



Freeze-Drying of Lactic Acid Bacteria: A Stepwise Approach for Developing a Freeze-Drying Protocol Based on Physical Properties

Fernanda Fonseca, Amélie Girardeau, Stephanie Passot

► To cite this version:

Fernanda Fonseca, Amélie Girardeau, Stephanie Passot. Freeze-Drying of Lactic Acid Bacteria: A Stepwise Approach for Developing a Freeze-Drying Protocol Based on Physical Properties. Willem F. Wolkers; Harriëtte Oldenhof. Cryopreservation and Freeze-Drying Protocols, 2180, Springer US, pp.703-719, 2021, Methods in Molecular Biology, 978-1-0716-0782-4. 10.1007/978-1-0716-0783-1_38 . hal-03824400

HAL Id: hal-03824400

<https://hal.science/hal-03824400>

Submitted on 21 Oct 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Freeze-drying of lactic acid bacteria: a stepwise approach for developing a freeze-drying protocol based on physical properties

Fernanda Fonseca¹, Amélie Girardeau¹ and Stéphanie Passot¹

¹UMR GMPA, INRA, AgroParisTech, Université Paris-Saclay, Thiverval-Grignon, France

Correspondance mail: fernanda.fonseca@inra.fr

Summary

Freeze-drying or lyophilization has become a reference process for preserving lactic acid bacteria. The development of stable freeze-dried lactic acid bacteria (LAB) requires maintaining the biological activity of the cells and the macroscopic porous structure while increasing the efficiency of the manufacturing process. Physical properties of protective solutions such as glass transition and collapse temperature, are key elements not only for process optimization but also for the stability of freeze-dried LAB. This chapter provides a stepwise approach for developing a protective formulation for long term preservation of LAB and an efficient freeze-drying process. Methods for determining glass transition and collapse temperatures of protective solutions and cell suspensions, as well as water activity and water content of freeze-dried products are described.

Key words: glass transition, collapse, lactic acid bacteria, lyoprotectants, freeze-drying, lyophilization, formulation, stabilizers

1. Introduction

From ancient times, lactic acid bacteria (LAB) have been used for preserving agricultural raw materials. Today LAB are of great importance for the food, health products (probiotics) and biotechnology industries. The market of concentrated cultures ready- to-used (starters) is continuously growing. The probiotic market is indeed expected to increase by 7% per year for the next five years, reaching \$69 billion USD by 2023 [1].

Freeze-drying is an essential and valuable preservation method to ensure the long-term stability of bioactive products. This method makes it possible to remove the majority of water at temperatures far below 0°C (usually between -40°C and -20°C) by sublimation, the phase transition from ice to water vapor. However, during this low-temperature desiccation process, bacteria are exposed to several environmental stresses (thermal, osmotic, mechanical, and oxidative), which can induce major cellular damages and lead to loss of viability and functional activities (acidification, production of aroma compounds, probiotic activities, etc) (*see Figure 1*). Various approaches are proposed to help bacteria overcome these stresses: (i) the addition of protective molecules before the stabilization process; (ii) the selection of operating conditions such as freezing kinetics, product temperature during freeze-drying, final water content and storage conditions; (iii) the adaptation of bacterial cells during the fermentation process. Changing the fermentation conditions enables the modulation of LAB membrane properties [2], and thus bacterial resistance [3–7]. Bacterial adaptation is however a time consuming and bacteria dependent approach and still does not replace formulation and freeze-drying optimization for delivering stable starters.

A well-designed freeze-drying cycle not only ensures physical and biological product stability, but also increases the efficiency of the manufacturing process. Therefore, as the most time consuming stage of the process, primary drying (ice sublimation) should be carried out at the maximum allowable product temperature (T_{max}) resulting from the operating conditions (shelf temperature and chamber pressure). If the product temperature is higher than T_{max} , the material will undergo viscous flow, resulting in loss of the pore structure, which is defined as the collapse phenomenon by Pikal and Shah [8]. Collapsed dried products generally have a high residual water content, a lengthy reconstitution time and may also display a loss of functional properties. For amorphous material, T_{max} is usually associated to the glass transition temperature of the maximally freeze-concentrated phase (T_g'). For simple binary aqueous solutions, collapse was shown to take place 1 to 3°C above T_g' [8–11]. However, when adding bacterial pellets to these solutions, collapse of the dried porous structure occurred at a

temperature about 10°C higher than T_g' [9,12]. The presence of LAB cells confers a significant robustness to the freeze-dried product, thus allowing the use of higher product temperatures during primary drying than expected from the protective solution.

Differential scanning calorimetry (DSC) and freeze-drying microscopy (FDM) are two complementary tools used to characterize the freeze-drying behavior of a solution [13]. DSC determines the phase changes occurring in a sample following cooling and heating, and it is the method of choice for measuring glass transition temperature in liquid (T_g') and solid (T_g) samples. By making it possible the direct microscopic observation of the porous dried structure following sublimation, FDM gives an estimation of the collapse temperature (T_{coll}).

The composition of the protective solution added to bacteria before the freeze-drying process affects T_g' , T_{coll} and T_g , thus constituting a key element for optimizing both the product quality and the process. **Table 1** summarizes the main protective molecules used for preserving LAB and their associated mechanisms of action. An appropriate choice of molecules is needed to prevent freezing- and drying-induced damage and to preserve biological activity during processing, storage and rehydration. The development of a protective solution must integrate the maximization of T_{max} . Consequently, the concentration of molecules lowering the T_{max} value should be reduced or still better if they can be replaced by molecules increasing the T_{max} value. **Table 2** summarizes the physical properties (T_g' and T_{coll}) of the most commonly used protective molecules for preserving lactic acid bacteria.

Many protective solutions and freeze-drying cycles have been separately developed on an empirical basis. However, it is today widely accepted that formulation and cycle development have to be performed concomitantly, on a more rational and scientific basis involving a deep characterization of the formulation's physical behavior. In the current chapter, a logical stepwise approach for developing a protective formulation for lactic acid bacteria that is compatible with an efficient freeze-drying process is described. In addition, methods are presented to determine physical properties of the freeze-dried product, glass transition temperature, water activity, and water content.

2. Materials

2.1 Differential scanning calorimeter (DSC)

1. A power compensation DSC Diamond (Perkin Elmer LLC, Norwalk, CT, USA) equipped with a liquid nitrogen cooling accessory (CryoFill, Perkin Elmer). Temperature calibration was done using cyclohexane (crystal-crystal transition at -87.1°C), mercury (melting point at -38.6°C) and n-octadecane (melting point at 27.8°C). This equipment is used for analyzing liquid samples.
2. A power compensation DSC Pyris 1 (Perkin Elmer LLC, Norwalk, CT, USA) equipped with a mechanical cooling system. Temperature calibration was done using cyclohexane (melting point at 6.5°C), n-octadecane (melting point at 27.8°C) and indium (melting point at 156°C). This equipment is used for analyzing freeze-dried samples.
3. 50 μL Perkin-Elmer DSC aluminum pans and covers.
4. Perkin Elmer universal crimper press.

2.2 Freeze-drying microscopy system

1. A Nikon Elipse E600 polarized microscope (Nikon, Japan) equipped with a 10 \times magnification objective.
2. A freeze-drying cryo-stage (FDCS 196, Linkam Scientific Instruments, Surrey, UK) equipped with a programmable temperature controller (TMS94, Linkam), a liquid nitrogen cooling system, a vacuum pump (Edwards E2M1.5, Linkam) and a Pirani gauge.
3. A Retiga 2000R digital monochrome camera (QImaging, Surrey, Canada).
4. 16-mm quartz cover slips, 13-mm glass cover slips and metallic spacers.

2.3 Freeze-dryer

1. A pilot scale freeze-dryer equipped with temperature controlled shelves (-50°C to 50°C), capacitive manometer for pressure control, and sensors for monitoring the product temperature (thermocouples) and the end of sublimation (Pirani gauge or moisture sensor or pressure rise test device) [14] (see **Note 1**).
2. A computer and software associated to the freeze-dryer allowing control of the equipment, data acquisition and visualization.
3. Suitable containers for freeze-drying the product (vials, trays, aluminum cups, etc).
4. Aluminum bags for conditioning the freeze-dried samples.
5. Equipment for sealing aluminum bags under vacuum.

2.4 Formulations for freeze-drying bacteria

1. Bacterial pellet obtained after fermentation and concentration steps. The protocol described by Fonseca et al. [15] can be followed to produce the bacterial pellet.
2. Protective molecules (*see Table 1*). A protective solution for preserving lactic acid bacteria usually involves three components: (i) a stabilizer that can form hydrogen bonds with proteins and polar head groups of membrane phospholipids and act as water substitute when water is removed during desorption of the unfrozen water (disaccharides for instance); (ii) a polysaccharide or polymer of high molecular weight that increase the glass transition temperature of the solution (T_g' higher than T_g' of disaccharides); (iii) an antioxidant molecule to limit oxidative reactions following storage of the freeze-dried product and (iv) in some cases additional molecules such as specific cryoprotective agents (amino acid, etc.) or surfactants. **Table 2** summarizes the physical properties (T_g' and T_{coll}) of the most commonly used protective molecules for preserving lactic acid bacteria.

2.5 Water activity and water content measurement of the freeze-dried product

1. A chamber continuously purged with dried air for manipulating hygroscopic dried samples.
2. a_w -meter labMaster-aw (Novasina, Precisa, Poissy, France).
3. Plastic cups for measuring water activity (Novasina, Precisa, Poissy, France).
4. Karl fisher titration method: Metrohom KF 756 apparatus (Herisau, Switzerland) with reagent Hydranal® Coulomat AG (Honeywell Fluka, Seelze, Germany).
5. Hydranal™ methanol dry (Honeywell Fluka, Seelze, Germany).
6. 1 mL Microliter™ syringe (Hamilton, Bonaduz, Switzerland).
7. 4 mL glass vials, septum and caps.
8. Mortar and pestle.

3. Methods

Figure 2 depicts a stepwise approach for developing a protective formulation for lactic acid bacteria that is compatible with an efficient freeze-drying process. This approach lies on the measurement of the formulation's physical properties (T_g' and T_{coll}).

3.1. Determination of the effect of cells and supernatant on the physical properties (T_{coll} and T_g')

1. Perform bacterial growth according to your own production protocol or to the protocol described by Fonseca et al. [15].
2. Prepare a protective solution (PS) composed of 200 g/L of sucrose and 9 g/L of NaCl (*see Note 2*).
3. Transfer the cell suspension to centrifugation flasks.
4. Centrifuge ($17000\times g$, 4°C , 30 min) to concentrate cell suspension, and remove the culture supernatant (*see Note 3*).
5. Mix cell pellets (CP) with the protective solution (PS) at a ratio of 1:2 (1 g of cells and 2 g of protective solution). Suspend cells thoroughly by using a spatula and a vortex mix until complete cell resuspension.
6. Determine the glass transition temperature of the maximally freeze-concentrated phase (T_g') of the protected bacterial suspension (mixture CP:PS) by using DSC. Briefly, place 15 μL of the suspension in 50- μL Perkin Elmer DSC sealed aluminum pan, and note the sample weight. Use an empty sealed aluminum pan as reference. Apply the following temperature program to the sample and the reference: cooling from 20°C to -100°C at $10^{\circ}\text{C}/\text{min}$, holding at -100°C for 1 min and heating from -100°C to 20°C at $10^{\circ}\text{C}/\text{min}$. Plot the first derivative of the heat flow recorded during the heating scan and identify the T_g' value associated to the higher heat capacity change ($T_g'_{\text{2}}$ in **Figure 3**, *see Note 4*). The characteristic glass transition temperature of samples is determined as the midpoint temperature of the heat capacity step associated to the glass transition and corresponds to the maximum of the heat flow's first derivative.
7. Determine the collapse temperature of the protected bacterial suspension (mixture CP:PS) by using freeze-drying microscopy. The method described by Fonseca et al. [12] can be used. Briefly:
 - (a) Put silicone oil on the temperature-controlled element of the freeze-drying cryo-stage and place the quartz cover slip on it.
 - (b) Place a drop of 5 μL of the protected bacterial sample in the middle on the quartz cover

slip, place around the drop a metallic spacer (to standardize the sample thickness) and place a glass cover slip overlapping the sample and the spacer.

(c) Cool the sample at 10°C/min to 10°C below the Tg' value of the solution (or to -40°C).

(d) Evacuate the system to the minimal pressure (~1 to 2 Pa).

(e) Maintain the temperature for a few minutes to obtain a freeze-dried layer large enough to observe its structure. The initial freezing pattern should be maintained (see **Figure 4a**).

(f) Increase the temperature progressively, step by step (steps of 1°C, 2°C or 5°C at 10°C/min) and observe the structure of the freeze-dried layer until transparent or light regions are visible within the dried region (Tcoll; see **Figure 4b,c**).

(g) By comparing the Tcoll and the Tg' values of the protected bacterial suspension, the effect of cell on collapse can be estimated: X °C = Tcoll – Tg'.

(h) By comparing the Tg' value of the protected bacterial suspension with the Tg' of a sucrose solution, the effect of the residual content of supernatant on Tg' can be estimated: Y °C = Tg' sucrose – Tg'.

3.2. Adjustment of the composition of the protective solution (PS)

1. If the collapse temperature is higher than -25°C, the protective solution can be selected for the freeze-drying process.
2. If the collapse temperature is lower than -25°C, the composition of the protective solution needs to be modified by addition of a molecular compound exhibiting higher collapse temperature.
3. Generate a new set of protective solutions including disaccharides and molecules with high Tg' (or Tcoll). Maintain the final concentration of the protective solution at about 200 g L⁻¹.
4. Measure the Tg' of the new set of protective solutions. The following equation can be used to have a first estimation of Tg' value of the solutions [16].

$$Tg'_M = \frac{\sum Tg'_i C_i}{\sum C_i}$$

With Tg'_M, the glass transition temperature of the maximally freeze-concentrated phase of the multi-component solution, and Tg'_i, and C_i, the Tg' value and the concentration of component i, respectively.

5. Select the PS with a Tg' higher than '-25-X+Y °C'.
6. Mix cell pellets (CP) with the protective solution (PS) at a ratio of 1:2 (1 g of cells and 2 g of protective solution). Suspend cells thoroughly by using a spatula and a vortex mix until complete cell resuspension.

7. Determine the collapse temperature of the protected bacterial suspension by using freeze-drying microscopy.
8. If the collapse temperature is higher than -25°C , the protective solution can be selected for the freeze-drying process (*see Note 5*).
9. If the collapse temperature is lower than -25°C , two options can be examined: (i) the modification of the composition of the protective solution by addition of molecules exhibiting higher collapse temperature, or (ii) the addition of a washing step of the bacterial pellet (CP) with saline solution before adding the protective solution (*see Note 6*).

3.3 Freeze-drying protocol

1. Prepare the protective solution at a total solute concentration of 400 g/L in saline solution and autoclave the solution at 120°C for 20 min. Store the protective solution at 4°C .
2. Prepare an antioxidant solution (sodium ascorbate) at a concentration of 10 g/L in saline solution and filter-sterilize the solution by passing it through a $0.2\ \mu\text{m}$ sterile membrane filter. Protect the solution from light (in a dark flask and/or cover with aluminum foil) and use immediately or store at 4°C for up to 1 day.
3. Aseptically mix equal volumes of protective and antioxidant solutions to obtain a protective solution with the following composition: 200 g/L total protective molecules, 9 g/L sodium chloride, and 5 g/L sodium ascorbate (*see Note 7*). Use immediately or store at 4°C for up to 1 day, protected from light.
4. Perform bacterial growth according to your own production protocol or to the protocol described by Fonseca et al. [15].
5. Aseptically transfer the cell suspension to sterile centrifugation flasks (*see Note 8*).
6. Centrifuge ($17000\times g$, 4°C , 30 min), and remove the supernatant (*see Note 3*).
7. Mix cell pellets (CP) with the protective solution (PS) at a ratio of 1:2 (1 g of cells and 2 g of protective solution). Suspend cells thoroughly by using a spatula and a vortex mix until complete cell resuspension. Maintain the cell sample at 4°C .
8. Aseptically aliquot the final protected bacterial suspensions into appropriate containers for freeze-drying (about 1 cm product fill depth).
9. Place the containers onto the precooled (at 4°C) shelves of a freeze-dryer (*see Note 9*).
10. Place calibrated thermocouples at the bottom of some containers and fix them with specific holders (rubber band for instance) (*see Note 10*).
11. Decrease shelf temperature to -50°C at 1 to $0.5^{\circ}\text{C}/\text{min}$ and hold this temperature until the sample temperature stabilizes to ensure complete solidification of the product (about 2 h).

12. After freezing the samples, cool the condenser to $<-65^{\circ}\text{C}$, switch on the vacuum pump, and decrease the chamber pressure to 10 Pa. Then increase the shelf temperature to -10°C at $1^{\circ}\text{C}/\text{min}$ to initiate the ice sublimation phase (*see Note 11*).
13. Monitor the process variables during the freeze-drying process (shelf, condenser, product temperatures and pressure) in order to detect any dysfunction and also to identify the end of sublimation. The end of sublimation is detected by following the decrease of the pressure measured by the Pirani Gauge. When the pressure value given by the Pirani Gauge reaches the pressure value given by the capacitance manometer, the sublimation step is finished (*see Figure 5*).
14. Increase the shelf temperature to 25°C at $0.25^{\circ}\text{C}/\text{min}$ to initiate the secondary drying phase and reduce the chamber pressure to minimal value (*see Note 12*).
15. After 10 h of desorption, break the vacuum by injecting dry air, vacuum-pack the freeze-dried product in aluminum bags and store at temperatures 20°C below the sample's T_g value (or at -20°C while waiting for T_g results).

3.4 Characterization of the freeze-dried product

1. Manipulate the freeze-dried sample inside a dry chamber, grind the freeze-dried cake into powder (*see Note 13*).
2. Measure the water activity: fill the plastic cup with powder until three-quarters full, place the cup in the a_w -meter previously conditioned at 25°C , close the equipment and start the measure (stability criteria for a_w measurement of 5 min).
3. Measure the glass transition temperature (T_g) of the freeze-dried sample by DSC: briefly place 10 mg of freeze-dried powder in 50- μL Perkin Elmer DSC sealed aluminum pan. Use an empty sealed aluminum pan as reference. Apply the following temperature program to the sample and the reference: cooling from 20°C to -10°C at $10^{\circ}\text{C}/\text{min}$, holding at -10°C for 1 min and heating from -10°C to 150°C at $10^{\circ}\text{C}/\text{min}$. Plot the first derivative of the heat flow recorded during the heating scan and identify the T_g . To eliminate interference from enthalpic relaxation, an additional cycle can be performed: heating from 20°C to a temperature few degrees higher than T_g at $10^{\circ}\text{C}/\text{min}$, cooling to -10°C at $10^{\circ}\text{C}/\text{min}$, holding at -10°C for 1 min and heating from -10°C to 150°C at $10^{\circ}\text{C}/\text{min}$.
4. Measure the water content using the Karl Fisher method.
Determination of the water content of methanol:
 - (a) Fill 5 vials with 2 mL of dry methanol.
 - (b) Take ~ 0.5 mL of methanol with the syringe in a vial, rinse the syringe with methanol,

take ~0.8 mL of methanol in the same vial (m_{blank}) and inject this quantity in the Karl Fisher titration vessel, note the measured quantity of water (x_{blank}).

(c) Repeat this procedure for the 4 remaining vials.

Determination of the water content of the freeze-dried sample:

(a) Put ~20 mg of powder (m_{powder}) in a 4 mL glass vial, add 2 mL of dry methanol ($m_{methanol}$), close the vial with the septum and stopper. Precisely note the weight of both the powder and methanol.

(b) Prepare 3 vials.

(c) Vortex the vials for 5 min (use a specific device for maintaining vials) (*see Note 14*).

(d) Take ~0.5 mL of sample with the syringe in a vial, rinse the syringe with the sample, take ~0.8 mL of sample in the same vial (m_{sample}) and inject this quantity in the Karl Fisher titration vessel, note the measured quantity of water (x_{sample}).

(e) Repeat this procedure for the 2 remaining vials.

The water content (WC, in %) is calculated using the following equation:

$$WC (\%) = \frac{\left(\frac{x_{sample}}{m_{sample}} - average \left(\frac{x_{blank}}{m_{blank}} \right) \right) m_{methanol}}{m_{powder}}$$

4. Notes

1. For freeze-drying LAB starters, freeze-dryer sterilization is not compulsory (like in pharmaceutical application). However, a thorough cleaning and disinfection procedure of the chamber, shelves and containers between batches is recommended to avoid cross contamination.
2. If the assessment of biological activity (viability, acidifying activity) is not required, the steps 2 to 5 can be carried out in non-aseptic conditions. Maintain the bacterial samples at 4°C (in water-ice mixture for instance) to limit cell degradation during all intermediate steps.
3. Different centrifugation forces and times can be applied according to specific LAB production protocol. The temperature should be kept between 2 and 4°C to limit cell degradation.
4. Aqueous solutions of protective molecules generally exhibit two thermal events characterizing the glass transition. They represent the glass transition temperature of the maximally freeze concentrated phase (the lower value, T_g' , $T_g'_{1}$ in **Figure 3**) [17] and the softening temperature at which the system exhibits an observable deformation (viscous flow in real time) under its own weight (the higher value, $T_g'_{2}$ in **Figure 3**) [18].
5. Further adjustment of the composition of the protective solution can be required according to the biological activity recovery after freeze-drying and storage. In any case, the concentration of solutes in the protective solution should not be higher than 250 g L⁻¹.
6. After the centrifugation step and the removal of culture supernatant, add an equivalent volume of saline solution, suspend cells thoroughly by using a spatula and a vortex mixer until complete cell resuspension, centrifuge and remove the supernatant. This operation can be repeated once.
7. Increasing the antioxidant concentration up to 10 g/L may be necessary, particularly for strains that are sensitive to oxidation reactions.
8. Use a laminar hood for transferring and manipulating cell samples. If a laminar hood is not available, work close to a gas burner.
9. Samples can be frozen outside the freeze-dryer (for instance in a -80°C freezer or by immersion in liquid nitrogen). In this case, the samples must be transferred to the freeze-drier after precooling the shelves to -50°C to avoid thawing the samples.
10. Place thermocouples at different locations in the freeze-dryer. If only one shelf is used, place them in the center of the container (and center of the shelf) and on the borders of the recipient (and the borders of the shelf) to take into account the heterogeneity of the product

temperature within the sample batch. The samples located at the border of the shelf receive an additional heat flow rate and display a product temperatures up to 4 °C higher than central samples [19,20].

11. The values of shelf temperature, chamber pressure and heating rate may require some adjustment dependent on the type of freeze-dryer, and the type of container. The selection of an optimal process condition for primary (sublimation) and secondary (desorption) steps can be guided by specific interactive software ([21], LyOptM software [22]).
12. The values of shelf temperature, heating rate and desorption time can be modified according to the properties of the formulation and bacterial sensitivity.
13. Place all the materials (plastic cups, vials, septum, stoppers, spatula, mortar and pestle, etc.) needed for manipulating the freeze-dried product and preparing sample for water activity, water content and glass transition temperature measurement in the chamber continuously purged with dried air, the day before the analysis.
14. A decantation time of several minutes may be needed in case of samples that are not soluble in methanol. Product residues must not be injected into the Karl Fisher titration vessel.

Acknowledgment

This work has received funding from the European Union's Horizon 2020 Marie Skłodowska-Curie research and innovation programme under grant agreement no. 777657.

Table 1

Summary of the main protective molecules used for long-term preservation of lactic acid bacteria (freezing, freeze-drying, and drying), associated with their protective mechanisms.

protective mechanism	group	example
preferential exclusion	monosaccharides	glucose, fructose, galactose
	disaccharides	sucrose, maltose, lactose, trehalose
	sugars	raffinose, pentaisomaltose, maltotriose
	polyols	glycerol, sorbitol, mannitol, inositol, xylitol
	polysaccharides	maltodextrin, inulin, FOS, GOS
	amino acids	glycine, sodium glutamate, betaine
water substitution	disaccharides	sucrose, maltose, lactose, trehalose
	sugars	raffinose, glucose, fructose, galactose
	polyols	mannitol, sorbitol, inositol
glassy state	monosaccharides	glucose, fructose, galactose
	disaccharides	sucrose, maltose, lactose, trehalose
	sugars	raffinose, pentaisomaltose, maltotriose
	polyols	glycerol, sorbitol, mannitol, inositol, xylitol
	polysaccharides	maltodextrin, inulin, FOS, GOS
antioxidant		vitamin C (ascorbic acid), sodium ascorbate, vitamin E, propyl gallate
surfactant		Tween 80

FOS: fructooligosaccharides; GOS: galactooligosaccharides

Table 2

Summary of the physical properties of solutions containing the main protective molecules used for long term stability of lactic acid bacteria determined by DSC (Tg') and FDM (Tcoll).

	concentration (%, w/w)	Tg' ₂ (°C)	Tg' ₁ (°C)	Tcoll (°C)
fructose	20%	-57	-44	-43
glucose	20%	-56	-44	-42
galactose	10%	-55	-42	-41
sucrose	20%	-46	-33	-31
trehalose	20%	-40	-29	-26
lactose	10%	-38	-28	-27
maltose	20%	-40	-29	-27
raffinose	20%	-37	-26	-24
kestose	20%	-38	-28	-26
nystose	20%	-38	-28	-23
fructofuranosylnystose	20%	-35	-23	-21
pentaisomaltose	20%	-30	-19	-18
inulin (from chicory)	10%	-30	-17	-15
FOS (from chicory)	10%	-27	-17	-15
glucidex 2	20%		-4	ND
glucidex 6	20%		-7	-3
glucidex 12	20%		-11	-8
glucidex 19	20%		-11	-10
sucrose:glucidex 6 mixture				
25:75 (%:%)	20%		-15	-11
50:50 (%:%)	20%	-32	-23	-17
75:25 (%:%)	20%	-43	-30	-25
sucrose in saline solution	20%	-50	-34	-32
CP:PS, ratio 1:2 (g:g)		-51	-36	-27

DSC: differential scanning calorimetry; FDM: freeze-drying microscopy; Tg': glass transition temperature of the maximally freeze-concentrated phase; Tcoll: collapse temperature; FOS: fructooligosaccharides; ND: not determined. Glucidex are maltodextrins of different DP kindly provided by Roquette Frères (Lestrem, France); CP: cell pellets obtained from cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 in mild whey medium [15]; PS: protective solution composed of 20 % (g/g) of sucrose in saline solution.

The solutions were prepared with demineralized water and the measurements were performed in triplicate according to the protocols described in the current chapter. The median values are reported in the table and the interquartile ranges were around 1°C.

References

1. Anonymous (2019) Probiotics Market by Application (Functional Food & Beverages [Dairy Products, Non-dairy Beverages, Infant Formula, Cereals], Dietary Supplements, Feed), Ingredient (Bacteria, Yeast), Form (Dry, Liquid), End User, and Region - Global Forecast to 2023 Report Code: FB 226. Markets and Markets
2. Fonseca F, Penicaud C, Tymczyszyn EE, Gomez-Zavaglia A, Passot S (2019) Factors influencing the membrane fluidity and the impact on production of lactic acid bacteria starters. *Appl Microbiol Biotechnol*, doi:10.1007/s00253-019-10002-1
3. Broadbent JR, Lin C (1999) Effect of heat shock or cold shock treatment on the resistance of *Lactococcus lactis* to freezing and lyophilization. *Cryobiology* 39:88–102
4. Hua L, Zhao WY, Wang H, Li ZC, Wang AL (2009) Influence of culture pH on freeze-drying viability of *Oenococcus oeni* and its relationship with fatty acid composition. *Food Bioprod Process* 87:56–61
5. Li C, Zhao JL, Wang YT, Han X, Liu N (2009) Synthesis of cyclopropane fatty acid and its effect on freeze-drying survival of *Lactobacillus bulgaricus* L2 at different growth conditions. *World J Microb Biot* 25:1659–1665
6. Schoug A, Fischer J, Heipieper HJ, Schnuerer J, Hakansson S (2008) Impact of fermentation pH and temperature on freeze-drying survival and membrane lipid composition of *Lactobacillus coryniformis* Si3. *J Ind Microbiol Biot* 35:175–181
7. Zhao WY, Li H, Wang H, Li ZC, Wang AL (2009) The Effect of Acid Stress Treatment on Viability and Membrane Fatty Acid Composition of *Oenococcus oeni* SD-2a. *Agric Sci China* 8:311–316
8. Pikal MJ, Shah S (1990) The collapse temperature in freeze drying: Dependence on measurement methodology and rate of water removal from the glassy state. *Int J Pharm* 62:165–186
9. Fonseca F, Passot S, Cunin O, Marin M (2004) Collapse Temperature of Freeze-Dried *Lactobacillus bulgaricus* Suspensions and Protective Media. *Biotechnol Prog* 20:229–238
10. MacKenzie AP (1974) Collapse during freeze-drying - qualitative and quantitative aspects. In Goldblith SA, Rey L, and Rothmayr WW (eds) *Freeze-drying and advanced food technology*. Academic Press, New York, 277–307
11. Sun WQ (1997) Temperature and viscosity for structural collapse and crystallization of amorphous carbohydrate solutions. *Cryoletters* 18:99–106
12. Fonseca F, Passot S, Lieben P, Marin M (2004) Collapse temperature of bacterial suspensions: the effect of cell type and concentration. *Cryoletters* 25:425–434
13. Passot S, Trélea IC, Marin M, Fonseca F (2010) The relevance of thermal properties for improving formulation and cycle development: Application to freeze-drying of proteins. In: Rey L and May JC (eds), *Freeze-Drying/Lyophilization of Pharmaceuticals and biological Products*, 3rd Edition ed. Informa Healthcare USA Inc, New York
14. Patel SM, Doen T, Pikal MJ (2010) Determination of end point of primary drying in freeze-drying process control. *AAPS PharmSciTech* 11:73–84

15. Fonseca F, Cenard S, Passot S (2015) Freeze-drying of lactic acid bacteria In: Wolkers WF, Oldenhof H (eds) Cryopreservation and freeze-drying protocols, Methods in Molecular Biology, Springer, 477–488
16. Fonseca F, Obert JP, Béal C, Marin M (2001) State diagrams and sorption isotherms of bacterial suspensions and fermented medium. *Thermochim Acta* 366:167–182
17. Ablett S, Izzard MJ, Lillford PJ (1992) Differential scanning calorimetric study of frozen sucrose and glycerol solutions. *J Chem Soc Faraday Trans* 88:789–794
18. Shalaev EY, Franks F (1995) Structural glass transitions and thermophysical processes in amorphous carbohydrates and their supersaturated solutions. *J Chem Soc Faraday Trans* 91:1511–1517
19. Scutella B, Bourles E, Plana-Fattori A, Fonseca F, Flick D, Trelea I-C, Passot S (2018) Effect of freeze dryer design on heat transfer variability investigated using a 3D mathematical model. *J Pharm Sci* 107:2098–2106
20. Scutellà B, Plana-Fattori A, Passot S, Bourles E, Fonseca F, Flick D, Trelea IC (2017) 3D mathematical modelling to understand atypical heat transfer observed in vial freeze-drying. *Appl Therm Eng* 126:226–236
21. Trelea IC, Passot S, Fonseca F, Marin M (2007) An interactive tool for the optimization of freeze-drying cycles based on quality criteria. *Dry Technol* 25:741–751
22. Trelea IC, Guillemin H (2009). LyOptM v.3: An interactive tool for freeze-drying cycle optimisation based on product quality criteria IDDN.FR.001.100008.000.R.P.2009.000.20700. UMR GMPA, Grignon.

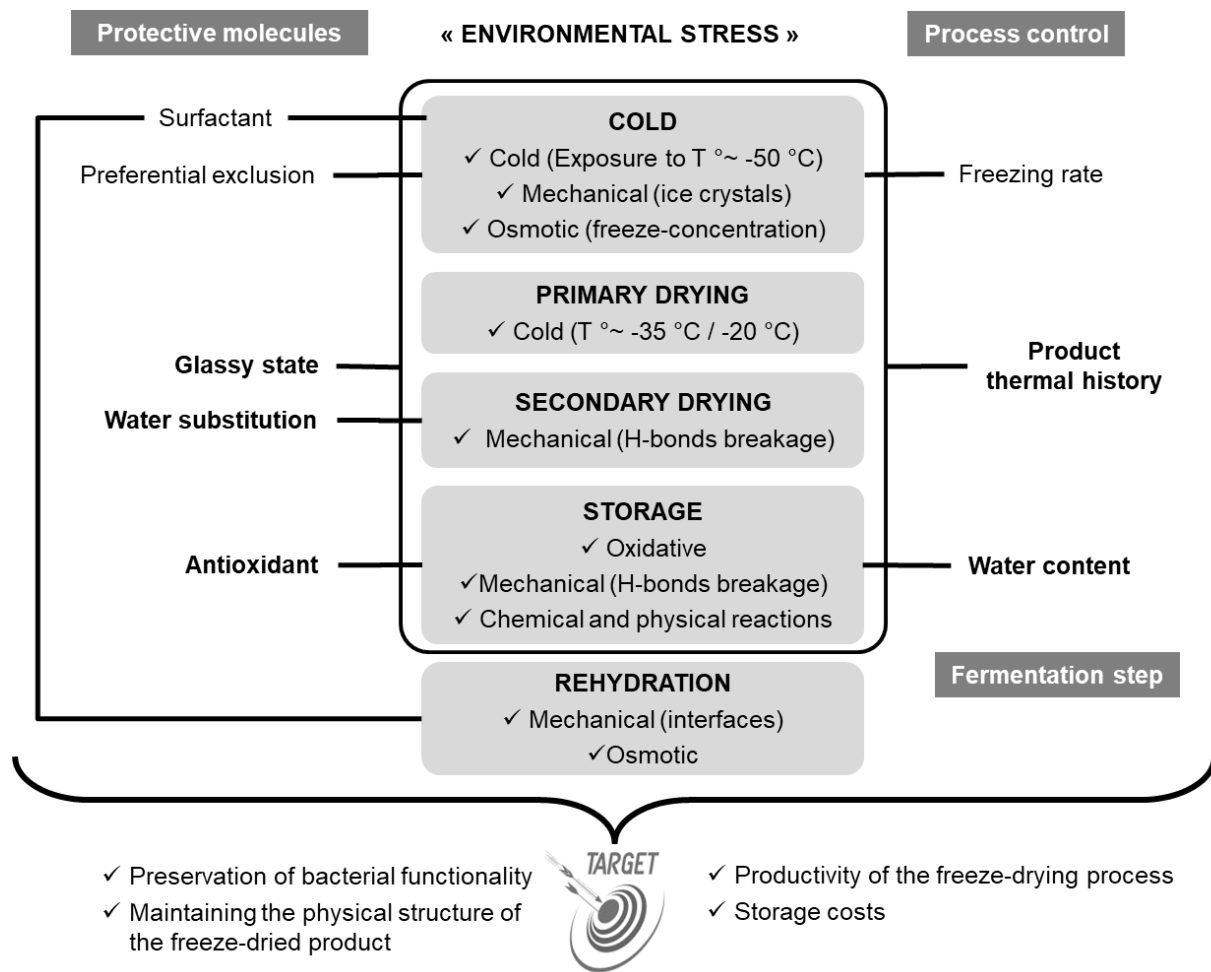


Fig. 1 Summary of the different stresses encountered by lactic acid bacteria during the different steps of the freeze-drying process and the main strategies for limiting these stresses.

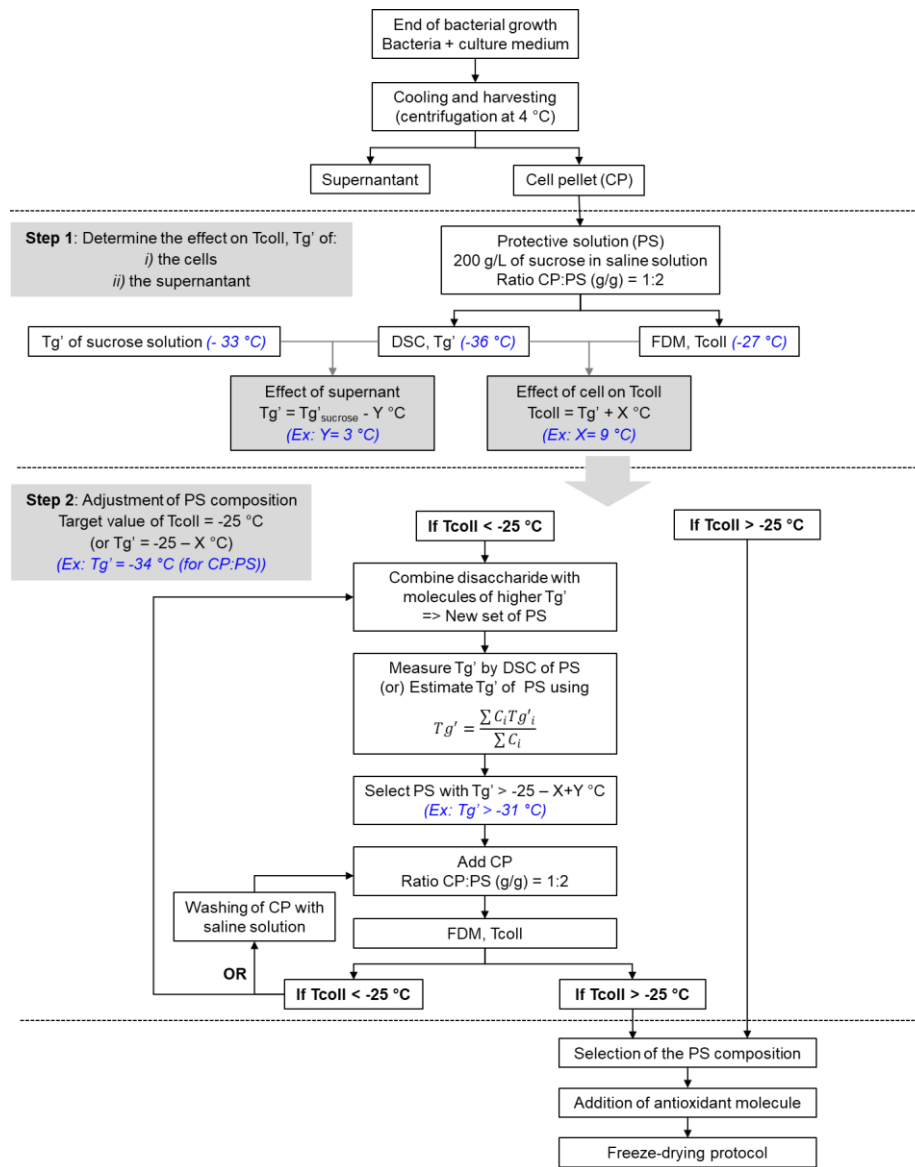


Fig. 2 Flowchart of the stepwise approach for developing a protective formulation for lactic acid bacteria that is compatible with an efficient freeze-drying process. CP: cell pellet; PS: protective solution; DSC: differential scanning calorimetry; FDM: freeze-drying microscopy; Tg': glass transition temperature of the maximally freeze-concentrated phase; Tcoll: collapse temperature.

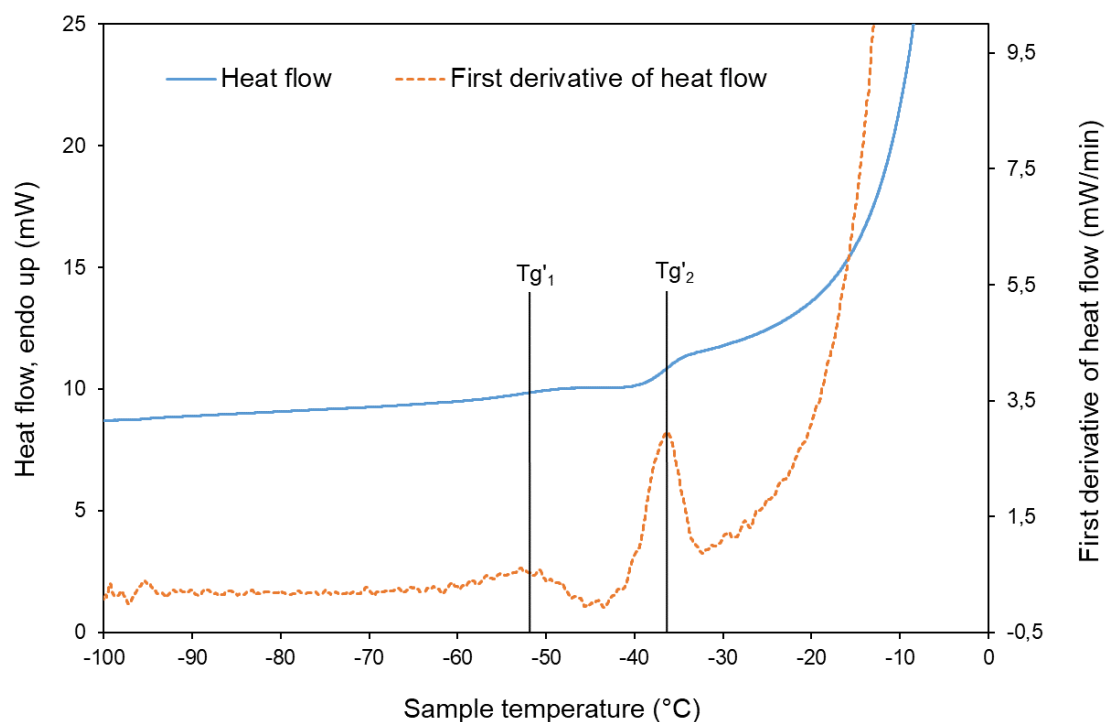


Fig. 3 DSC heating scan (10°C/min) of the frozen solution involving cell pellet (CP) of *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 and protective solution (PS) of 20% of sucrose in saline solution (ratio CP:PS of 1:2, g/g) Tg'_1 and Tg'_2 are the midpoint of glass transition temperatures.

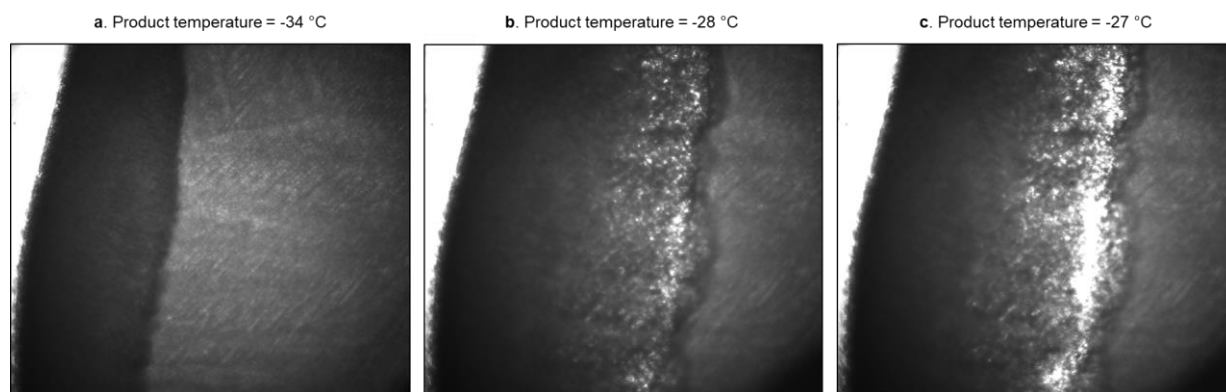


Fig. 4 Microscopic observations of the freeze-drying of a sample containing a cell pellet (CP) of *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 and a protective solution (PS) composed of 20% of sucrose in saline solution (ratio CP:PS of 1:2, g/g). (a) Structure retention in the dried region at -34°C. (b) Beginning of collapse in the dried region at -28 °C. (c) Overall loss of structure in the dried region (collapse) at -27°C.

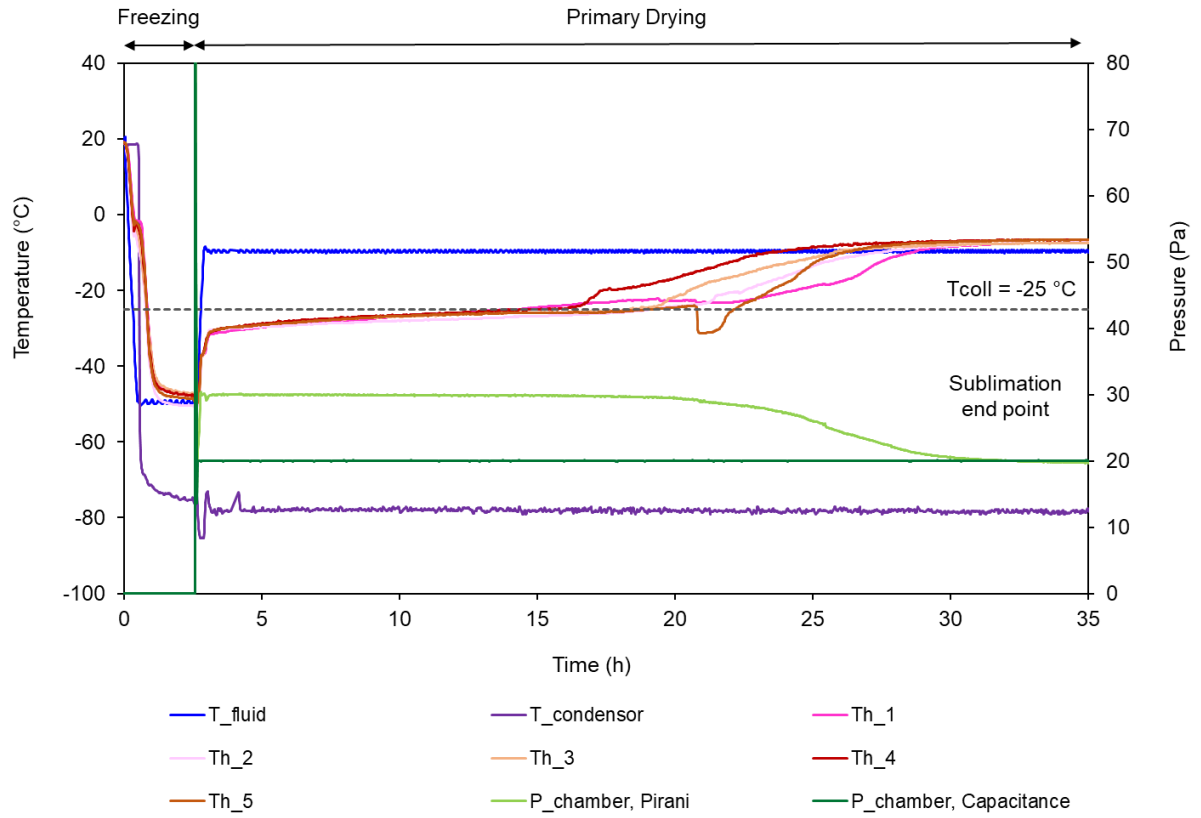


Fig. 5 Evolution of process variables with time during the freezing and primary drying steps, including fluid (shelf) temperature, cold trap temperature, product temperature in different locations (Th1-Th5), and chamber pressure (Pirani gauge and capacitance manometer).