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Chickpeas' cooking wastewater as an alternative source of galacto-oligosaccharides for improving the freeze-dried resistance of lactic acid bacteria

Stéphanie Passot¹, Stéphanie Cenard¹, Pascale Lieben¹, Sarrah Ghorbal¹, Gonçalo Nuno Martins², Paula Cristina Castilho², Fernanda Fonseca^{1*}

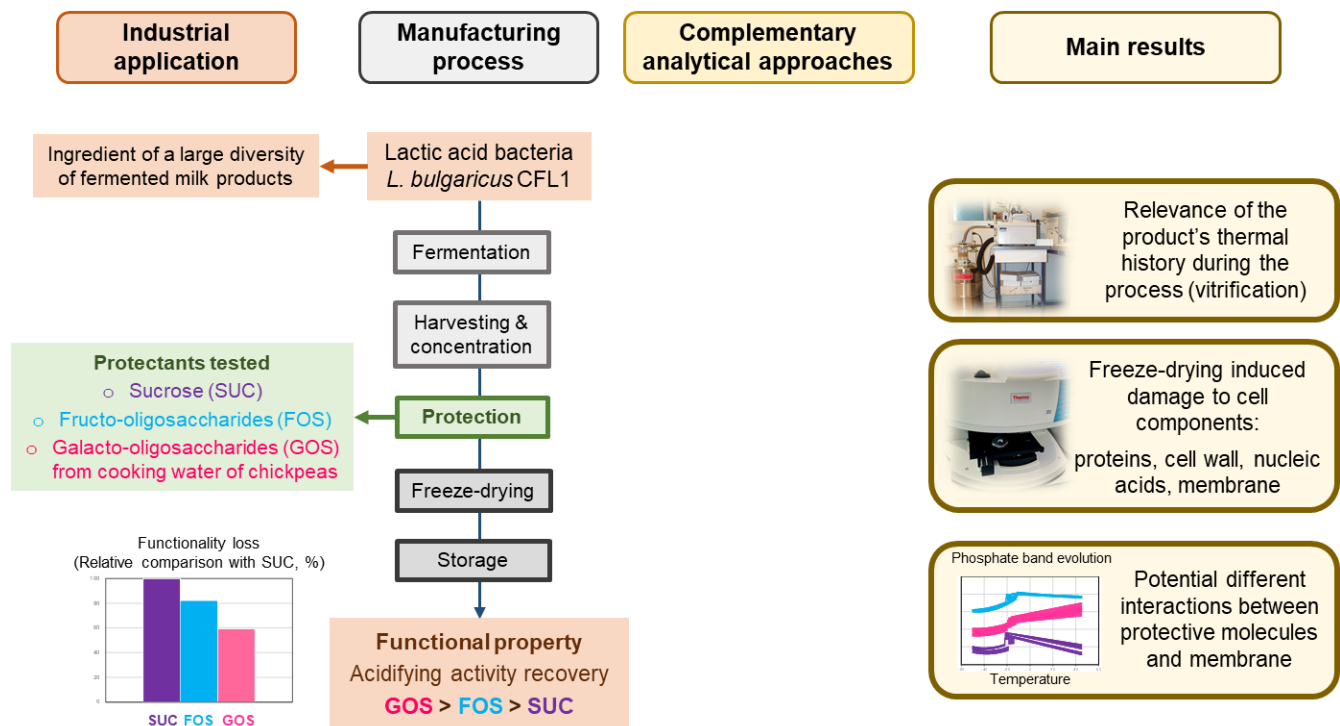
¹ Université Paris-Saclay, INRAE, AgroParisTech, UMR SayFood, F-91120 Palaiseau, France

² CQM – Centro de Química da Madeira, Universidade da Madeira, Campus da Penteada, 9020-105 Funchal, Portugal.

*Correspondance mail: fernanda.fonseca@inrae.fr

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Graphical abstract



Abstract

Oligosaccharides, well-known for their prebiotic properties are reported as promising protective carbohydrates for ensuring the long-term stability of dried lactic acid bacteria and probiotics. This study examines the mechanisms involved in the freeze-drying and storage protective abilities of an extract from chickpea's cooking wastewater containing galacto-oligosaccharides, a fructo-oligosaccharide inulin-derived product, and sucrose, on *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1, a strain highly sensitive to stabilization steps. Fourier transform infrared micro-spectroscopy analysis demonstrated significant molecular changes in cell components after freeze-drying regardless of the protectant used. The freeze-drying led to damage to proteins, peptidoglycans of the cell wall, nucleic acids, and induced membrane integrity losses. The highest resistance to freeze-drying observed with the galacto-oligosaccharides extract was associated with its ability to immobilize cells in a glassy matrix presenting a glass transition temperature well above the desorption temperature, and its interaction with the phospholipids of the bacterial membrane.

Keywords

Oligosaccharides, prebiotics, probiotics, FTIR spectroscopy, glass transition; freeze-drying, membrane integrity

1. Introduction

Lactic acid bacteria (LAB) starters are widely used as biological agents for producing fermented food (meat, vegetables, fruit, and dairy products) and health-beneficial products. The market for starter cultures is incessantly growing due to the development and use of plant-origin fermentation substrates, and their ability to convert by-products of green chemistry. Starter cultures are delivered in the form of ready-to-use and highly concentrated bacterial suspensions to food companies or directly to consumers. Their production and commercialization require the application of successive operations: fermentation, concentration, and preservation. Freezing and freeze-drying are processes of choice when it comes to the long-term preservation of sensitive strains. They are based on the decrease in water activity to strongly slow down or inhibit the damaging reactions and to maintain adequate biological, physical, and chemical stability of the product during long-term storage. However, during freezing and, more importantly, freeze-drying, bacteria are exposed to environmental stresses (thermal, osmotic, oxidative, and mechanical), often resulting in a loss of viability and functional properties (acidifying activity, probiotic activity, etc.). Regardless of the preservation strategy, increasing the LAB quality seems essential for improving the environmental performance of the starter's production system¹. Therefore, the shelf life increase of freeze-dried starters represents an important societal and economic challenge.

To maximize bacterial survival after the process and the gastrointestinal tract, an efficient strategy is to embed the cells in a matrix containing protective molecules. Although skim-based formulations are still studied^{2,3}, they can lead to stability problems due to their contribution to the early Maillard reaction (nonenzymatic browning)⁴ and/or lactose crystallization during the manufacturing and storage of LAB concentrates. Non-reducing disaccharides (e.g., sucrose and trehalose) and polymers (e.g., maltodextrin) have increasingly replaced skim milk for freeze-drying LAB⁵⁻⁷. They afford protection according to two main protection mechanisms: vitrification and water replacement^{8,9}. The use of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) as protective formulations for LAB has increased in recent years¹⁰⁻¹³. They are composed of sugars of different sizes (degrees of polymerization), thus potentially protecting through vitrification and water replacement¹⁴. Oligosaccharides present the added interest of being well-recognized prebiotics (i.e., “non-digestible food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract”¹⁵)¹⁶.

In this work, we investigated the effect of a GOS extract produced from chickpeas cooking wastewater¹⁷ and a commercial FOS on the response of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 to freeze-drying and storage. Sucrose was used as the reference protective formulation. *L. bulgaricus* CFL1 was chosen as a model strain due to its sensitivity for freezing, freeze-drying, and storage^{7,18} and the importance of *L. delbrueckii* subsp. *bulgaricus* strains in the yoghurt market. To better understand the mechanisms of protection and identify sites of cellular damage, deep characterization of protected bacterial suspensions was applied using a multiscale approach (matrix, cell, and cell membrane). Physical (glass transition temperature and water content of the matrix), biochemical (Fourier transform infrared characteristics), and biophysical (phospholipid organization and membrane integrity) properties were related to the biological activity (culturability, acidifying activity, viability) quantified at different steps of the manufacturing process (freezing, freeze-drying, and two weeks of storage at 28°C). Moreover, pilot fermentation and freeze-drying conditions were applied to identify improved preservation conditions relevant to the industry.

2. Materials and Methods

The experimental approach used in this work and the main parameters investigated are presented in **Figure 1**.

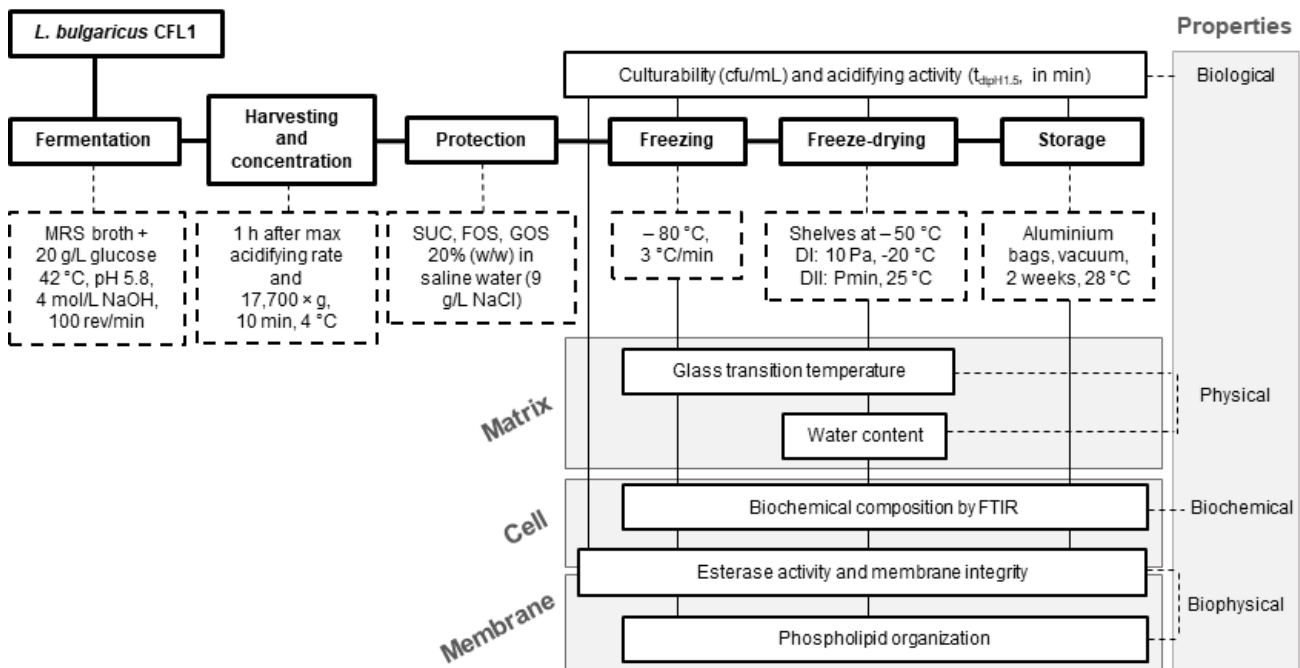


Fig.1 Diagram of the experimental approach used in this study, including production conditions, protective solutions and different measurements performed to characterize the bacterial concentrates at different steps of the process.

2.1. Freeze-dried *L. bulgaricus* CFL1 production and storage

2.1.2. Bacterial strain and culture conditions

Lactobacillus delbrueckii subsp. *bulgaricus* CFL1 (CIRM-BIA; Rennes, France) was used in this study. 1 mL of inocula stored at -80 °C was thawed at 42 °C before the inoculation into 10 mL of Man, Rogosa, and Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France), which constituted the first preculture. A second preculture was performed similarly and was used for inoculating a 2 L bioreactor containing 1.8 L of culture medium. The initial bacterial concentration in the bioreactor was about 10⁶ CFU/mL.

The culture medium was composed of MRS broth (Biokar, Diagnostics, Beauvais, France) supplemented with 20 g/L glucose (VWR, Leuven, Belgium), to avoid depletion of the carbon source. It was sterilized by filtration (0.22-µm polyethersulfone filter, Stericap PLUS, Millipore Express®, Merck KGaA, Darmstadt, Germany).

The fermentation was performed at 42 °C and pH 5.8, with an agitation speed of 100 rev/min. The pH was controlled by the automatic addition of a 4 mol/L NaOH solution.

2.1.3. Harvesting, concentration, and protection

Cells were harvested 1 h after attaining the maximum acidification rate (obtained from the first derivative of the NaOH consumption curve). *L. bulgaricus* CFL1 cells were concentrated by centrifugation (Avanti® J-E centrifuge; Beckman

Coulter, Fullerton, CA, USA) at $17,700 \times g$ for 10 min at 4 °C. Cell pellets were re-suspended in the protective formulations at a ratio of 1:2 (concentrated cell suspension: protective formulation, g/g). Three different protective agents were tested: sucrose (VWR, Leuven, Belgium), a fructo-oligosaccharide inulin-derived product (Orafti® P95, hereafter called FOS) (Beneo Orafti; Tienen, Belgium), and an extract from chickpea's cooking wastewaters containing galacto-oligosaccharides (produced by the CQM—Centro de Química da Madeira, Universidade da Madeira, Portugal), hereafter called GOS). The compositions of the FOS and GOS used in this study are presented in **Table 1**. The protective solutions composed of 20% (w/w) of either sucrose, FOS, or GOS and 9 g/L of NaCl were previously sterilized at 121 °C for 20 min.

Table 1. Composition of oligosaccharides as g/100 g of dried extracts

	DP1	Suc/DP2	DP2	DP3	DP4	DP5	DP6	DP7	Other
FOS	3	5		25	29	18	14	6	
GOS	4		18*	21	14	2**			41***

DP: degree of polymerization; FOS: fructo-oligosaccharides (Orafti® P95, Beneo Orafti; Tienen, Belgium) (composition from Romano et al. ¹²); GOS: galacto-oligosaccharides produced by the CQM—Centro de Química da Madeira (Universidade da Madeira, Portugal) from chickpeas cooking (boiling) wastewaters (C-BW, hereafter called GOS) (composition from Martins et al. ¹⁷); Suc: sucrose; * raffinose family DP2, possibly melibiose; ** DP \geq 5; ***Probably starch.

2.1.4. Freeze-drying and storage

Protected cell suspensions were freeze-dried in one-millilitre cryo-tubes (Sarstedt, Nümbrecht, Germany) (0.5 mL filled volume) as previously described ¹⁹. At the end of the freeze-drying cycle, the cryotubes were taken out of the freeze-drier, manually capped, and packed under a vacuum in multilayer aluminium bags. The bags were either stored at -80 °C before freeze-dried characterization or placed at 28 °C for two weeks for storage experiments. Three fermentations were carried out to generate three independent batches (biological replicates) of freeze-dried lactic acid bacteria.

2.2. Biological activity of *L. bulgaricus* CFL1 concentrates

The cell culturability and acidifying activity were used to characterize the biological activity of *L. bulgaricus* CFL1 concentrates. The measurements were carried out before and after freezing, after freeze-drying, and after two weeks of storage at 28 °C (**Figure 1**). Before analysis, frozen samples were thawed at 42 °C in a water bath for 2 min, and freeze-dried samples were rehydrated in sterilized saline water (9 g/L NaCl) with 5 min shaking and 5 min holding at ambient temperature.

2.2.1. Culturability measurements

The agar plate count method was used to determine bacterial cell concentration. Appropriate sample dilutions in saline water were inoculated in the bulk of MRS agar (Biokar Diagnostics; Beauvais, France) plates. Colonies were enumerated after 48 h of anaerobic (GENbox anaer, bioMerieux; Marcy l'Etoile, France) incubation of plates at 42 °C. Plates

containing between 30 and 300 colonies were kept for cell concentration calculation (in CFU/mL). Plate count measurements were performed in triplicate.

2.2.2. Specific acidifying activity measurements

The CinAc system (AMS, Guidonia, Italia) was used to measure the acidification activity of *L. bulgaricus* CFL1 suspensions²⁰. Acidifying activity was measured as previously described¹⁹. The time necessary to obtain a decrease of 1.5 pH units ($t_{dpH1.5}$, in min). was the descriptor used to characterize the acidifying activity of bacterial suspensions. The higher the $t_{dpH1.5}$ value was, the longer the latency phase was, and the lower the acidification activity was. Moreover, the specific acidifying activity t_{spe} (in min/log (CFU/mL)) was calculated as the ratio of $t_{dpH1.5}$ to the corresponding log of the cell concentration (Eq. 1)²¹. Consequently, the descriptor t_{spe} gives measurements of the biological activity of LAB, including its physiological state and viability.

$$t_{spe} = \frac{t_{dpH1.5}}{\log(CFU.mL^{-1})} \quad (\text{Equation 1})$$

The specific acidifying activity was measured after protection (before freezing, t_{spe_BF}), freezing (t_{spe_F}), freeze-drying (t_{spe_FD}), and two weeks of storage at 28°C (t_{spe_S}).

The loss of specific acidification activity following freeze-drying (Δt_{spe_FD}) and storage (Δt_{spe_S}), as well as the rate of specific acidification activity loss during storage (k_{spe} , in t_{spe} /day) were also calculated (Eq. 2-4).

$$\Delta t_{spe_FD} = t_{spe_FD} - t_{spe_BF} \quad (\text{Equation 2})$$

$$\Delta t_{spe_S} = t_{spe_S} - t_{spe_FD} \quad (\text{Equation 3})$$

$$k_{spe} = \frac{\Delta t_{spe_S}}{\text{days of storage}} \quad (\text{Equation 4})$$

An increase in the Δt_{spe_FD} and Δt_{spe_S} values corresponds to an increased loss of acidifying activity during freeze-drying and storage, respectively. A high value of k_{spe} was related to a rapid decrease in specific acidification activity during storage.

2.3. Physical properties of *L. bulgaricus* CFL1 concentrates

2.3.1. Water content measurements

The water content of freeze-dried samples was determined after freeze-drying by the Karl Fisher titration method using a Metrohm 756 KF titrator (Metrohm AG, Herisau, Switzerland). About 20 mg of powder were mixed with 3 mL of dry methanol and titrated with Hydranal Coulomat AG (Honeywell Fluka, Seelze, Germany) until reaching the endpoint. The water content was measured in triplicate on samples obtained from two biological replicates.

2.3.2. Glass transition temperature measurements

The glass transition temperatures of the protected cell concentrates after freeze-thawing (Tg' , in °C) and the freeze-dried powder (Tg , in °C) were determined by differential scanning calorimetry (DSC) as previously described⁵. Tg and Tg' values were obtained from two and three biological replicates, respectively.

2.4. Biochemical characterization of *L. bulgaricus* CFL1 cells by FTIR micro-spectroscopy

The biochemical characterization of *L. bulgaricus* CFL1 concentrates in an aqueous environment was performed at different stages of the stabilization process (after freezing, freeze-drying, and two weeks of storage at 28 °C) using FTIR micro-spectroscopy in transmission mode. The equipment, including an FTIR microscope (Nicolet iN10, Thermo Fisher Scientific; Madison, WI, USA) and a specific demountable homemade sample holder for liquid samples is described in previous work^{22,23}. Before analysis, freeze-thawed and rehydrated-freeze-dried samples were washed three times in a saline solution to remove any trace of the protective solution. Spectral measurements and pre-processing were performed according to the method detailed elsewhere^{22,23}. The average amide I / II area ratio of 2.3 was used for determining the water subtraction scaling factor for frozen, freeze-dried, and stored cell concentrates, regardless of the formulation.

Post-processing of water-subtracted spectra was performed with the Unscrambler® X software package (Version 10.2, CAMO Software AS; Oslo, Norway) on the spectral regions containing information on fatty acyl chains of the bacterial membrane (the 3016 – 2800 cm⁻¹ region), cell proteins, particularly amide I and amide II bands (the 1800 – 1370 cm⁻¹ region), amide III bands of proteins, nucleic acids, and cell wall components (the complex region of 1370 – 975 cm⁻¹). To normalize and correct the baseline of spectra an extended multiplicative scatter correction procedure was used in each of the spectral regions. The post-processed spectra were statistically analysed by principal component analysis (PCA) using the Unscrambler® X software package to evidence data variance (score plots) and peak positions of interest (loading plots). The principal absorption bands were assigned using data from the literature on bacteria.

The difference between scores after freeze-drying and freezing was calculated to evaluate the extent of biochemical modifications induced by each protectant.

To identify the effect of the protective molecules further treatment on the infrared spectra was performed^{5,23}. For each protective formulation, step of the process, and biological replicate, the second-order derivative of the average spectrum was calculated using a Savitzky-Golay algorithm (third-degree polynomial and a 9-point smoothing factor) in the 1800 – 1370 cm⁻¹ and 1370 – 975 cm⁻¹ spectral regions. For each wavenumber (ν_i) corresponding to the peaks observed in the second-order derivative spectra, the relative variation (r_{ν_iFTIR}) of the peak's height (h) compared to the freezing condition was determined as follows:

$$r_{\nu_iFTIR} = \frac{h_{\nu_iFreeze-drying\ or\ 2\ weeks\ of\ storage}}{h_{\nu_iFreezing}} - 1$$

2.5. Esterase activity and membrane integrity of *L. bulgaricus* cells

Esterase activity and membrane integrity were assessed using a CyFlow Space cytometer (Sysmex-Partec; Villepinte, France) as previously described by Guerrero et al.²³, using carboxyfluorescein diacetate (cFDA, ChemChrome V8, BioMérieux; Marcy l'Etoile, France) and propidium iodide (PI, Sigma-Aldrich; L'isle d'Abeau, France) as fluorescent probes.

2.6. Membrane phospholipid organization of *L. bulgaricus* cells

Measurements were carried out on a Nicolet Magna 750 FTIR spectrometer (Thermo Fisher Scientific; Madison, WI, USA) equipped with an MCT detector and a variable temperature sample holder (Specac Ltd.; Orpington, Kent, UK), selon le protocol described by Guerrero et al.²³ The changes in membrane phospholipid organization were evaluated by monitoring the vibration band position of two specific chemical groups during cooling (or freezing): (i) the $\nu_{sym}CH_2$ arising from phospholipid acyl chains (i.e., related to the central part of the bilayer), and (ii) the $\nu_{asym}PO_2^-$ -involving

phosphate groups of the external surface of the bilayer. The $\nu_{\text{sym}}\text{CH}_2$ and $\nu_{\text{asym}}\text{PO}_2^-$ peaks, at 2850 and 1220 cm^{-1} , respectively, were plotted as a function of temperature. The $\nu_{\text{sym}}\text{CH}_2$ plots were fitted with a curve based on an asymmetric sigmoid transition function. The first-order derivative was calculated, and its maximum was taken as the lipid phase transition temperature resulting from freezing (T_s , in $^\circ\text{C}$). The ice nucleation temperature (T_n , in $^\circ\text{C}$) was also determined from the upshift of the water ($\nu_{\text{H}_2\text{O}}$) combination band (OH libration and bending modes). Results were obtained from three biological replicates (with no technical replicates).

2.7. Statistical analyses

Culturability, specific acidifying activity, water content, glass transition temperature, membrane integrity, and membrane organisation data were compared using the nonparametric Kruskal-Wallis and the post-hoc Conover Iman tests. All tests were performed on medians using XLSTAT 19.6 (Addinsoft; Paris, France) and a significance level of 95 % (p -value < 0.05).

3. Results and discussion

3.1. Influence of the protective formulations on the resistance of *L. bulgaricus* CFL1 to freezing, freeze-drying, and storage

The freeze-drying process induced the highest reduction of culturability (0.56 - 1.40 log (CFU/mL)) and increase of t_{spe} value (32.7 – 55.3 min/log (CFU/mL)), regardless of the protectant considered (**Fig. 2**). The GOS formulation exhibited the highest acidifying activity (lowest t_{spe} value) after freeze-drying, whereas the lowest acidifying activity (highest t_{spe} value) involved the SUC formulation.

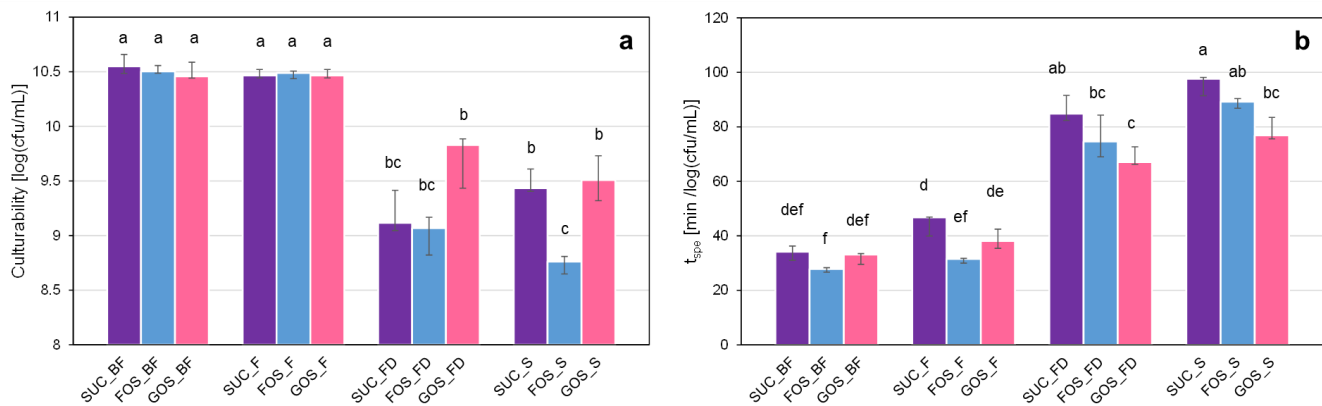


Fig. 2 Culturability (log (CFU/mL)) (a) and specific acidifying activity (in min/log (CFU/mL)) (b) of *L. bulgaricus* CFL1 concentrates protected with three protective solutions, before freezing (BF), after freezing (F), after freeze-drying (FD) and after 2 weeks of storage at 28 $^\circ\text{C}$. Data are the medians of three biological replicates and at least three technical replicates; error bars represent interquartile ranges. SUC: sucrose; FOS: fructo-oligosaccharides; GOS: galacto-oligosaccharides

Freezing and two weeks of storage at 28 $^\circ\text{C}$ did not significantly affect the culturability and specific acidifying activity of each formulation (same groups between FD and S, p -values < 0.05) (**Fig. 2**). However, culturability after storage was significantly lower with FOS compared to SUC and GOS formulations (**Fig. 2a**). The slight loss of specific acidifying activity observed for *L. bulgaricus* CFL1 freeze-dried samples protected with sucrose following two weeks of storage at 28 $^\circ\text{C}$ (SUC, Δt_{spe_S} , k_{spe} , **Table 2**) is consistent with the low rate of loss of specific acidifying activity ($k_{\text{spe}} < 2 t_{\text{spe}}/\text{day}$) reported by Passot et al. ⁷, exhibiting similar residual water content, and stored in a glassy state.

Table 2. Glass transition temperatures of bacterial concentrates (Tg') of *L. bulgaricus* CFL1 containing three different protective solutions, glass transition temperature (Tg) and water content (%H₂O) of the corresponding freeze-dried samples, loss of specific acidifying activity (Δt_{spe}) during freeze-drying and storage, and rate of loss of acidifying activity (k_{spe}) during two weeks of storage at 28 °C

	Tg'	Tg	%H₂O	Δt_{spe}		k_{spe}
	(°C)	(°C)	(g/100 g)	(min/(log(CFU/mL)))		(min/(log(CFU/mL)))/day
	F	FD	FD	FD	S	S
SUC	-35.8 ^b	32.1	3.8 ^a	55.3 ^a	5.1 ^b	0.34 ^a
IQR	0.6	2.7	0.4	4.7	6.3	0.42
FOS	-36.9 ^b	19.2	4.1 ^a	49.2 ^a	17.5 ^b	1.17 ^a
IQR	0.1	4.2	0.8	14.9	11.8	0.79
GOS	-34.5 ^a	70.1	2.0 ^b	32.7 ^a	9.8 ^b	0.66 ^a
IQR	1.0	2.1	0.4	1.2	1.4	0.09

Data presented are medians of three (Tg', %H₂O, Δt_{spe} , k_{spe}) or two (Tg) biological replicates (and at least 3 technical replicates); IQR: interquartile ranges. Superscript letters (a, b) represent statistical differences (at the 95 % confidence level) between the three protective solutions, for each parameter (Tg', %H₂O, Δt_{spe} , k_{spe}); BF: before freezing; F: freeze-thawed samples; FD: freeze-dried and rehydrated samples; S: freeze-dried samples stored two weeks at 28 °C and rehydrated for the analysis; SUC: sucrose; FOS: fructo-oligosaccharides; GOS: galacto-oligosaccharides.

$\Delta t_{spe_FD} = t_{spe_FD} - t_{spe_BF}$, corresponds to the loss of specific acidifying activity following freeze-drying.

$\Delta t_{spe_S} = t_{spe_S} - t_{spe_FD}$, corresponds to the loss of specific acidifying activity following storage.

$k_{spe} = \Delta t_{spe_S} / \text{days of storage (14)}$, corresponds to the rate of loss of specific acidifying activity during storage.

3.2. Identification of sites of cellular damage induced by the freeze-drying process

FTIR micro-spectroscopy was used to investigate the evolution of the biochemical composition of the bacterial populations after the different steps of the process (freezing, freeze-drying and storage). In addition to direct observation of mean FTIR spectra of frozen and freeze-dried protected cells (**Fig. 3a and 3b**), different data treatments were applied to identify the effect of the process or the composition of the protective formulation: principal component analysis (PCA, **Supplementary data file Fig.S1**), calculation of the PC1 scores difference between freeze-drying and freezing conditions (**Fig. 3c and 3d**), and of the relative variation of the second derivative peak's height (t_{0FTIR} , **Fig. 3e and 3f**).

Table 3 summarizes the assignments of the vibrational bands displayed in **Fig 3e and 3f**.

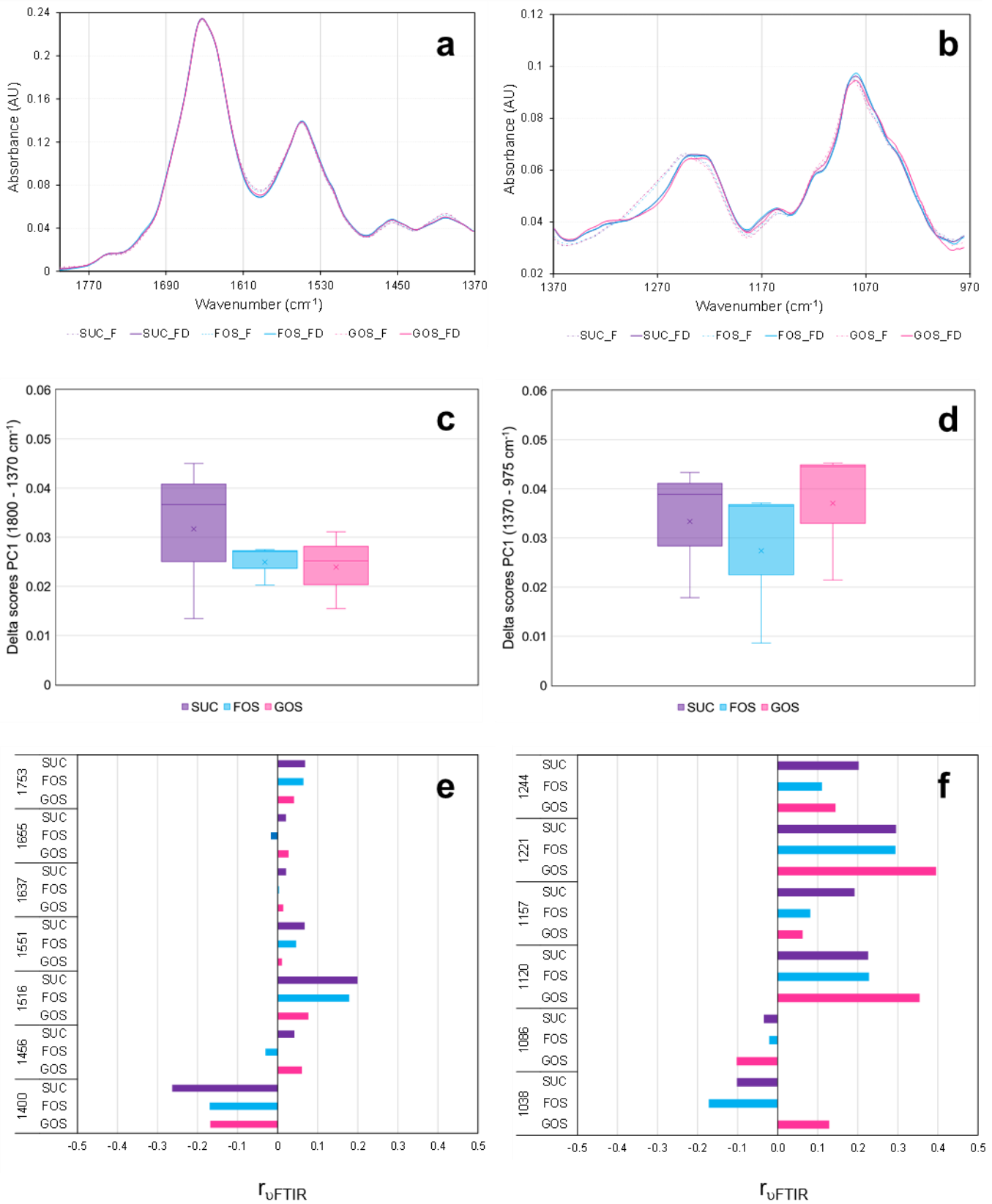


Fig. 3 Biochemical modifications observed by FTIR micro-spectroscopy due to the freeze-drying process, in the **a, b, c** 1800-1370 cm⁻¹ region and **d, e, f** 1370-975 cm⁻¹ region. **a, d**: Mean FTIR spectra of frozen (dotted lines) and freeze-dried (continuous lines) cells protected with sucrose (SUC, purple), fructo-oligosaccharides (FOS, blue) and galacto-oligosaccharides (GOS, pink). **b, e**: scores' differences according to the PC1 of the principal component analysis (PCA) characterizing the freeze-drying effect on the biochemical composition, according to the formulation. **c, f**: Changes in the peak height (r_{FTIR}) of the infrared vibrational bands.

The PCA analysis in the 1800 – 1370 cm^{-1} and 1370 – 975 cm^{-1} spectral regions (**Supplementary data file Fig.S1**) revealed that the freeze-drying process induced more changes in the infrared spectra than the composition of the protective formulation (SUC, FOS, GOS) or the storage step. Regardless of the spectral region and the formulation, the cluster of frozen cells was separated from the clusters of freeze-dried cells along PC1 (74 and 65 % of the total variance, respectively). The second principal component (PC2) accounted for concentrates' heterogeneity with no significant discrimination among formulations (13 and 19 % of the total variance, respectively). PCA analysis was also performed, including the spectra of bacterial stored samples and the clusters of freeze-dried and stored cells overlapped for each formulation. By focusing on r_{vFTIR} values higher than 0.1 (10 % of variation) (**Fig. 3e and 3f**), the principal targets of cellular damage induced by freeze-drying process appeared to be proteins (1221, 1400, 1516, 1244 cm^{-1}), nucleic acids (1121, 1120, 1244 cm^{-1}), and the cell wall components (1221, 1120, 1400, 1244, 1157, 1038 cm^{-1}). Furthermore, the direct observation of the FTIR spectra (fig. 3b), evidenced a significant change in the amide III region (1300 – 1250 cm^{-1}) induced by freeze-drying. FTIR studies on different model proteins previously reported modification of the amide III region due to thermal ²⁴ or chemical ²⁵ denaturation. The authors attributed the modification to changes in alpha-helix and irregular or random coil protein structures. When considering the preservation of lactic acid bacteria, few authors have evidenced alteration of the secondary protein structures ^{26,27}, DNA conformation ¹¹, and cell wall peptidoglycans ³ following stabilization processes. Recently, Guerrero et al. ^{5,23} reported modifications in the protein structures amide II and amide III after freeze-drying of *L. salivarius* CECT5713 cells. However, freeze-drying damage was more pronounced for *L. salivarius* CECT5713, involving additional vibrational bands of carbohydrates of the cell wall and nucleic acids and exhibiting r_{vFTIR} values much higher than *L. bulgaricus* CFL1 (up to 1.8 and 0.4, respectively).

The composition of the protective formulation (SUC, FOS, and GOS) appeared to have a very limited effect on the FTIR features (**Fig 3c and 3d**). SUC exhibited the highest modification in the 1800 – 1370 cm^{-1} spectral region containing mainly information on protein components, whereas similar modifications were observed for the three formulations in the 1370 – 975 cm^{-1} region. Furthermore, the GOS formulation seemed to provide the best protective ability in the amide II proteins' region (1551 and 1516 cm^{-1} , **Fig. 3e**). In agreement with these FTIR outcomes, flow cytometry results based on the activity of an intracellular enzyme (esterase) (**Fig. 4**) showed that the GOS formulation exhibited the lowest proportion of dead cells after freeze-drying.

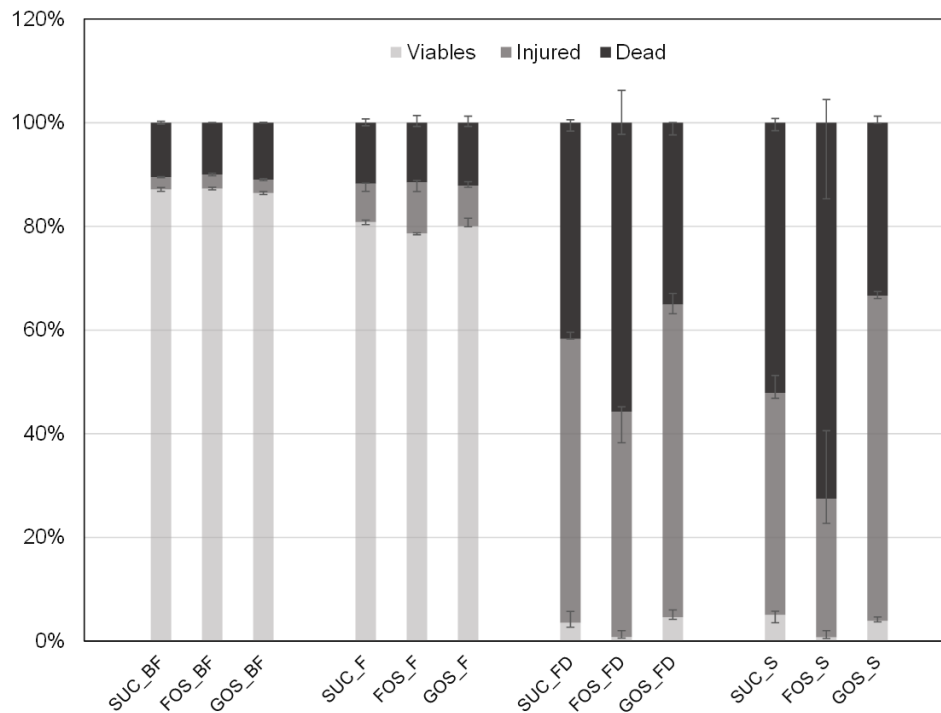


Fig. 4 Relative percentages of viable, injured, and dead cells of *L. bulgaricus* CFL1 concentrates protected with three protective solutions, before freezing (BF), after freezing (F), after freeze-drying (FD) and after 2 weeks of storage at 28 °C). The evolution of percentages was evaluated by flow cytometry combined with dual staining (cFDA-PI). Data are the medians of three biological replicates and at least 3 technical replicates; error bars represent interquartile ranges. SUC: sucrose; FOS: fructo-oligosaccharides; GOS: galacto-oligosaccharides.

The membrane is generally considered the first target of damage following the freeze-drying process due to its location between the external environment and the intracellular medium. The analysis of the FTIR spectra in the spectral region containing specific information on the bacterial membrane (3016 – 2800 cm⁻¹) revealed no modification induced by the freeze-drying process and the storage step. However, when examining the flow cytometry results (**Fig. 4**), a significant loss of membrane integrity (percentage of PI-stained cells: injured + dead cells) was observed after freeze-drying. More than 95% of the bacterial population exhibited membrane damage regardless of the protective formulation. The GOS formulation led to the lowest, but still high, loss of membrane integrity (95.4 % compared to 99.2 % and 96.5 % for the SUC and FOS formulation, respectively).

3.3. Investigation of the protection mechanisms taking place during freeze-drying and storage

Adding protective molecules is an efficient strategy to improve the survival and functionality recovery of LAB after freeze-drying and following storage. Their protective effect has been associated with i) the bacteria immobilization in a glassy matrix^{5,7,23,28-31} and ii) the membrane stabilization by its interaction with small molecules (e.g. disaccharides)^{8,9,32-36}. However, the sufficiency of a glassy state for stability and the mechanism of interaction between small molecules and cell membranes are still controversial. Therefore, in this work, we investigated the role of the glass transition temperature of the bacterial concentrates on *L. bulgaricus* CFL1 stability and the mechanism of cell membrane interactions with the protective formulations.

3.3.1. Glass transition temperature role

The physical state of *L. bulgaricus* CFL1 concentrated suspensions protected with SUC, GOS, and FOS formulations was characterized by measuring the glass transition temperature of the maximally freeze-concentrated phase (T_g'), as well as the glass transition temperature (T_g) and water content (%H₂O) of freeze-dried powders (**Table 2**).

During the freeze-drying, the product temperature (T_p) values were approximately -32°C during sublimation and 25°C during desorption. Therefore, the FOS samples never reached the glassy state ($T_g' = -37^\circ\text{C} < -32^\circ\text{C}$, $T_g = 19^\circ\text{C} < 25^\circ\text{C}$). They remained in the rubbery state all along the freeze-drying and storage steps, which could explain the high loss of acidifying activity observed after freeze-drying and the lowest storage stability (highest value of k_{spe} , Table 2). Conversely, for the GOS and SUC formulations, glassy matrices were obtained during the freeze-drying process. The GOS formulation, providing the best bacterial protection during freeze-drying, exhibited the highest T_g' and T_g values. Although the GOS formulation was also in a viscous state during sublimation ($T_p < T_g'$), it exhibited the lowest value of $(T_p - T_g')$. As a result, the time that samples remained in a viscous state during the process (until enough water was removed to reach a glassy state) was probably shorter for GOS than SUC samples. The GOS capacity to limit *L. bulgaricus* CFL1 inactivation during freeze-drying and storage can be related to the substantial difference of $42\text{--}45^\circ\text{C}$ observed between the glass transition temperature and the desorption and storage temperatures (25°C and 28°C), in line with previous work^{5,23,37}.

However, the SUC formulation provided the best storage stability (lowest k_{spe} value) with a temperature difference ($T_s - T_g$) of only 4°C , suggesting that other preservation mechanisms than vitrification (forming glassy matrices) may account for the protective role of sucrose observed in the present work. Disaccharides are indeed known for stabilizing membranes, but the mechanism by which this is accomplished is still controversial, and most reported works involve model systems of lipids^{34,35,38,39} and proteins^{40–44}.

Recent works propose that the preservation capacity of some FOS on bacteria¹² or some GOS on liposomes¹⁴, relates to the ability of larger oligosaccharides to increase the T_g of the glassy matrix and mono and disaccharides (the smallest sugars) to replace water molecules on lipid membranes.

The high polysaccharide ($\sim 41\%$ starch) and intermediate disaccharide ($\sim 20\%$ DP2) contents of GOS appeared to be the best balance in terms of component size for the protection of *L. bulgaricus* CFL1. However, the presence of reducing sugars in the FOS (monosaccharides) and GOS (galactose and possibly melibiose) formulations may certainly have an impact on the storage stability due to the possible Maillard reactions⁴⁵.

3.3.2. Cell membrane interactions with the protective formulations

To further investigate the preservation mechanisms of sugars and to identify potential interactions with the cell membrane, the changes in membrane phospholipid organization at the core and surface levels were evaluated using FTIR spectroscopy.

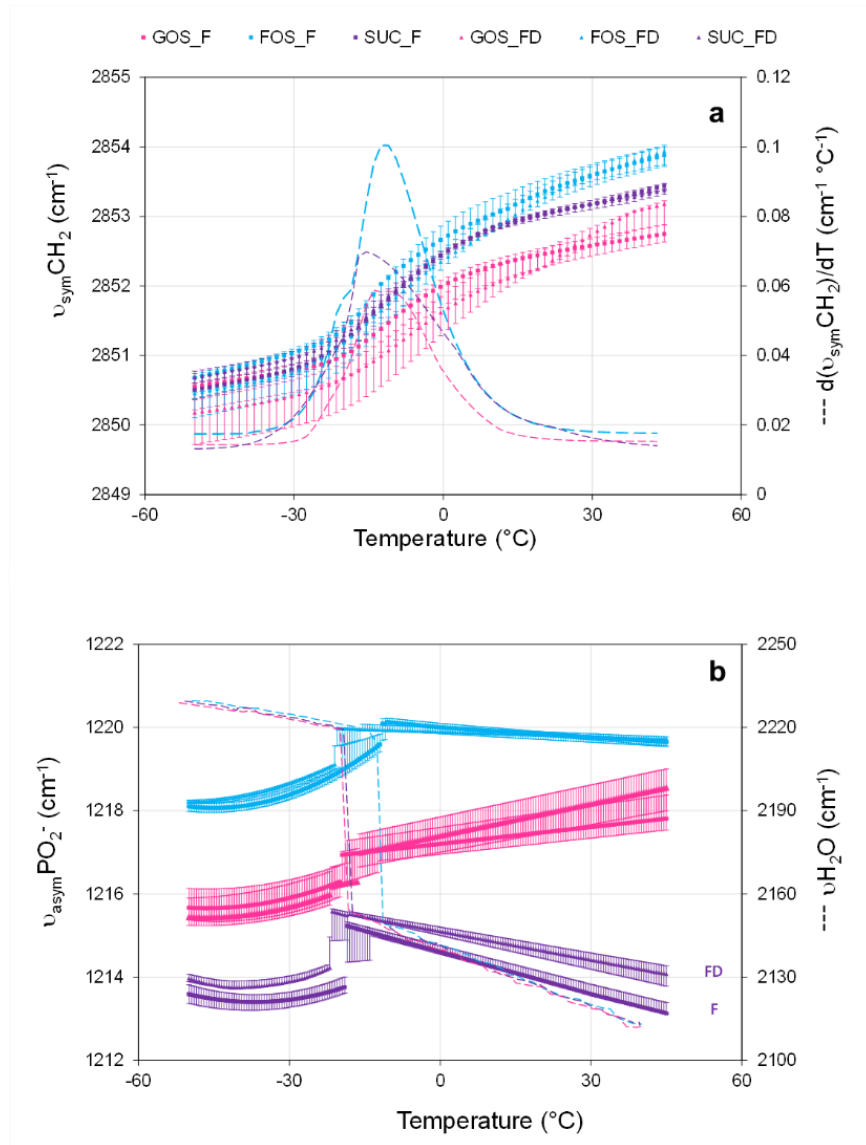


Fig. 5 Effect of freeze-drying on the peak position of the symmetric CH_2 stretching (a) and the asymmetric PO_2^- (b) vibration band arising from *L. bulgaricus* CFL1 cell concentrates with three protective solutions (SUC, FOS, GOS) after freezing (F) and freeze-drying (FD). In panel a, dotted lines indicate the first derivatives of the $\nu_{\text{sym}}\text{CH}_2$ curves, and the maximum of each one corresponds to the lipid transition temperature (T_s) from a liquid-crystalline to a gel phase. In panel b, dotted lines correspond to the H_2O libration and bending band ($\nu_{\text{H}_2\text{O}}$), and the shifts from approx. 2150 to 2220 cm^{-1} indicate ice nucleation. SUC: sucrose; FOS: fructo-oligosaccharides; GOS: galacto-oligosaccharides. Data are the medians of three biological replicates; error bars represent interquartile ranges.

When considering the behaviour of the membrane bilayer's core (**Fig. 5a**), the $\nu_{\text{sym}}\text{CH}_2$ shifted to lower frequencies as temperature decreased, regardless of the formulation considered. This reflects the lipid phase transition of the membrane from a relatively disordered fluid lipid state (usually named the liquid crystalline phase) to an ordered rigid lipid state (usually named the gel phase)^{46,47}. *L. bulgaricus* CFL1 samples protected with GOS and FOS presented the lowest and highest frequency variation following the membrane lipid phase transition from 42 to -42 °C (2.2 cm^{-1} for GOS and 3.3 cm^{-1} for FOS). However, neither the temperature at which this transition takes place during freezing (T_s -12/-13 °C) nor the peak wavenumber ($\nu_s\text{CH}_2$ at T_s from 2851.3 to 2851.9 cm^{-1}) were significantly affected by the protective solution or by the freeze-drying process.

At the level of the external bilayer's surface (**Fig. 5b**), the asymmetric PO_2^- peak position (~ 1218 cm^{-1}), which informs about the strength of P=O bonds and indirectly about the state of phospholipid headgroups hydrogen bonding and their

interactions with the protective molecules^{37–40}, evidenced different behaviour according to the formulation. Regardless of the temperature, FOS and SUC concentrates exhibited the highest and lowest asym PO_2^- wavenumber positions, respectively (p -values < 0.05). The temperature decrease resulted in a linear elevation of the PO_2^- band position for the FOS and SUC formulations until ice nucleation occurred. Upon ice nucleation, a sharp wavenumber downshift was observed, which was less extensive for FOS (0.8 cm^{-1}) compared to SUC formulation (1.2 cm^{-1}) (p -value < 0.05). The GOS formulation presented intermediate frequency values between FOS and SUC, but a band position diminution with decreasing temperatures and the smallest downshift upon ice nucleation (0.55 cm^{-1}).

The low frequency of the PO_2^- band for the SUC condition indicates a weak strength of the P=O bonds, and a low phospholipid headgroup packing, which, in turn, suggests an increase in the interaction of the phospholipid headgroup with the extracellular protective medium (sucrose). This interaction could be attributed either to H-bonding to sucrose molecules (direct interaction by water replacement)^{48–51}, or to water molecules (sucrose exclusion from the hydrated membrane surface)^{33,34,52}. A dual nature of sugar-membrane interactions, which depends on sugar concentration, could explain these opposing views⁵³. Applying small-angle scattering and thermodynamic measurements to model lipid membranes and sugars, Andersen et al.⁵³ showed that at low concentrations ($< 0.2 \text{ M}$), sugars bind quite strongly to the lipid bilayer, while at higher concentrations, such as the sucrose concentration used in the present work (0.58 M), the sugars become progressively excluded from the surface of the membrane.

Either by direct interaction or exclusion, the PO_2^- frequency shifts observed in this work may be related to the mono- and disaccharide content of each formulation, in line with previous works^{54,55}. The SUC and FOS samples with the highest (100 %) and lowest (8 %) small sugar contents ($\text{DP} \leq 2$) exhibited the highest and lowest PO_2^- sugar interactions, respectively. The GOS formulation, with an intermediate small sugar content (20 %) induced an intermediate packing of phospholipid headgroups and level of interaction with sugars. Increasing the proportion of the sugar components with $\text{DP} > 2$ in the protective formulation resulted in decreasing the hydrogen bonding interaction between the bacterial membrane and the extracellular medium, in agreement with previous work on liposomes^{54,56}. Furthermore, the freeze-dried sample protected with sucrose exhibited a slight but significant shift to higher frequency, indicating less H-bonding with PO_2^- groups and higher packing of phospholipid headgroups compared to the frozen samples (**Fig. 5b**). This result indicates modifications with potential damage to membrane functions.

In conclusion, the extract from the wastewater of chickpeas cooking containing galacto-oligosaccharides appears promising as a protectant for ensuring freeze-dried stability of *L. bulgaricus* CFL1 and the potential application of circular bio-economy principles. The protective ability of GOS was related to a high content of polysaccharides, which provided a glassy matrix and enough disaccharide content for preserving bacterial membranes. This work highlighted the relevance of the product's thermal history during freeze-drying to the bacterial recovery after stabilization. Nevertheless, regardless of the formulation investigated (sucrose, FOS, and GOS), freeze-drying induced a significant loss of culturability and acidifying activity, which was ascribed to damage to proteins, membrane, nucleic acids, and peptidoglycans of the cell wall. Furthermore, monitoring the PO_2^- asymmetric stretching vibration position with temperature by FTIR discriminated protective formulations, thus appearing as a relevant tool to investigate potential interactions between bacterial membrane and protective molecules.

Abbreviations used: LAB: lactic acid bacteria; GOS: galacto-oligosaccharides; FOS: fructo-oligosaccharides; SUC: sucrose; FTIR: Fourier transform infrared; MCT-detector: mercury-cadmium-telluride detector; DSC: differential scanning calorimetry; T_g: glass transition temperature; T_p: product temperature; cFDA: carboxyfluorescein diacetate; PI: propidium iodide; PCA: principal component analysis; T_s: lipid phase transition temperature following freezing; T_n: nucleation temperature; BF: before freezing; F: freezing; FD: freeze-drying; S: storage; t_{spe}: specific acidifying activity; k_{spe}: rate of loss of specific acidifying activity.

Supporting information

Additional information includes the principal component analysis (PCA) (score plots and loadings) of FTIR spectra in two spectral regions: 1800 – 1370 cm⁻¹ and 1370 – 975 cm⁻¹, of frozen and freeze-dried *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 cells, protected with SUC, FOS and GOS.

Author contribution

Conceptualization and methodology FF, SP, SC; experiments SC, PL (FTIR spectroscopy), SG (flow cytometry), FF (T_g, T_g' measurement), GM and PC (production of GOS sample); formal analysis SC, PL, SG, FF; data curation and statistical treatment: FF, SP, SC; writing original draft of the paper: FF; writing review SP, FF; final review and editing: all; supervision and project administration FF, SP, PC; resources and funding acquisition FF, SP, PC.

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Data availability

The datasets generated during and/or analyzed during the current study are available in the Data INRAE repository (<https://doi.org/10.57745/N9VZCS>)

Declarations

Ethics approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest: The authors declare no competing interest.

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Supporting information

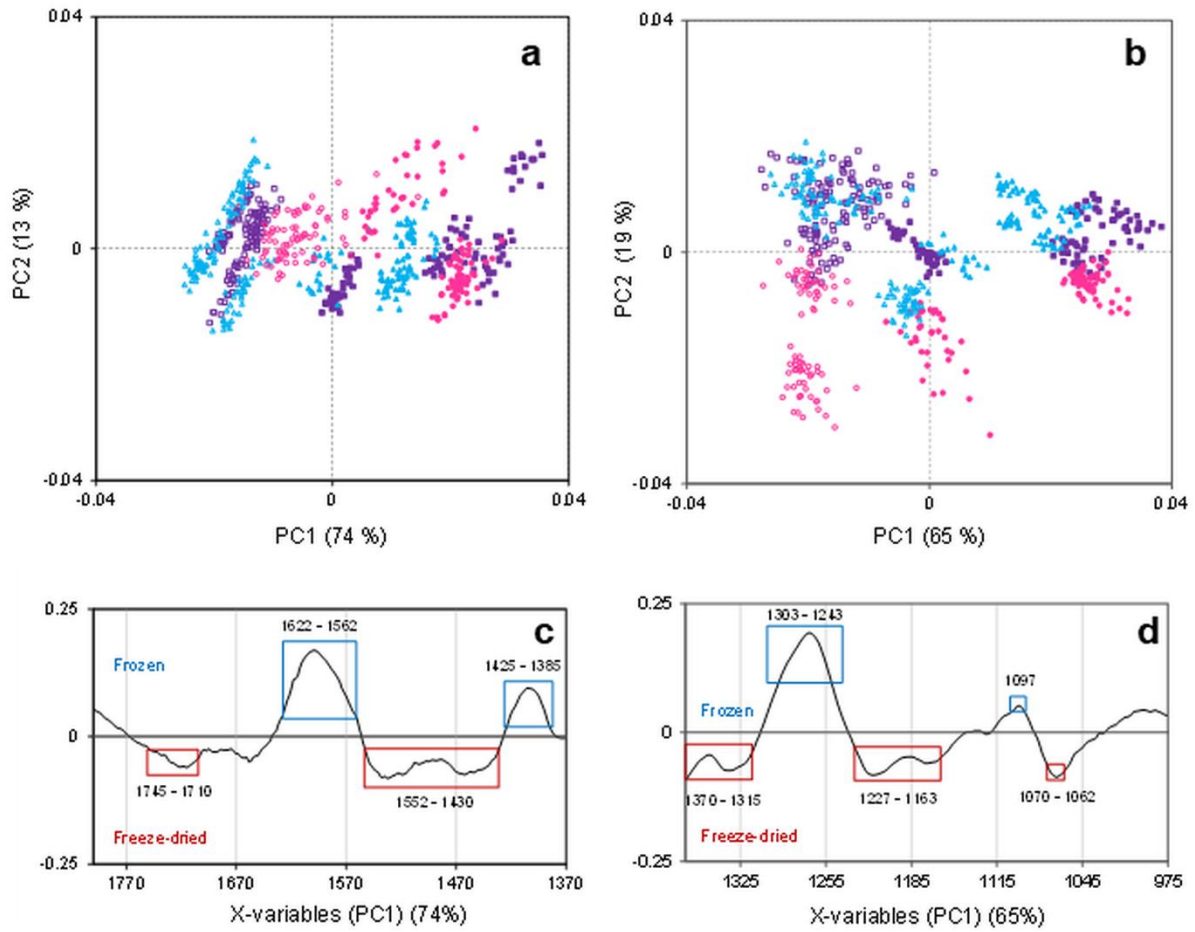


Figure S1 Principal component analysis (PCA) of FTIR normalized and corrected spectra of frozen (filled symbols) and freeze-dried (empty symbols) *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 cells in an aqueous environment, in the **a, c** 1800–1370 cm⁻¹ and **b, d** 1370–975 cm⁻¹ region. **a** PC1 versus PC2 score plot explaining 74 and 13% of the variance, respectively. **b** PC1 versus PC2 score plot explaining 65 and 19% of the variance, respectively. **c, d** Loading plots of the PC1. Positive peaks characterized frozen cells, whereas negative peaks characterized freeze-dried cells of *L. bulgaricus* CFL1 strain. Three protectants were used: sucrose (purple squares), fructo-oligosaccharides (Orafti® P95, Beneo Orafti; Tienen, Belgium) (blue triangles), galacto-oligosaccharides from chickpeas cooking (boiling) wastewaters (produced by the CQM—Centro de Química da Madeira (Universidade da Madeira, Portugal) (pink circles).