Food Research International 90 (2016) 251–258

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Role of mono- and oligosaccharides from FOS as stabilizing agents during freeze-drying and storage of *Lactobacillus delbrueckii* subsp. *bulgaricus*

Nelson Romano [a], Carolina Schebor [b], Pablo Mobili [a], Andrea Gómez-Zavaglia [a,*]

[a] Center for Research and Development in Food Cryotechnology (CIDCA, CCT-CONICET La Plata), RA1900 La Plata, Argentina
[b] Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, UBA, Ciudad Universitaria, CABA, RA1428, Argentina

ARTICLE INFO

Article history:
Received 10 September 2016
Received in revised form 28 October 2016
Accepted 2 November 2016
Available online 05 November 2016

Keywords:
Fructo-oligosaccharides mixtures
Vitreous transition temperature
Protective effect
Lactobacilli
Freeze-drying
Storage

ABSTRACT

The aim of this work was to assess the role of mono- and oligosaccharides present in fructo-oligosaccharides (FOS) mixtures as protective agents during freeze-drying and storage of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333.

Different FOS mixtures were enzymatically obtained from sucrose and further purified by removing the monosaccharides produced as secondary products. Their glass transition temperatures (T_g) were determined at 11, 22 and 33% relative humidity (RH). Bacterial cultures were freeze-dried in the presence of 20% w/v solutions of the studied FOS. Their protective effect during freeze-drying was assessed by bacterial plate counting, and by determining the *lag* time from growth kinetics and the uptake of propidium iodide (PI). Plate counting during bacterial storage at 4 °C, and 11, 22 and 33% RH for 80 days completed this rational analysis of the protective effect of FOS.

Purification of FOS led to an increase of T_g in all the conditions assayed. Microorganisms freeze-dried in the presence of non-purified FOS were those with the shortest *lag* times. Bacteria freeze-dried with pure or commercial FOS (92% of total FOS) showed larger *lag* times (8.9–12.6 h). The cultivability of microorganisms freeze-dried with non-purified FOS and with sucrose was not significantly different from that of bacteria before freeze-drying (8.74 ± 0.14 log CFU/mL). Pure or commercial FOS were less efficient in protecting bacteria during freeze-drying. All the protectants prevented membrane damage. The cultivability of bacteria freeze-dried with FOS decayed <1 logarithmic unit after 80 days of storage at 11% RH. When storing at 22 and 33% RH, pure and commercial FOS were those that best protected bacteria, and FOS containing monosaccharides were less efficient.

The effect of FOS on bacterial protection is the result of a balance between monosaccharides, sucrose and larger FOS in the mixtures: the smallest sugars are more efficient in protecting lipid membranes, and the larger ones favor the formation of vitreous states.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Lactic acid bacteria have an important role in food and biotechnology industries, as they are widely used as starters for the manufacturing of food and probiotic products. Considering that benefits of probiotic consumption can be obtained when at least 6–7 log CFU of viable microorganisms per gram of product are present at the end of shelf-life (Aquilina et al., 2013; Phuapaiboon et al., 2013; Tripathi & Giri, 2014; Hill et al., 2014), adequate preservation processes are necessary to minimize viability and functionality losses, and stabilize microorganisms during storage.

Freeze-drying is one of the most widely used processes to preserve lactic acid bacteria (Fonseca, Cenard, & Passot, 2015; Meng, Stanton, Fitzgerald, Daly, & Ross, 2008; Morgan, Herman, White, & Vesey, 2006). However, the dehydration involved in the process leads to structural damages, which in turn, result in a decrease of bacterial viability (Tymczyszyn, Gómez-Zavaglia, & Disalvo, 2007; Tymczyszyn et al., 2008). To avoid these problems, using protective compounds becomes mandatory and sugars are generally used for this purpose (Carvalho et al., 2004).

There are two accepted hypotheses to explain the protective effect of sugars. One of them proposes that sugars can replace water molecules during dehydration and maintain biological structures in hydrated conditions (Crowe, Hoekstra, & Crowe, 1992; Leslie, Israeli, Lighthart,

Abbreviations: FOS, fructo-oligosaccharides; DP, degree of polymerization; DP3, degree of polymerization equal to three; DP4, degree of polymerization equal to four; DP5, degree of polymerization equal to five; HPLC, high performance liquid chromatography; DSC, differential scanning calorimetry; T_g, glass transition temperature; PI, propidium iodide; RH, relative humidity; M_n, average molecular weight; Tm, membrane phase transition temperature; DPPC, dipalmitoyl phosphatidylcholine; T, temperature of storage.

* Corresponding author at: Calle 47 y 116, 1900 La Plata, Buenos Aires, Argentina.
E-mail address: angoza@qui.uc.pt (A. Gómez-Zavaglia).

http://dx.doi.org/10.1016/j.foodres.2016.11.003
0963-9969/© 2016 Elsevier Ltd. All rights reserved.

Crowe, & Crowe, 1995; Santivarangkna, Higl, & Foerst, 2008). The other hypothesis is based on the capacity of sugars to form glassy matrices (vitrification) in which the high viscosity and low molecular mobility constraints molecular interactions (Tymczyszyn, Gerbino, Illanes, & Gómez-Zavaglia, 2011; Tymczyszyn et al., 2012). Glass transitions are dependent on the water content and occur at a given temperature. Hence, for a successful storage samples shall remain in an amorphous state, that is below the vitreous transition temperature (T_g) (Higl et al., 2007; Miao et al., 2008). These two hypotheses are not excluding. In fact, it has been reported that vitrification is a necessary but not sufficient condition for a good protection (Crowe, Carpenter, & Crowe, 1998; Oldenhof, Wolkers, Fonseca, Passot, & Marin, 2005). In this regard, some authors suggest that the conjoint use of high T_g polysaccharides with small sugars having not so high T_g but that interact with membranes (*e.g.*, sucrose or glucose) may be a good strategy for bacterial stabilization (Oldenhof et al., 2005).

Fructo-oligosaccharides (FOS) are well recognized prebiotics, that is, "non-digestible food components that beneficially affect the host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson & Roberfroid, 1995). They are used in infant formula and other functional food products (Romano, Tymczyszyn, Mobili, & Gómez-Zavaglia, 2015, chap. 10). From a chemical point of view, they are mixtures of small chain oligosaccharides composed of fructose units linked by (2 → 1)-β-glycosidic bonds and a single D-glucosyl unit at the non-reducing end. They can be synthesized from sucrose using fructosyltransferases (β-fructofuranosidase, EC 3.2.1.26 or β-D-fructosyltransferase, EC 2.4.1.9) as biocatalysts (Vega & Zuniga-Hansen, 2011, 2012, 2014; Romano, Santos, Mobili, Vega, & Gómez-Zavaglia, 2016). This process leads to the obtaining of mixtures containing FOS of degrees of polymerization (DP) ranging from 2 to 6 (Crittenden & Playne, 2009). As most of the commercial fructosyltransferases usually have both fructosyltransferase and hydrolase activities, monosaccharides (*e.g.*: glucose and fructose) are also produced as result of the enzymatic reaction. Therefore, their removal (generally using glucose oxidase or chromatographic methods) contributes to increase the production throughput of FOS and thus, enhance their prebiotic properties (Vega & Zuniga-Hansen, 2014).

The protective effect of FOS has been scarcely addressed. In this regard, the interaction of short chain FOS [*e.g.*, 1-kestose (DP3), nystose (DP4), 1^F-fructofuranosylnystose (DP5)] with model systems (liposomes) has demonstrated to be size and species dependent (Hincha, Popova, & Cacela, 2006; Hincha et al., 2007; Vereyken, Chupin, Demel, Smeekens, & De Kruijff, 2001; Vereyken, Chupin, Hoekstra, Smeekens, & De Kruijff, 2003; Vereyken et al., 2002). The stabilizing effect of FOS during freeze-drying and spray-drying of lactic acid bacteria has been reported more recently (Golowczyc et al., 2011; Romano et al., 2015, chap. 10). However, the effect of FOS composition, particularly the role of the monosaccharides resulting from the enzymatic synthesis, has not been addressed hereto. To this aim, a rational study including FOS of different compositions, with and without monosaccharides becomes necessary.

In this work, FOS of different compositions were enzymatically synthesized using sucrose as initial substrate. The monosaccharides produced during enzymatic reactions were removed using an activated charcoal column. The composition of the obtained products was determined by HPLC, before and after removing monosaccharides. The T_g of the obtained mixtures were determined at different relative humidities. The protective effect of FOS during freeze-drying of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333 [a strain very sensitive to any kind of stress (Tymczyszyn et al., 2007, 2008, 2011, 2012)] was assessed by bacterial plate counting, by determining the *lag* time from growth kinetics (indicator of global damage), and by determining membrane damage using SYTO 9® and propidium iodide (PI) fluorescent probes. Plate counting during bacterial storage at 4 °C for 80 days allowed a comprehensive analysis of the protective effect of FOS during storage.

2. Materials and methods

2.1. Materials

Viscozyme L was donated by Blumos SA-Chile. 1-Kestose (DP3), nystose (DP4) and 1^F-fructofuranosylnystose (DP5) standards were purchased from Wako Chemicals (Richmond, VA, USA). Sucrose, glucose and fructose were obtained from Sigma Chemical (St. Louis, MO, USA). Commercial FOS were obtained from Orafti Beneo p95 (Mannheim, Germany), and LiCl, KCH_3COO and $MgCl_2$, from Anedra, (Buenos Aires, Argentina). Activated charcoal was supplied by Cicarelli (Santa Fe, Argentina) in a granular form, with 1.5 mm mean particle diameter. Ethanol was obtained from Anedra (Buenos Aires, Argentina). MRS broth was obtained from Difco (Detroit, MI, USA) and the dye mixture containing SYTO 9® and PI 1:1, from BacLight L-7012 (Molecular Probes, Eugene, Oregon, USA).

2.2. Methods

2.2.1. Synthesis of FOS

40% w/v sucrose solutions prepared in distilled water were used as substrate for the enzymatic synthesis. 4% v/v Viscozyme L (56 FU/mL; FU: fructosyltransferase units) was used as biocatalyst and the pH was adjusted to 5.5 with 2 M NaOH, according to Romano et al. (2016). The enzymatic reaction was performed at 50 ± 1 °C in 25 mL Erlenmeyer flasks with stirring (100 rpm). One fructosyltransferase unit was the amount of enzyme required to transfer 1 μmol of fructose per minute at pH 5.5, 50 °C and 100 rpm stirring. The reactions were stopped by heating the products (obtained as syrups) at 100 °C for 2 min. Samples were collected throughout the synthesis after 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 and 24 h of incubation.

The composition of the synthesized FOS was analyzed by HPLC in a Perkin-Elmer Series 200 equipment (Massachusetts, USA) with refractive index detector and autosampler. Commercial FOS were also included for comparison. A BP-100 Ag + (300 × 7.8 mm) chromatographic column for carbohydrate analysis (Benson Polymeric, Reno, NV, USA) was used. The column is composed of a stable high cross-linked styrene-divinylbenzene copolymer resin in the silver form that can resolve saccharides as large as DP7.

Column and detector temperatures were maintained at 50 °C and 40 °C, respectively. Once collected, samples were filtered through 0.22 μm Millipore Durapore membranes (Billerica, MA, USA) and eluted with Milli-Q water (mobile phase) at a flow-rate of 0.4 mL/min. Chromatograms were integrated using Total Chrom software (version 6.3.1, Perkin Elmer, USA).

The composition of samples was determined by assuming that the area of each peak was proportional to the weight percentage of the respective sugar on the total sugar mass (Boon, Janssen, & van der Padt, 1999). The accuracy of such assumption was checked by making a material balance. External standards of fructose, glucose, sucrose, 1-kestose (DP3), nystose (DP4) and 1^F-fructofuranosylnystose (DP5) were used to determine their retention times and check the linear range of the measurements.

2.2.2. Purification of the obtained FOS

2.2.2.1. Preparation of the column. To purify the synthesized FOS (that is, to remove monosaccharides) activated charcoal was used as adsorbent. Before filling the column, charcoal was washed and autoclaved to remove particles and air from the pores. Then, 180 g activated charcoal were loaded into a glass column of 300 mm × 44.8 mm internal diameter (IVA, Buenos Aires, Argentina), previously filled with Milli-Q pure water. To remove the fines and air bubbles, and equilibrate the charcoal inside the column, 4 L of Milli-Q water were pumped with a peristaltic pump (Gilson, Middleton, WI, USA) at a higher flow-rate than that used to purify FOS (18 mL/min).

2.2.2.2. FOS purification. The activated charcoal column was loaded with 200 mL of FOS syrups at a flow rate of 18 mL/min. The syrups were recirculated until the equilibrium between the sorbent and the moving phase was obtained (about 3 h). To remove the non-adsorbed sugars, 6 L of Milli-Q pure water were passed through the column. The retained sugars were then recovered by elution with a gradient of ethanol as follows: 1 L of 2.5% v/v ethanol and 1 L of 5% v/v ethanol for monosaccharides' desorption (these fractions were discarded), 1 L of 15% v/v ethanol and 2 L of 20% v/v ethanol for FOS elution. All experiments were performed at 25 °C. The fractions collected in the desorption-phase were evaporated at 60 °C with a rotary evaporator (Büchi Rotavapor, Flawil, Switzerland), to remove ethanol. Concentrated sugars were quantified by HPLC as explained in Section 2.2.1, and then freeze-dried.

2.2.3. Glass transition temperatures

20% w/v solutions were prepared using the synthesized FOS before and after purification, the commercial FOS and crystalline sucrose. 1 mL of the obtained solutions was transferred into 5 mL glass vials and frozen at −80 °C for 48 h. Freeze-drying was carried out on a Heto FD4 equipment (Heto Lab Equipment, Denmark) operating with the condenser at −45 °C at a chamber pressure of 0.04 mbar. The freeze-drying process lasted for 48 h.

Glass transitions of the freeze-dried samples were determined by differential scanning calorimetry (DSC) (onset values, heating rate: 10 °C/min) using a Q100 calorimeter (TA Instruments, New Castle, DE, USA), calibrated with indium, lead and zinc. Hermetically sealed 40 μL medium pressure pans were used (an empty pan served as reference). An average value of at least two replicates is reported. The standard deviation for the glass transition temperature measurement was ±1 °C.

2.2.4. Bacterial strains and growth conditions

Lactobacillus delbrueckii subsp. *bulgaricus* CIDCA 333 was isolated from a commercial starter (Gomez-Zavaglia, Abraham, Giorgieri, & De Antoni, 1999). The strain was maintained frozen at −80 °C in 120 g/L non-fat milk solids.

2.2.5. Preparation of samples for freeze-drying

Cultures grown in MRS broth (Difco, Detroit, MI, USA) (de Man, Rogosa, & Sharpe, 1960) at 37 °C overnight to obtain approximately 5.5×10^8 CFU/mL (stationary phase) were harvested by centrifugation at 4000 ×*g* for 10 min. The pellets were washed twice with 0.85% w/v NaCl and resuspended in the same volume of 20% w/v aqueous solutions of FOS (commercial and synthesized before and after purification) or sucrose, previously sterilized using 0.2 μm sterile filters, or in 0.85% w/v NaCl (control).

2.2.6. Freeze-drying procedure

One milliliter of each suspension was transferred into 5 mL glass vials and frozen in liquid nitrogen (−196 °C). Freeze-drying was carried out on a Heto FD4 equipment (Heto Lab Equipment, Denmark) operating with the condenser at −45 °C at a chamber pressure of 0.04 mbar. The freeze-drying process lasted for 48 h.

2.2.7. Determination of the lag time

Freeze-dried microorganisms were rehydrated at 25 °C in 1 mL of 0.85% w/v NaCl. The rehydrated microorganisms were inoculated in MRS broth (2% inoculum) and incubated at 37 °C. Acidification kinetics were followed by determining the pH every 60 min for each condition assayed.

2.2.8. Determination of membrane damage

Flow cytometry was used to determine viable and damaged microorganisms after each freeze-drying treatment. For each assay, the freeze-dried microorganisms were rehydrated at 25 °C in 1 mL 0.85% w/v NaCl, and bacterial suspensions were adjusted to 1×10^6 bacteria/mL. 100 μL of the dye mixture containing SYTO 9® and PI (1:1 ratio; final concentration 0.5 mg/mL) were added to 1 mL of the bacterial suspensions, and incubated for 2 min at 25 °C in the dark.

Determinations were carried out with a FACS Calibur instrument using the CellQuest software (Becton Dickinson, Mountain View, CA, USA). For each sample 10,000 events were collected, the event rate being <300 events/s. All parameters were collected as logarithmic signals. FL1 channel (530 nm) was used to set the green fluorescence of SYTO and FL3 channel (650), to set the red fluorescence of PI. Mixtures of thermally dead cells (80 °C for 30 min) and freshly harvested cells were stained with SYTO and PI both in double-staining assays. They were used as controls to set the flow cytometer detectors and compensation, to differentiate three regions: Q1 (damaged and dead bacteria): PI+ and SYTO−; Q2 (debris): PI− and SYTO−; and Q3 (viable bacteria): PI− and SYTO+. The percentage of each population was determined as [i / (Q1 + Q3)] / 100, where i is Q1 or Q3. Non-fluorescent debris (Q2) were excluded (Hiraoka & Kimbara, 2002).

2.2.9. Storage experiments

The freeze-dried samples obtained in Section 2.2.6 were stored for 80 days at 4 °C in atmospheres of LiCl, KCH_3COO and $MgCl_2$, giving relative humidities (RHs) of 11, 22 and 33%, respectively. Cultivability was determined immediately after freeze-drying, after equilibration at the storage RHs (*ca.* 7–10 days), and then, every 15 days. For each determination, samples were re-hydrated in 1 mL 0.85% w/v NaCl. Bacterial suspensions were serially diluted, plated on MRS agar, and incubated at 37 °C for 48 h in aerobic conditions.

2.2.10. Reproducibility of results

All experiments were performed on duplicate samples using three independent cultures of bacteria. The relative differences were reproducible irrespective of the cultures used. Analysis of variance (ANOVA) was carried out using the statistical program Infostat v2009 software (Córdoba, Argentina). Differences were tested with paired sample t tests, and if $P < 0.05$ the difference was considered statistically significant.

3. Results

The composition of FOS obtained throughout the enzymatic synthesis is provided in Table S1. As some of the products had similar compositions, only those showing the largest differences (those obtained after 2.5 and 24 h of synthesis) were selected for the remaining assays. They were then purified to remove monosaccharides, and their composition before and after purification, together with that of commercial FOS is shown in Table 1. The concentration of total FOS before purification was 55–56%, and after purification, 86–87% for both the 2.5 and the 24 h reactions. DP3 and DP4 were the main FOS present in the reaction medium after 2.5 h of synthesis. After 24 h synthesis, DP3, DP4 and DP5 were predominant, and small amounts of DP6 were also present. The composition of commercial FOS was more heterogeneous, DP3 and DP4 being the major components, with lower contribution of DP5 and DP6, and small amounts of DP7.

Table 1
Composition of the FOS used as protectants.

	Synthesis				Commercial
	2.5 h	2.5 h (pure)	24 h	24 h (pure)	
Monosaccharides (%)	23	2	35	4	3
Sucrose (%)	21	12	10	9	5
DP3 (%)	44	59	11	22	25
DP4 (%)	10	24	27	44	29
DP5 (%)	2	3	15	20	18
DP6 (%)	0	0	2	1	14
DP7 (%)	0	0	0	0	6
Total FOS (%)	56	86	55	87	92

Table 2 shows the T_g of the investigated FOS at 11, 22 and 33% RH, together with that of sucrose, used for comparison. As expected, the values of T_g decreased as soon as the RH increased. Purification of FOS led to an increase of T_g at all the three RH. Pure FOS obtained after 24 h synthesis were those showing the highest T_g values. T_g values of pure FOS obtained after 2.5 h synthesis were slightly lower, and similar to those of commercial FOS. In all cases, the T_g values of pure and commercial FOS were higher than those of sucrose.

In a further step, *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 was freeze-dried in 20% w/v solutions of FOS and sucrose. The acidification kinetics obtained after freeze-drying (Fig. 1) were adjusted according to Eq. (1):

$$pH(t) = \frac{pH_0 - pH_f}{1 + \frac{t}{c}^{p}} + pH_f \quad (1)$$

where t is the time in hours, pH_0 is the pH of the culture medium at time equal to 0, pH_f is the pH once attained the stationary phase, c is the time corresponding to the inflection point and p is an exponential fitting factor. The *lag* time was calculated as the intersection between the tangent line at $t = c$ and pH_0. The acidification rate during the exponential phase was calculated as the module of the slope of the tangent line. The values of the *lag* time, acidification rate and final pH for all the growth kinetics are shown in Table 3. Microorganisms freeze-dried in the presence of non-purified FOS (both after 2.5 and 24 h of synthesis) were those with the shortest *lag* times: 5.6 and 6.0 h, respectively (full up-triangles and full circles in Fig. 1). These values were lower than those corresponding to the *lag* time of microorganisms freeze-dried in the presence of sucrose: 8.5 h (full squares). In turn, bacteria freeze-dried with purified FOS had larger *lag* times: 8.9 h for microorganisms dehydrated with FOS obtained after 2.5 h, and 12.6 h for those dehydrated with FOS obtained after 24 h (open up-triangles and open circles). Commercial FOS, containing very low amounts of monosaccharides, had similar *lag* times (10.2 h, open down-triangles) than purified FOS. The acidification rates followed the same pattern as the *lag* times: FOS containing monosaccharides, and sucrose were those with the highest acidification rates whereas pure and commercial FOS showed the lowest ones. The final pH was around 4.10–4.20 in all the conditions assayed, exception made of bacteria freeze-dried without protectants, which did not attain the stationary phase after 50 h incubation (Fig. 1).

Table 4 depicts the viable cell counts and percentage of damage (measured as PI uptake) after the different treatments. Non-treated microorganisms and microorganisms freeze-dried without protectants were used as controls. The cultivability of microorganisms freeze-dried with non-purified FOS and with sucrose was not significantly different from that of non-treated microorganisms ($P > 0.05$). On the contrary, the cultivability of bacteria freeze-dried with pure or commercial FOS was significantly lower than that of the controls ($P < 0.05$). Pure FOS obtained after 24 h synthesis provided the lowest protection to microorganisms during freeze-drying. Humidification procedures resulted in a cultivability decrease of *ca.* 1 logarithmic unit in all cases. The PI uptake provided information about membrane integrity. All the protectants prevented damage, but in a different extent. Pure FOS and sucrose were the most efficient ones, and the efficiency of non-purified and commercial FOS was significantly lower ($P < 0.05$).

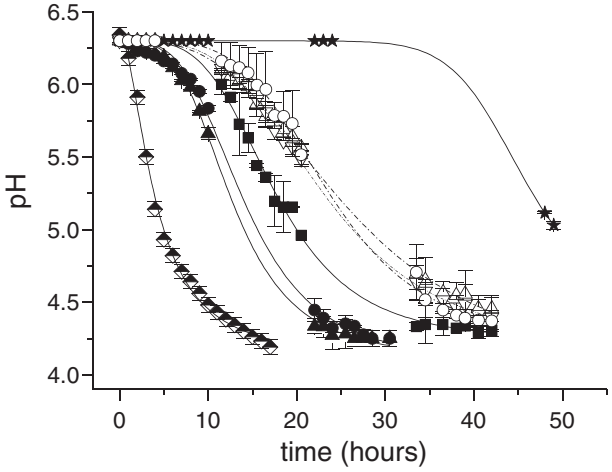

Fig. 1. Growth kinetics of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333 freeze-dried in the presence of: FOS obtained after 2.5 h of synthesis (full up-triangle), pure FOS obtained after 2.5 h of synthesis (open up-triangle), FOS obtained after 24 h of synthesis (full circle), pure FOS obtained after 24 h of synthesis (open circle), commercial FOS (open down triangle), sucrose (full square). Microorganisms freeze-dried in the absence of protectants: full stars; non-freeze-dried microorganisms: half open diamond. Dash dot lines: regressions corresponding to pure FOS obtained after 2.5 and 24 h of synthesis and to commercial FOS. Solid lines: regressions corresponding to the remaining conditions.

Fig. 2 shows the loss of cultivability of microorganisms stored at 11, 22 and 33% RH at 4 °C along time. To analyze the effect of FOS just during storage, the cultivability obtained after equilibration (Table 4) was considered as N_0, and the log N/N_0 *vs* time was plotted for each RH. Storage at 11% RH was the best condition for all the protectants (Fig. 2A). In this condition, the cultivability of bacteria freeze-dried with FOS decayed < 1 logarithmic unit after 80 days. The composition of FOS did not lead to significant differences on bacterial cultivability during storage ($P > 0.05$). When storing at 22 and 33% RH, pure and commercial FOS (opened symbols) were those that best protected bacteria, and FOS containing monosaccharides (non-purified FOS) were less efficient (Fig. 2B and C). It must be pointed out that FOS containing monosaccharides led to sticky samples when stored at 33% RH for 30 days.

4. Discussion

Although the protective capacity of FOS can be explained on the basis of their carbohydrate nature, their stabilizing effect during freeze-drying or spray-drying has only been proved empirically (Golowczyc et al., 2011; Romano et al., 2015, chap. 10). Moreover, no rational studies have been performed to analyze whether the presence of monosaccharides in the FOS mixtures enhance or decrease the protective effect of FOS.

Table 2
Vitreous transition temperatures of FOS and sucrose at different relative humidities (RH).

FOS/sucrose	T_g		
	11% RH	22% RH	33% RH
2.5 h	30.79 °C	15.28 °C	0.56 °C
2.5 h (pure)	49.06 °C	39.72 °C	28.55 °C
24 h	20.06 °C	13.60 °C	−6.10 °C
24 h (pure)	53.65 °C	43.92 °C	30.58 °C
Commercial	46.92 °C	37.49 °C	27.88 °C
Sucrose	40.28 °C	31.28 °C	14.58 °C

Table 3
Kinetic parameters of microorganisms grown in MRS broth after freeze-drying in the presence of FOS or sucrose.

FOS/sucrose	*lag* time (h)	Medium acidification rate (pH units/h)	pH (final)	R^2
2.5 h	5.6	0.15	4.11	0.999
2.5 h (pure)	8.9	0.07	4.02	0.999
24 h	6.0	0.14	4.07	0.997
24 h (pure)	12.6	0.09	4.20	0.997
Commercial	10.2	0.08	4.17	0.999
Sucrose	8.5	0.12	4.22	0.997
Without protectant	37.1	0.11	4.56	1
Control (non-freeze-dried)	0.44	0.25	4.07	0.996

Table 4
Logarithm of CFU/mL of microorganisms recovered and % of PI uptake before and after freeze-drying and humidification at different RH in the presence of 20% w/v FOS of different compositions. Different letters (a, b, c and d) denote statistically significant differences ($P < 0.05$).

Treatment	Log CFU/mL	% PI uptake
Control (non-treated microorganisms)	8.74 ± 0.14 (a)	5.33 ± 1.87 (a)
Freeze-drying with FOS 2.5 h	8.68 ± 0.00 (a)	23.39 ± 3.54 (b)
Humidification at 11% RH	7.65 ± 0.04	n.d.[a]
Humidification at 22% RH	7.98 ± 0.75	
Humidification at 33% RH	8.10 ± 0.71	
Freeze-drying with FOS 2.5 h (pure)	6.28 ± 0.10 (b)	17.71 ± 1.34 (c)
Humidification at 11% RH	5.84 ± 0.00	n.d.
Humidification at 22% RH	5.54 ± 0.00	
Humidification at 33% RH	5.67 ± 0.04	
Freeze-drying with FOS 24 h	8.66 ± 0.10 (a)	21.76 ± 0.27 (b)
Humidification at 11% RH	7.79 ± 0.02	n.d.
Humidification at 22% RH	8.27 ± 0.71	
Humidification at 33% RH	7.79 ± 0.02	
Freeze-drying with FOS 24 h (pure)	5.76 ± 0.19 (c)	17.14 ± 0.33 (c)
Humidification at 11% RH	4.70 ± 0.07	n.d.
Humidification at 22% RH	4.71 ± 0.05	
Humidification at 33% RH	4.98 ± 0.07	
Freeze-drying with commercial FOS	6.40 ± 0.13 (b)	23.49 ± 3.54 (b)
Humidification at 11% RH	5.40 ± 0.00	n.d.
Humidification at 22% RH	5.07 ± 0.10	
Humidification at 33% RH	5.24 ± 0.28	
Freeze-drying with sucrose	8.58 ± 0.20 (a)	17.69 ± 0.76 (c)
Humidification at 11% RH	8.58 ± 0.00	n.d.
Humidification at 22% RH	8.14 ± 0.03	
Humidification at 33% RH	8.58 ± 0.05	
Freeze-dried in the absence of protectants	2.79 ± 0.16 (d)	87.62 ± 1.55 (d)

[a] n.d.: not determined.

The analysis of the protective effect of FOS of different compositions during freeze-drying and during storage, allowed a comprehensive understanding of their protective role on each step of the preservation process. We succeeded in obtaining these mixtures by collecting the reaction products at different times of incubation. Although DP3 is the oligosaccharide produced with the highest efficiency regardless the initial concentration of sucrose (Romano et al., 2016), its relative contribution to the total FOS depends on the contribution of the other FOS present in the mixture. It must be pointed out that the enzymatic production of short chain FOS is a complex process involving different reactions of synthesis (transfructosylation) and hydrolysis that occur simultaneously both in parallel and in series, through consecutive sets of disproportionation reactions (Vega & Zuniga-Hansen, 2014). In these reactions, the FOS synthesized in the first steps act as fructosyl donors and acceptors leading to a simultaneous production of FOS with DP immediately higher (DPn + 1) and lower (DPn − 1) than those of the FOS acting as reagents (Jung, Yun, Kang, Lim, & Lee, 1989). When the availability of sucrose (DP2) is high, as occurs at the beginning of the enzymatic reaction, the reaction is displaced to the production of DP3 (DP2 + 1), with release of glucose (DP2 − 1). Once attained the maximum sucrose conversion, DP3 acts as both fructosyl donor and acceptor to produce DP4. This latter acts as fructosyl donor and acceptor to yield DP5, only when DP3 starts decreasing (Vega & Zuniga-Hansen, 2014). In this work, we were able to obtain DP6 upon prolonged times of incubation (24 h) (Table S1). This oligosaccharide is usually present in FOS obtained by hydrolysis of polysaccharides (*e.g.*, inulin), but had never been reported as a product of the enzymatic synthesis of FOS. However, together with the production of DP6, an increase of the monosaccharides' concentration was also observed, which results from the decrease of the transfructosylation/hydrolysis ratio (Tables 1 and S1).

To analyze the protective effect of the different FOS on bacterial freeze-drying and storage, the mixtures with the largest composition differences (those obtained after 2.5 and 24 h synthesis) were selected. The products obtained after 2.5 h of synthesis were those combining a high sucrose conversion, high concentration of DP3 and relatively low concentrations of DP4. The reaction products obtained after 24 h of

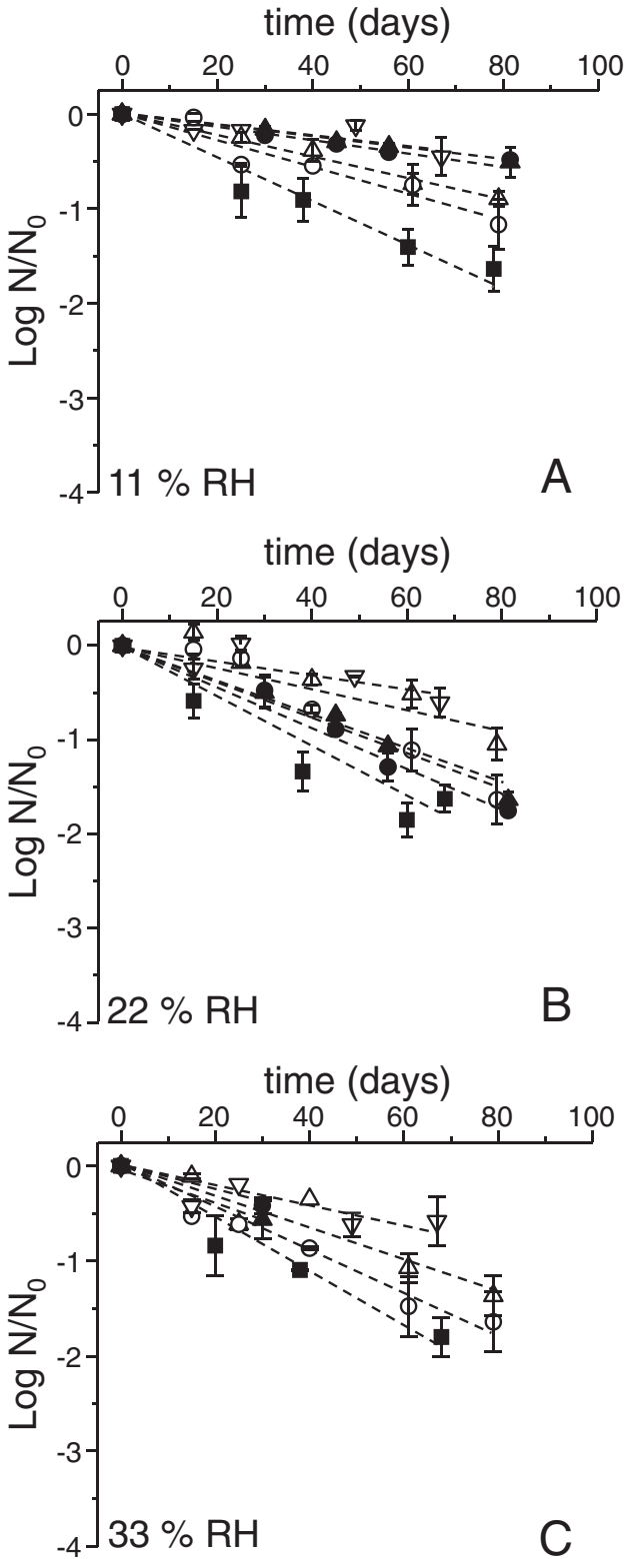

Fig. 2. Loss of cultivability of freeze-dried *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 as a function of the time of storage at 4 °C. A: bacteria equilibrated at 11% RH; B: bacteria equilibrated at 22% RH; C: bacteria equilibrated at 33% RH. N = CFU/mL after storage; N_0 = CFU/mL after equilibration (Table 4). Dashed lines correspond to linear regressions. Bacteria stabilized with: FOS obtained after 2.5 h of synthesis (full up-triangle), pure FOS obtained after 2.5 h of synthesis (open up-triangle), FOS obtained after 24 h of synthesis (full circle), pure FOS obtained after 24 h of synthesis (open circle), commercial FOS (open down triangle), sucrose (full square). Microorganisms freeze-dried in the absence of protectants could not be plate counted because of their low cultivability already after freeze-drying (2.79 ± 0.16) (see Table 4).

synthesis had a more heterogeneous composition, including DP3, DP4, DP5 and DP6. The monosaccharides present in both mixtures were removed using a charcoal column, thus leading to two further FOS (named "pure FOS") (Table 1). Hence, five different FOS' compositions were investigated: as obtained and purified FOS resulting from 2.5 and 24 h of synthesis, and FOS obtained from hydrolysis of inulin (commercial FOS). These latter FOS had higher concentrations of DP6 and DP7, and were comparable to pure FOS as almost no monosaccharides were present in the mixture (Table 1).

Removing monosaccharides from FOS led to a noticeable increase in the T_g of the mixtures (Table 2). Pure FOS obtained after 24 h of synthesis were those with the highest T_g at all the RH assayed, followed by those obtained after 2.5 h of synthesis and the commercial ones. Their T_gs were between 6 and 16 °C higher than those of sucrose (used as a reference). It is well-known that T_g increases with the molecular weight and reaches a limiting value at moderate molecular weights. In this regard, Fox and Flory (1950) defined an equation that relates the number-average molecular weight (M_n) with the T_g:

$$T_g(M_n) \approx T_{g,\infty} - K/M_n$$

As pure FOS were mainly composed of DP3, DP4 and DP5, the total contribution of these three oligosaccharides in the mixture (86% for both pure FOS and 72% for commercial FOS, Table 1) can be related with their higher T_gs. The contribution of DP6 and DP7 in commercial FOS (20% as a whole) might also increase the T_g of the mixture (Table 1). Non-purified FOS had much lower T_g because of the presence of high concentrations of monosaccharides (23 and 35% for 2.5 and 24 h synthesis FOS, respectively) (Roos & Karel, 1991).

The chemical heterogeneity of the selected FOS provided a broad spectrum of thermophysically different oligosaccharides to thoroughly evaluate their effect on both the freeze-drying and the storage processes.

The dehydration involved in freeze-drying processes leads to structural damages at different levels, which can be disregarded if the efficiency of FOS as protectants is evaluated just by plate counting. For this reason, acidification kinetics carried out on freeze-dried microorganisms provided information about the bacterial capacity to repair damages and recover after freeze-drying (Fig. 1). Determining the *lag* time and the PI uptake gave evidences about global and membrane damages, respectively (Tables 3 and 4). It was observed that bacteria freeze-dried in the presence of non-purified FOS obtained after 2.5 and 24 h of synthesis (containing *ca.* 44–45% of monosaccharides + sucrose, Table 1) showed the shortest *lag* times and the highest acidification rates (Table 3). This performance was better than the one obtained using sucrose as protectant (used as reference). On the contrary, pure and commercial FOS led to larger *lag* times and lower acidification rates (Table 3). Within this latter group, a lower bacterial activity (larger *lag* times) could also be related with a higher contribution of larger FOS in the mixtures. Hence, pure FOS obtained after 2.5 h of synthesis (59% DP3, 24% DP4) led to shorter *lag* times than pure FOS obtained after 24 h of synthesis and commercial FOS, in which DP4 + DP5 + DP6 accounted 65 and 61%, respectively, with a considerably lower amount of DP3 (22–25%) (Table 1).

The results of the *lag* times and acidification rates indicate as a whole, that the presence of monosaccharides in the mixture was necessary to preclude global damages during freeze-drying. This protective effect decreased as soon as the DP increased, as shown by the weaker protective capacity of the pure FOS containing higher concentrations of larger FOS (sum of DP4, DP5 and DP6) (Tables 1 and 3).

Regarding the PI uptake, although all the protectants were efficient in precluding membrane damage (in comparison with bacteria freeze-dried without protectants), pure FOS and sucrose were significantly more efficient than non-purified and commercial FOS ($P < 0.05$) (Table 4). The capacity of sucrose to interact with lipid membranes by replacing water molecules is well known (Crowe et al., 1998) and is one of the mechanisms explaining its protective capacity during dehydration processes (water replacing hypothesis) (Crowe et al., 1998). On the other hand, DP3, DP4 and DP5 were in higher percentages in pure FOS (86–87%) than in non-purified ones (53–56%) and commercial FOS (72%) (Table 1). Considering the greater efficiency of pure FOS to protect bacterial membranes (Table 4), it can be conjectured that the higher content of DP3, DP4 and DP5 allows a stronger interaction with the polar head groups of lipids. In this sense, Crowe et al. (1998) reported that the interaction of sugars with lipid membranes is progressively more intense along the series glucose-trehalose (DP2)-raffinose (DP3), leading to a decrease of the phase transition temperature (Tm) of dipalmitoyl phosphatidylcholine (DPPC) membranes. Hincha, Zuther, and Heyer (2003) found that sucrose (DP2), raffinose (DP3), stachyose (DP4) and verbascose (DP5) stabilize membranes, by preventing fusion and leakage of egg phosphatidylcholine liposomes, progressively better with increasing DP. This stabilization was explained on the basis of the greater capacity of higher DP sugars to interact with lipid membranes, and also to their higher T_g. The presence of FOS larger than DP5 (as occurs in commercial FOS, DP6 + DP7 accounting 20%) seems to weaken their capacity to interact with lipid membranes (Tables 1 and 4).

The protective effect of FOS on bacterial cultivability was also evaluated directly after freeze-drying, after equilibration at different RH and during storage at 4 °C. This approach allowed determining the effect of protectants on each step of the process in an independent way (Table 4 and Fig. 2). When comparing bacterial cultivability immediately after freeze-drying, it was observed that non-purified FOS and sucrose were significantly more efficient than pure and commercial FOS (Table 4). The difference between non-purified and pure FOS could be due to the presence of significant amounts of sucrose and monosaccharides in the former. Although monosaccharides have low T_g, they are able to interact with lipid membranes and replace water molecules, thus having a stabilizing effect (Crowe et al., 1992, 1998). For this reason, their use in combination with certain polysaccharides (*e.g.*: maltodextrin) has been suggested as a strategy to improve bacterial stability (Oldenhof et al., 2005).

During storage, bacteria stabilized at 11% RH were those with the best performance (Fig. 2). At this RH, no significant differences were observed in the protective effect of the five investigated FOS mixtures. The protective effect of pure and commercial FOS during storage can be ascribed to their higher T_g (Table 2). The efficiency of non-purified FOS during storage at 11% RH can be explained considering that: a) the storage temperature (T) (4 °C) was below their T_g, thus ensuring the storage in a glassy state; b) the moderate concentration of sucrose in non-purified FOS (especially in those obtained after 2.5 h synthesis) (Table 1) can also contribute to bacterial protection. The importance of vitrification was better observed when bacteria were stored at 22 and 33% RH. At 22% RH, although T was below T_g (Table 2), non-purified FOS showed a worse performance than their pure counterparts (Fig. 2B). This behavior can be explained considering the lower value of T-Tg (parameter directly related with the storage stability) in non-purified FOS (Roos & Karel, 1991). In turn, at 33% RH, the stickiness of samples ($T > T_g$) precluded the evaluation of their efficiency (Table 2 and Fig. 2C).

The effect of the investigated protectants on all the analyzed parameters is summarized in Fig. 3, making it easier to judge, at a glance, the influence of FOS composition on their protective capacity. The parameters associated to the effect of FOS during freeze-drying (*lag* time and acidification rate in MRS broth, PI uptake and loss of cultivable cells) are shown in the upper half of the figure, and those associated to the effect of FOS during storage (loss of cultivability after 60 days of storage at 11 and 33% RH and remnant cultivability after such storage), on the lower half. The values depicted on each corner of the octagon represent the best performance for each parameter. Hence, the closest to the corner, the better. Although non-purified FOS showed a better performance during freeze-drying and storage at 11% RH, their lower T_g precluded their use as glassy matrices during storage at 33% RH.

Because vitrification is a necessary but not sufficient condition for a good protection (Romano et al., 2015, chap. 10), different authors

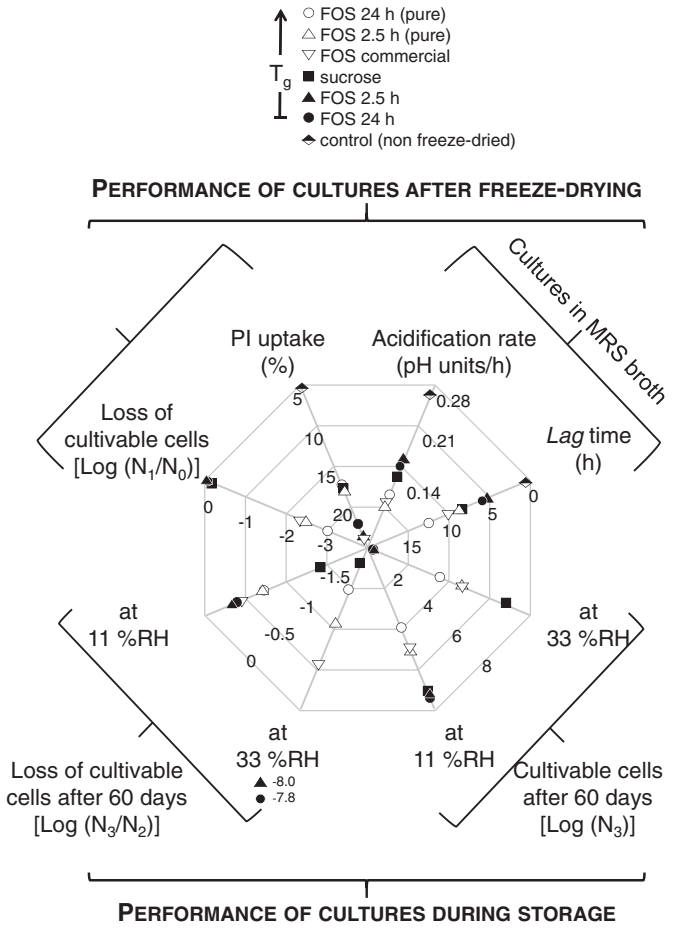

Fig. 3. Schematic representation disclosing the effect of FOS on each of the parameters evaluated (located on each corner of the octagon). The scales are presented on each axis, the more external the value the better its performance for bacterial activity or preservation. The parameters related with the freeze-drying process were plotted in the upper half of the Figure. They are: *lag* time and acidification rate in MRS broth, PI uptake, loss of cultivability immediately after freeze-drying. N_1 is the cultivability after freeze-drying and N_0, the cultivability before the process. The parameters related with storage were plotted in the lower half of the octagon. They are: loss of cultivability after 60 days of storage at 11 and 33% RH, and remnant cultivability after such storage. N_2 is the cultivability at the beginning of storage (time equal to 0) and N_3, the cultivability after 60 days of storage. Symbols represent bacteria freeze-dried in the presence of: FOS obtained after 2.5 h of synthesis, purified (open up-triangle) or not (full up-triangle); FOS obtained after 24 h of synthesis, purified (open circle) or not (full circle); commercial FOS (open down-triangle), sucrose (full square). Fresh bacteria (half full diamond) were used as controls. The arrow near the symbols' reference indicates the increase of T_g for the saccharides under analysis.

have proposed the conjoint use of polysaccharides with high T_g (*e.g.*, maltodextrin, starch) with small sugars having not so high T_g but that interact with membranes (*e.g.*, glucose or sucrose) as an adequate protection strategy (Hincha et al., 2007; Oldenhof et al., 2005; Crowe et al., 1998). For this reason, it can be concluded that the effect of FOS on bacterial protection results from a balance between monosaccharides, sucrose and larger FOS in the mixtures: the smallest sugars are more efficient in protecting lipid membranes, and the largest ones favor the formation of vitreous states.

5. Conclusions

In this work, we rationalized the role of the mono- and oligosaccharides present in FOS mixtures during freeze-drying and storage of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333. The presence of monosaccharides and sucrose was important to stabilize bacteria during freeze-drying. However, the presence of these compounds in the mixtures led to a decrease in the T_g, which favored the formation of sticky states (non-desirable during storage). On the contrary, larger oligosaccharides were less efficient during freeze-drying, but their higher T_g had a stabilizing effect during storage. From the results obtained, it can be concluded that the effect of FOS in bacterial protection is the result of a balance between monosaccharides, sucrose and larger oligosaccharides. The smaller sugars are more efficient in protecting lipid membranes, and the largest ones favor the formation of glassy states.

Supplementary data to this article can be found online at doi:10.1016/j.foodres.2016.11.003.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

N.R. did the experimental work. C.S. determined and analyzed the vitreous transition temperatures in the context of this work. A.G.-Z. and P.M. coordinated the work (analysis of results, discussion and writing of the manuscript). All authors have approved the final version of the manuscript.

Acknowledgments

This work was supported by the Argentinean Agency for the Scientific and Technological Promotion (ANPCyT) (Projects PICT/2014/0912) and the Argentinean National Research Council (CONICET) (PIP 2015-2017 11220150100636CO). Eng. Javier Lecot, Daniel Russo, Lic. Maria Cecilia Alvarez, Claudio Reyes and Lib. Diana Velasco are acknowledged for technical assistance. P.M., C.S. and A.G.-Z. are members of the research career CONICET. N.R. is doctoral fellow from CONICET.

References

Aquilina, G., Bach, A., Bampidis, V., Bastos, M. L., Flachowsky, G., Gralak, M. A., ... Westendorf, J. (2013). Scientific opinion on the safety and efficacy of Probiotic LACTINA® (*Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus bulgaricus*, *Lactobacillus lactis*, *Streptococcus thermophilus* and *Enterococcus faecium*) for chickens for fattening and piglets. *EFSA Journal*, *11*, 3170–3183.

Boon, M. A., Janssen, A. E. M., & van der Padt, A. (1999). Modelling and parameter estimation of the enzymatic synthesis of oligosaccharides by β-galactosidase from *Bacillus circulans*. *Biotechnology and Bioengineering*, *64*, 558–567.

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P. M., Malcata, F. X., & Gibbs, P. (2004). Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze-dried *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Biotechnology Progress*, *20*, 248–254.

Crittenden, R., & Playne, M. J. (2009). Prebiotics. In L. YK, & S. Salminen (Eds.), *Handbook of probiotics and prebiotics* (pp. 535–581) (2nd ed.). John Wiley.

Crowe, J. H., Hoekstra, F. A., & Crowe, L. M. (1992). Anhydrobiosis. *Annual Review of Physiology*, *54*, 579–599.

Crowe, J. H., Carpenter, J. F., & Crowe, L. M. (1998). The role of vitrification in anhydrobiosis. *Annual Review of Physiology*, *60*, 73–103.

de Man, J. C., Rogosa, M., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology*, *23*, 130–135.

Fonseca, F., Cenard, S., & Passot, S. (2015). Freeze-drying of lactic acid bacteria. *Methods in Molecular Biology*, *1257*, 477–488.

Fox, T. G., & Flory, P. J. (1950). Second-order transition temperatures and related properties of polystyrene. I. Influence of molecular weight. *Journal of Applied Physics*, *21*, 581–591.

Gibson, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota-introducing the concept of prebiotics. *Journal of Nutrition*, *125*, 1401–1412.

Golowczyc, M. A., Gerez, C. L., Silva, J., Abraham, A. G., De Antoni, G. L., & Teixeira, P. (2011). Survival of spray-dried *Lactobacillus kefir* is affected by different protectants and storage conditions. *Biotechnology Letters*, *33*, 681–686.

Gomez-Zavaglia, A., Abraham, A. G., Giorgieri, S., & De Antoni, G. L. (1999). Application of polyacrylamide gel electrophoresis and capillary gel electrophoresis to the analysis of *Lactobacillus delbrueckii* whole-cell proteins. *Journal of Dairy Science*, *82*, 870–877.

Higl, B., Kurtmann, L., Carlsen, C. U., Ratjen, J., Forst, P., Skibsted, L. H., ... Risbo, J. (2007). Impact of water activity, temperature, and physical state on the storage stability of *Lactobacillus paracasei* ssp. *paracasei* freeze-dried in a lactose matrix. *Biotechnology Progress*, *23*, 794–800.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., ... Sanders, M. E. (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term

probiotic. *Nature Reviews Gastroenterology & Hepatology*. http://dx.doi.org/10.1038/nrgastro.2014.66 (advance online publication (June 2014)).
Hincha, D. K., Zuther, E., & Heyer, A. G. (2003). The preservation of liposomes by raffinose family oligosaccharides during drying is mediated by effects on fusion and lipid phase transitions. *Biochimica et Biophysica Acta, 1612*, 172–177.
Hincha, D. K., Popova, A. V., & Cacela, C. (2006). Effects of sugars on the stability of lipid membranes during drying. In A. Leitmannova Liu (Ed.), *Advances in planar lipid bilayers and liposomes. Vol. 3*. (pp. 189–217). Amsterdam: Elsevier.
Hincha, D. K., Livingston, D. P., Premakumar, R., Zuther, E., Obel, N., Cacela, C., & Heyer, A. G. (2007). Fructans from oat and rye: Composition and effects on membrane stability during drying. *Biochimica et Biophysica Acta, 1768*, 1611–1619.
Hiraoka, Y., & Kimbara, K. (2002). Rapid assessment of the physiological status of the polychlorinated biphenyl degrader *Comamonas testosteroni* TK102 by flow cytometry. *Applied and Environmental Microbiology, 68*, 2031–2035.
Jung, K. H., Yun, J. W., Kang, K. R., Lim, J. Y., & Lee, J. H. (1989). Mathematical model for enzymatic production of fructo-oligosaccharides from sucrose. *Enzyme Microbial Technology, 11*, 491–494.
Leslie, S., Israeli, E., Lighthart, B., Crowe, J. H., & Crowe, L. M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology, 61*, 3592–3597.
Meng, X. C., Stanton, C., Fitzgerald, G. F., Daly, C., & Ross, R. P. (2008). Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chemistry, 106*, 1406–1416.
Miao, S., Mills, S., Stanton, C., Fitzgerald, G. F., Roos, Y. H., & Ross, R. P. (2008). Effect of disaccharides on survival during storage of freeze dried probiotics. *Dairy Science and Technology, 88*, 19–30.
Morgan, C. A., Herman, N., White, P. A., & Vesey, G. (2006). Preservation of micro-organisms by drying: A review. *Journal of Microbiological Methods, 66*, 183–193.
Oldenhof, H., Wolkers, W., Fonseca, F., Passot, S., & Marin, M. (2005). Effect of sucrose and maltodextrin on the physical properties and survival of air-dried *Lactobacillus bulgaricus*: An *in situ* Fourier transform infrared spectroscopy study. *Biotechnology Progress, 21*, 885–892.
Phuapaiboon, P., Leenanon, B., & Levin, R. (2013). Effect of *Lactococcus lactis* immobilized within pineapple and yam bean segments, and jerusalem artichoke powder on its viability and quality of yogurt. *Food and Bioprocess Technology, 6*, 2751–2762.
Romano, N., Tymczyszyn, E., Mobili, A., & Gómez-Zavaglia, A. (2015). Prebiotics as protectants of lactic acid bacteria. In W. RR, & P. VR (Eds.), *Bioactive foods in promoting health: Probiotics, prebiotics, and synbiotics (2nd ed.). Part 1: Prebiotics in health promotion*. (pp. 155–164). Academic Press, Elsevier.
Romano, N., Santos, M., Mobili, P., Vega, R., & Gómez-Zavaglia, A. (2016). Effect of sucrose concentration on the composition of enzymatically synthesized short-chain fructo-oligosaccharides as determined by FTIR and multivariate analysis. *Food Chemistry, 202*, 467–475.
Roos, Y., & Karel, M. (1991). Phase transitions of mixtures of amorphous polysaccharides and sugars. *Biotechnology Progress, 7*, 49–53.
Santivarangkna, C., Higl, B., & Foerst, P. (2008). Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. *Food Microbiology, 25*, 429–441.
Tripathi, M. K., & Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods, 9*, 225–241.
Tymczyszyn, E. E., Gómez-Zavaglia, A., & Disalvo, E. A. (2007). Effect of sugars and growth media on the dehydration of *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Journal of Applied Microbiology, 102*, 845–851.
Tymczyszyn, E. E., Díaz, M. R., Pataro, A., Sandonato, N., Gómez-Zavaglia, A., & Disalvo, E. A. (2008). Critical water activity for the preservation of *Lactobacillus bulgaricus* by vacuum drying. *International Journal of Food Microbiology, 128*, 342–347.
Tymczyszyn, E. E., Gerbino, E., Illanes, A., & Gómez-Zavaglia, A. (2011). Galacto-oligosaccharides as protective molecules in the preservation of *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Cryobiology, 62*, 123–129.
Tymczyszyn, E. E., Sosa, N., Gerbino, E., Hugo, A. A., Gómez-Zavaglia, A., & Schebor, C. (2012). Effect of physical properties on the stability of *Lactobacillus bulgaricus* in a freeze-dried galacto-oligosaccharides matrix. *International Journal of Food Microbiology, 155*, 217–221.
Vega, R. J., & Zuniga-Hansen, M. E. (2011). Enzymatic synthesis of fructooligosaccharides with high 1-kestose concentrations using response surface methodology. *Bioresource Technology, 102*, 10180–10186.
Vega, R. J., & Zuniga-Hansen, M. E. (2012). Potential application of commercial enzyme preparations for industrial production of short-chain fructooligosaccharides. *Journal of Molecular Catalysis B: Enzymatic, 76*, 44–51.
Vega, R. J., & Zuniga-Hansen, M. E. (2014). A new mechanism and kinetic model for the enzymatic synthesis of short-chain fructooligosaccharides from sucrose. *Biochemical Engineering Journal, 82*, 158–165.
Vereyken, I. J., Chupin, V., Demel, R. A., Smeekens, S. C. M., & De Kruijff, B. (2001). Fructans insert between the headgroups of phospholipids. *Biochimica et Biophysica Acta, Biomembranes, 1510*, 307–320.
Vereyken, I. J., Chupin, V., Islamov, A., Kuklin, A., Hincha, D. K., & De Kruijff, B. (2002). The effect of fructan on the phospholipid organization in the dry state. *Biophysical Journal, 85*, 3058–3065.
Vereyken, I. J., Chupin, V., Hoekstra, F. A., Smeekens, S. C. M., & De Kruijff, B. (2003). The effect of fructan on membrane lipid organization and dynamics in the dry state. *Biophysical Journal, 84*, 3759–3766.