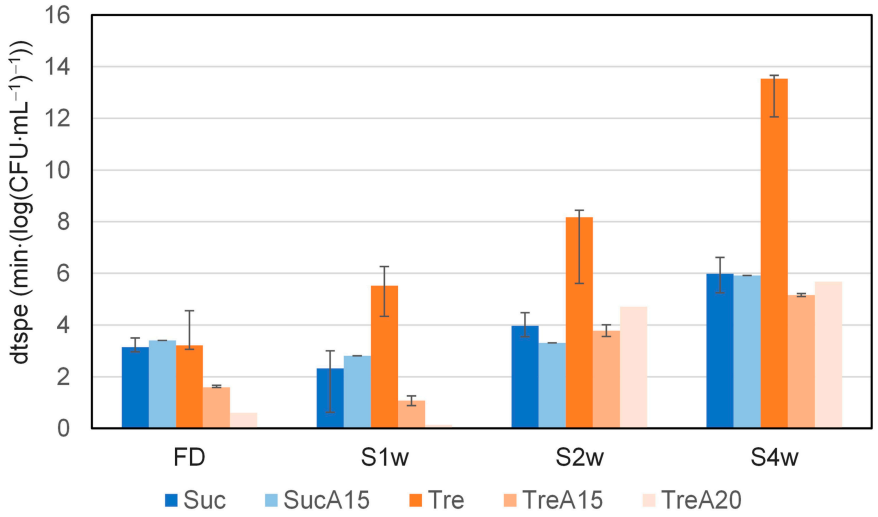


Figure 5. Specific acidifying activity losses (dtspe, in min·(log(CFU·mL^{-1})$^{-1}$)) of *C. maltaromaticum* CNCM I-3298 following freeze-drying (FD) and storage at 25 °C (one (S1w), two (S2w) and four (S4w) weeks). Except for TreA20, data are medians of at least two biological replicates; error bars represent interquartile ranges. The error bars are absent for the formulation TreA20 because it was tested in only one batch. Formulation compositions are detailed in Table A1.

Increasing the concentration of sodium ascorbate to 20 g·L^{-1} provided no additional benefit, consistent with the concentration-dependent effect reported for spray-dried *Limosilactobacillus reuteri* KUB-AC5 [45]. This result suggests that 15 g·L^{-1} is the maximum concentration to protect *C. maltaromaticum* cells, avoiding the pro-oxidant activity previously observed in other bacteria at higher concentrations [65].

Both sucrose (Suc) and trehalose + ascorbate (TreA15) formulations remained in a glassy state at 25 °C, with Tg values exceeding the storage temperature (Tg_Suc = 32 °C; Tg_TreA15 = 58 °C). These values were, as expected, lower than those of pure compounds due to residual water activity (aw_Suc = 0.15; aw_TreA15 = 0.05) and traces of culture medium.

The water activity of the sucrose formulation was within the previously reported optimal range for LAB (0.1–0.24) [35], corresponding to the presence of small amounts of water at the monolayer level. Under these hydration conditions, which promote physical stabilization through optimal water activity and reduced molecular mobility, the oxidation rate of the sucrose formulation is expected to be minimal [35,66,67].

In addition, according to the fast-dynamics hypothesis proposed by Pikal et al. [61] and Ciceroni et al. [64], sucrose would be more efficient than trehalose in reducing local molecular mobility below Tg, thereby limiting degradation reactions of cell functions. Moreover, the residual water content of the sucrose formulation may also behave as an adequate diluent to slow local mobility processes and increase the barrier properties of the glassy matrix [68]. We propose that adding low concentrations of molecules such as sodium ascorbate to trehalose may further slow such dynamics. However, this interpretation should be treated with caution, as its main limitation is the lack of water activity measurements on all formulations.

The differences in the second-derivative peak height (Δv *FTIR*) of the FTIR spectra of the two formulations, after freeze-drying (Equation (9)) and after storage (Equation (10)), were calculated to identify the main bands affected by the addition of the antioxidant (Figure S5, Supplementary Materials). FTIR analyses showed that the addition of the antioxidant to the trehalose formulation (TreA15) reduced spectral differences in the fingerprint region (1300–995 cm^{-1}) compared to the reference sucrose, suggesting a limitation of oxidative damage to cell wall components and nucleic acids. These results should be treated as preliminary, as it was not possible to carry out any biological replicates, and no direct measurement of oxidative damage was performed.

Finally, lowering the storage temperature to 4 °C, as explored in one fermentation, improved stability after one month of storage for both sucrose and TreA15 formulations, with dtspe values reduced by 40% and 72%, respectively (Figure 6). As a first approximation to predict stability over longer periods, linear models were applied, yielding coefficients of determination (R^2) higher than 0.86 for both formulations at 4 °C and 25 °C (Equation (4), Figure S6, Supplementary Materials).

At first glance, TreA15 appeared to exhibit a slightly lower loss of activity (dtspe_TreA15, 1 month = 0.7 min·(log(CFU·mL^{-1}))$^{-1}$), corresponding to an approximately 15% reduction compared with sucrose after six months at 4 °C. However, when considering an interquartile range (IQR) of 30% for these data—calculated as the average IQR observed across all formulations and storage times—only a temperature effect could be identified. No significant differences between formulations can therefore be inferred (Figure 6).

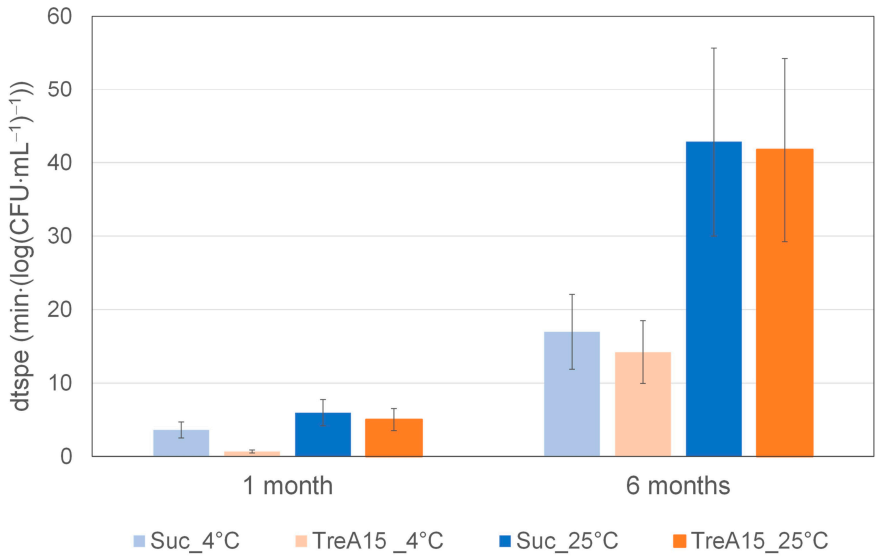


Figure 6. Specific acidifying activity losses (dtspe, in min·(log(CFU·mL^{-1})$^{-1}$)) of *C. maltaromaticum* CNCM I-3298 following 1 month of storage at 25 °C and predicted values for 6 months storage calculated from the rate of loss of specific acidifying activity (Figure S4, Supplementary Materials). To estimate the uncertainty of the model used for the stability prediction, error bars extending ±15% from the dtspe value are included, corresponding to the average IQR (30%) observed across Suc and TReA15 formulations and storage times. Formulation compositions are detailed in Table A1.

4. Conclusions

In conclusion, this study identified sucrose as the most efficient single protectant for preserving *C. maltaromaticum* CNCM I 3298 by freeze-drying. A formulation combining trehalose and sodium ascorbate at 15 g·L^{-1} showed comparable performance to sucrose; however, due to its higher glass transition temperature (Tg), it may be more suitable for industrial applications involving exposure to elevated temperatures (e.g., non-refrigerated

transport or storage in hot climates where refrigeration is unavailable or unreliable), conditions that can lead to losses in functionality. Although formulation optimization is generally strain-dependent, these two formulations can be considered good starting points.

Based on the literature, the superior protective role of the sucrose formulation was ascribed to distinct physical properties, in particular its ability to reduce local molecular mobility below Tg, thus limiting chemical degradation reactions such as oxidation and deamidation. Adding sodium ascorbate to trehalose may also slow these fast dynamics.

Biochemical analysis using FTIR micro-spectroscopy revealed that freeze-drying caused the most damage, affecting nucleic acids, proteins, and cell wall components. Storage induced additional modifications to protein secondary structures.

Further studies including direct measurements of molecular mobility and oxidative damage are needed to clarify the relative roles of added antioxidants and residual water content in LAB stability.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app16136782/s1, Table S1: Assignments of the infrared vibrational bands displayed in Figure 5 in the 1800–975 cm^{-1} regions of the infrared spectra of *C. maltaromaticum* CNCM I-3298 cells protected with different formulations and the associated main biomolecules; Figure S1: Method used to calculate the relative variation (ΔνFTIR) of the peak's height (hνi): (a) Mean second derivative of the normalized spectrum of the frozen (F), freeze-dried (FD), and stored for four weeks at 25 °C (S4w) *C. maltaromaticum* CNCM I-3298 cells, on the 1370–975 cm^{-1} region, showing the peaks (*) where the ΔνFTIR parameter has been evaluated. (b) Zoom on the 1040–1025 cm^{-1} region showing the calculation of the relative variation (ΔνFTIR) of the 1032 cm^{-1} peak's height; Figure S2: Specific acidifying activity (tspe, in min·(log(CFU·mL^{-1})$^{-1}$)), acidifying activity (tm, in min) and culturability (log(CFU·mL^{-1}) of *C. maltaromaticum* CNCM I-3298 before freezing (BF), after freezing (F), and following freeze-drying (FD) and one (S1w), two (S2w) and four (S4w) weeks of storage at 25 °C. Data are medians of 5–7 biological replicates; error bars represent interquartile ranges. For each variable and step of the process, letters (a, b, c, d) represent statistical differences at a 95% confidence level between samples. Formulation compositions are detailed in Table A1; Figure S3: Principal component analysis (PCA) of FTIR normalized and corrected spectra of *C. maltaromaticum* CNCM I-3298 cell populations protected with four different formulations, frozen (F), freeze-dried (FD), and 4 weeks of storage at 25 °C, in the 3050–2800 cm^{-1} region. PC1 versus PC2 score plots explain 48 and 9% of the variance, respectively; Figure S4: Changes observed in the relative variation (ΔνFTIR) of the infrared vibrational bands in the 1800–975 cm^{-1} region, in *C. maltaromaticum* CNCM I-3298 cell populations protected with the trehalose based formulations (Tre, TreIT12 and TreG2), compared to the samples protected with sucrose (Suc): (a) after freeze-dried (FD) and (b) after storage for four weeks at 25 °C (FD + S4wΔνFTIR values of the two biological replicates are plotted separately: panel (a) and (c) for one replicate, and (b) and (d) for the second replicate. Only peaks that exhibited Δv *FTIR*, values larger than 10% of the highest Δv *FTIR* value (absolute value of 3×10^{-5} in this study) for at least one of the samples are displayed. Formulation compositions are detailed in Table A1; Figure S5: Changes observed in the relative variation (ΔvFTIR) of the infrared vibrational bands in the 1800–975 cm^{-1} region, in *C. maltaromaticum* CNCM I-3298 cell populations protected with trehalose plus antioxidant (TreA15) and trehalose alone (Tre), compared to the samples protected with sucrose (Suc) after storage for four weeks at 25 °C (FD + S4w). Only peaks exhibiting Δv *FTIR* values greater than 10% of the maximum Δv *FTIR* value (absolute value of 3×10^{-5} in this study) for at least one sample are shown. Formulation compositions are detailed in Table A1; Figure S6: Evolution of the specific acidifying activity (tspe) *C. maltaromaticum* CNCM I-3298 freeze-dried samples, protected with sucrose (Suc) and trehalose plus antioxidant (TreA15), during 24 days of storage at 4 °C and 25 °C. The regression coefficients (R^2) of the linear fit for the curves were higher than 0.86 in all cases. Formulation compositions are detailed in Table A1.

Author Contributions: Conceptualization, F.F. and S.P.; methodology, P.L., S.C., S.P. and F.F.; validation, X.W., S.C., P.L. and F.F.; formal analysis, P.L. and F.F.; investigation, X.W., P.L., S.C., F.F. and S.P.;

resources, S.P. and F.F.; data curation, X.W., P.L., S.C., S.P. and F.F.; writing—original draft preparation, F.F.; writing—review and editing, X.W., P.L., S.C., S.P. and F.F.; visualization, F.F.; supervision, F.F.; project administration, F.F.; funding acquisition, S.P. and F.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Author Xavier Wood is employed by the company Beiersdorf (Beiersdorfstrasse 3, 22529 Hamburg, Germany). The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

LAB	Lactic acid bacteria
FTIR	Fourier transform infrared
Tg	Glass transition temperature
aw	Water activity
PCA	Principal component analysis

Appendix A. Principal Characteristics of the Formulations Investigated

Table A1. Composition of protective formulations investigated.

Name	Composition	Concentration ($g \cdot L^{-1}$) *
Suc	Sucrose	200
Tre	Trehalose	200
TreG2	Trehalose Glucidex® 2	100 100
TreIT12	Trehalose Glucidex® IT 12	100 100
TreA15	Trehalose Sodium ascorbate	200 15
TreA20	Trehalose Sodium ascorbate	200 20
SucA15	Sucrose Sodium ascorbate	200 15

* In saline solution (9 $g \cdot L^{-1}$ NaCl).

Table A2. Molecular weight and glass transition temperature of disaccharides and maltodextrins used in this study in protective formulations.

Name	Mw (g·moL^{-1})	Tg (°C)	Reference (Tg Values)
Sucrose	342	60–75	[69–71]
Trehalose	342	113–155	[69,70]
Glucidex® IT 12	~1480 *	127	[72]
Glucidex® 2	~8600 *	180	[72]

Mw: molecular weight; Tg: glass transition temperature of dehydrated powder (aw ~0); * Castro et al. (2016) [73].

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